Effect of exogenous chitosan, salicylic acid, and their combination on some physiological parameters of *Citrullus colocynthis* (L.) under drought stress

Zohreh Azin[1], Abbasali Emamjomeh[2 & 3]*, Sedigheh Esmaeilzadeh Bahabadi[4], Parisa Hasanein[4]

1. Department of Horticulture, Faculty of Agriculture, University of Zabol, Zabol, Iran
2. Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Zabol, Zabol, Iran
3. Department of Bioinformatics, Faculty of Science, University of Zabol, Zabol, Iran
4. Department of Biology, Faculty of Science, University of Zabol, Zabol, Iran

Abstract

Drought stress is alarmingly on the rise over the past decades. Growth regulators including salicylic acid (SA) and chitosan are being successfully used to protect plants against biotic and abiotic stresses. To investigate the effect of SA, chitosan, and their combination on biochemical traits and cucurbitacin contents in *Citrullus colocynthis* (L.) under different levels of drought stress, four irrigation levels (control (100% Field capacity: FC), 75, 50, and 25% FC) were used along with three different treatments by administering SA and chitosan (150 mg L^{-1}), and their combinations. Drought stress significantly increased the amount of sugar, proline, lipid peroxidation, and the activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) while decreasing growth parameters, protein, leaf relative water content (RWC), chlorophyll, and carotenoid contents. Although SA could increase some biochemical traits including sugar, chlorophyll, and carotenoid contents only in severe level of drought stress, chitosan and the combined treatment exerted beneficial effects in almost all levels of irrigation. Furthermore, combination of SA and chitosan induced more protective effects compared to only chitosan treatment in increasing proline and the activities of SOD and CAT. Combined treatment was also effective in increasing cucurbitacin B, C and L contents but not cucurbitacin E. Combination of SA and chitosan showed the major impact on improving physiological parameters and cucurbitacin contents of *C. colocynthis* and therefore could be a potential candidate to protect the plant against adverse effects of drought stress.

Keywords: Antioxidant enzymes, drought stress, *Citrullus colocynthis*, cucurbitacin, salicylic acid

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Introduction

Plant growth and productivity is severely affected by drought stress. Plants respond to drought stress at molecular, metabolic, and physiological levels. The responses depend on species and genotype, the length and severity of water deficit, the age, and stage of development (Barnabás et al., 2008).

Drought stress causes serious physiological and biochemical dysfunctions

* *Corresponding Author*
E-mail Address aliimamjomeh@uoz.ac.ir
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including ion disequilibrium, stomatal closure, reduction in cellular expansion and growth, and membrane instability (Farouk and Amany, 2012; Miao et al., 2015). Various abiotic stresses lead to excessive production of reactive oxygen species (ROS) causing oxidative damage and cell death (Sharma et al., 2012). Scavenging or detoxification of excess ROS is achieved by an efficient antioxidative system including the enzymatic and non-enzymatic antioxidants (Noctor and Foyer, 1998). The enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). Ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, and various antioxidant secondary metabolites serve as potent non-enzymatic antioxidants (Gill and Tuteja, 2010). Various methods have been used to improve drought tolerance such as introduction of drought-resistant genes to a host plant and cultivation of drought-tolerant varieties of crop plants (Budak et al., 2013).

Several studies have reported the application of plant growth regulators (PGR) as an effective method for inducing drought resistance (Farooq et al., 2010). Salicylic acid (SA) is a phenolic compound which participates in the regulation of physiological processes and induces stress tolerance in plants (Rivas-San Vicente and Plasencia, 2011). The effect of exogenous SA depends on different factors including the species and developmental stage of plant, the concentration of SA, and the mode of application (Horváth et al., 2007). Barely seedlings under drought stress combined with the application of SA showed higher dry mass, net CO2 assimilation, and antioxidative enzyme activities (Habibi, 2012). SA at 300 mg L^{-1} increased antioxidant enzymes, sugar, proline, protein, and essential oil content in *Lippiacitri odora* under drought stress (Dianat et al., 2016). Chitosan, a cationic polysaccharide is known to play a key role in defense responses (Mandal, 2010). It is involved in some systems of signal transduction to protect plants against oxidative stress (Guan et al., 2009) and to stimulate plant growth (Farouk et al., 2008; Farouk et al., 2011). Foliar spray of chitosan resulted in higher vegetative growth and improvement in fruit quality of pepper, radish, and cucumber (Farouk et al., 2008; Ghoname et al., 2010).

Citrullus colocynthis (L.) is an important medicinal plant, traditionally used as an antidiabetic medication in tropical and subtropical countries (Diwan et al., 2000). *C. colocynthis* contains active compounds such as saponins, alkaloids, and glycosides (Diwan et al., 2000). The main constituents are highly oxidized steroid compounds called cucurbitacins (Seger et al., 2005). There are a variety of cucurbitacin compounds such as cucurbitacin A, B, C, D, E, F, I, and L(Hatam et al., 1989).

Drought may cause significant changes in metabolite yields and compositions of medicinal crops (Bettaieb et al., 2009; Pirbalouti et al., 2014). There are few reports on the effect of SA and chitosan on medicinal plants under drought stress. Because of the medical importance of *C. colocynthis* in Iran, one of the goals of this study was to evaluate the impact of drought stress on some biochemical and physiological parameters in this plant. Furthermore, cucurbitacin as a main triterpene derivative in *C. colocynthis* plays a crucial role in inhibiting cancer cells (Saeed et al., 2019; Abdulridha et al., 2019). Therefore, we also measured production of this metabolite under the effects of different stimulators and drought stress. Also, to the best of our knowledge there has been no previous report regarding the effects of foliar application of SA and chitosan on *C. colocynthis* under water stress. Therefore, the purpose of this study was to investigate the effects of drought stress on some physiological and biochemical parameters, cucurbitacins content, and determination of the role of SA and chitosan in mitigation of water deficit stress in *C. colocynthis* Plant.

Materials and Methods

To investigate the effect of SA, chitosan and their combination on biochemical traits and cucurbitacin contents in *Citrullus colocynthis* (L.) under different levels of drought stress, four irrigation levels (control (100% Field capacity: FC), 75, 50, and 25% FC) along with three different treatments by administration of SA and chitosan (150 mg L^{-1}), and their combinations were used.

Plant culture and treatments

Seeds of *C. colocynthis* were directly planted in the field, in plots (4 m width by 5 m length) with 50 cm line spacing. The experiment was performed in a randomized block design factorial experiments (2 × 3) with three replications. The irrigation treatments consisted of four levels based on field capacity (FC): control (100% FC), 75, 50, and 25% FC. The interaction effects of water and chitosan and SA treatments were determined. Chitosan was dissolved in 1% acetic acid solution for 3 h and SA was dissolved in ethanol 70%. These solvents did not cause changes in responses and showed no difference with control (data not shown). The chitosan and SA (150 mg L^{-1}) foliar application were performed 3 times at plant establishment stage, middle phase of vegetative growth, and flowering stage. After five months, the shoots of plants were gathered and immediately frozen in liquid nitrogen and stored at -80 ℃ for subsequent analysis.

Growth parameters

Dry weights (g) and plant height (cm) were recorded and leaf area (cm^2) was calculated using a Leaf Area Meter (DELTA-T, ENGLAND) at the end of experiment.

Leaf relative water content (RWC)

RWC was estimated by weighing (g) fresh leaves (FW) and then immersing it in water until the weight of leaves was constant; then, water saturated leaves (SW) were weighed and dried for 72 hours at 70 ℃ for dry weight (DW) determination and the percentage of leaf relative water content (RWC) was calculated as follows:

$$\text{RWC\%} = \frac{Fresh\ Weight - Dry\ Weight}{Saturated\ Weight - Dry\ Weight} \times 100$$

Determination of photosynthetic pigment contents

Content of chlorophyll a, chlorophyll b, and carotenoids were determined according to the methods of Cock et al. (1976).

Briefly, the leaves were cut in small pieces and immersed in 80% acetone solvent to extract pigments. The extraction solvent was diluted and measured with spectrophotometer at 663, 645 and 440.5 nm. The content of pigments was calculated by equation:

$$C_a = 0.0127 \times D663 - 0.00269 \times D645$$

$$C_b\ (\text{mg g}^{-1}\ \text{fresh leaf}) = 0.0299 \times D645 - 0.00468 \times D663$$

$$C_{car}\ (\text{mg g}^{-1}\ \text{fresh leaf}) = 0.004695 \times D440.5 - 0.000268\ (C_a + C_b)$$

where, C_a and C_b (mg g^{-1} fresh leaf) are contents of chlorophyll a and b; and C_{car} (mg g^{-1} fresh leaf) is content of carotenoid.

Determination of soluble carbohydrate and protein content

Total soluble sugar content was determined using anthrone reagent and glucose as standard (Roe, 1955). Briefly, 0.1 g fresh weight of leaf tissue powder was ground and extracted with 2.5 ml of 80% (v/v) ethanol at 90 ℃ for 60 min, followed by centrifugation at 10 000 *g* at 4 ℃ for 10 minutes. The process was repeated for complete extraction. Soluble carbohydrate contents were expressed as mg g^{-1} DW.

Protein content was estimated spectrophotometrically by Bradford method (Bradford, 1976). Briefly, samples (0.5 g) were homogenized in 2.5 ml of 50 mM phosphate buffer (pH 7) containing 1 M ethylenediamine tetra acetic acid (EDTA), 1 mM phenyl methyl sulfonyl fluoride (PMSF), and 1% polyvinyl pyrrolidone (PVP). The homogenate solution was centrifuged at 20 000 g at 4 ℃ for 20 min and the supernatant was used for measurement of total soluble protein that was expressed as mg g^{-1} FW.

Determination of proline content

Proline content of leaves was determined according to Bates et al. (1973). Fresh leaves (0.1 g) were homogenized in 10 mL aqueous 3% sulfosalicylic acid and the homogenate was filtered. The filtrate (2 mL) was mixed with 2 mL fresh acid ninhydrin solution and 2 mL glacial acetic acid and then with 4 mL toluene. The absorbance of the colored solutions was measured at 520 nm.

Determination of lipid peroxidation

The level of lipid peroxidation was measured in terms of Malondialdehyde (MDA) concentration that was determined by the method of Hodges et al. (1999). Mature fresh leaves (0.15 g) were homogenized with 2 ml of ice-cold 50 mM phosphate buffer (pH 7.8) and 5 ml of 0.5% thiobarbituric acid (TBA). The mixture was heated in a water bath shaker at 100 ℃ for 10 min, and quickly cooled in an ice bath. The samples were centrifuged at 3,000 g for 15 min. The absorbance was measured at 532 nm.

Determination of antioxidant enzymes SOD and CAT

SOD and CAT were assayed by the method described by Sankar et al. (2007) with few modifications. For determination of SOD activity, leaves (0.1 g) were homogenized in 3 mL of phosphate buffer (50 mM, pH 7.8) containing 1% polyvinyl polypyrrolidone. The homogenate was then centrifuged for 15 min at 10,000 g. Reaction mixture consisted of extraction buffer (0.1 mM EDTA; 50 mM Na_2CO_3; pH 10.2), 75 mM nitroblue tetrazolium chloride (NBT), 12 mM L-methionine, 4 mM riboflavin, and 0.2 mL the enzyme extract. The amount of enzyme that resulted in 50% inhibition of the rate of NBT reduction at 560 nm was defined as one unit of SOD activity, expressed against mg protein of the extract.

For measurement of CAT activity, 0.1 g fresh leaves were extracted in 3 mL of sodium phosphate buffer (25 mM; pH 6.8) and centrifuged (12,000 rpm) for 20 min. The reaction mixture contained 1.0 ml of 50 mM Tris–Hydrochloric acid buffer (pH 7.0), 0.2 ml of 200 mM H_2O_2, 0.1 ml of enzyme extract and 1.7 ml deionized water. Activity of CAT was monitored by measuring the decrease in absorbance at 240 nm due to H_2O_2 consumption, against mg protein.

Cucurbitacin determination

Dried leaves of *C. colocynthis* (60 g) were extracted with methanol in a Soxhlet apparatus for 24 hours. The extract was evaporated under vacuum. The residue was dissolved in methanol for the GC/MS analysis. GC–MS analyses were carried out on a Hewlett Packard 5890 GC–MS system equipped with silica ultra-performance cross-linked methyl silicone column (50 m length 0.2 mm i.d.; film thickness 0.25 mL). The temperature was raised from 100 to 280 ℃ at 4 ℃/min, carrier gas helium with a linear velocity of 1 mL /min, ionization energy 70 eV, scan time 1 s, and mass range 40 - 400 amu (Li et al., 2009).

Statistical Analysis

The experimental design was split-plots in Randomized Complete Block (RCBD) design with three replications. The experiment was performed in Research Field of University of Zabol, Iran. Two studied factors were drought stress as main plot with three levels (25%, 50%, 75%, and 100% Field capacity as control) and treatment as sub-plots with four levels (control, SA, chitosan, and SA + chitosan). Duncan multiple range test was applied to compare the means of treatments in 1% level.

Results

Effects of SA, chitosan, and SA/chitosan combined treatment on vegetative growth of C. colocynthis under drought stress

Drought stress significantly decreased dry weight and length of the treated plants in comparison with the control plants (Figs. I. a and b). Both SA and chitosan treatments alone as well as in combination were effective in increasing dry weight and plant length in all levels of irrigation except the first level (irrigation equal to 100% field capacity). The best results were achieved by combined treatment of SA and chitosan at the third and fourth levels of drought stress (Figs. I. a and b). Similar trend was observed in leaf area (Fig. I. c). There were no detectable differences between SA, chitosan, and combined treatment in leaf area increase.

Effects of SA, chitosan and SA/chitosan combined treatment on RWC of C. colocynthis under drought stress

Changes in RWC under drought stress are shown in Fig. I. d. Increased drought stress (75% - 25% of FC) tended to decrease relative water content. The results of this study showed that use of SA,

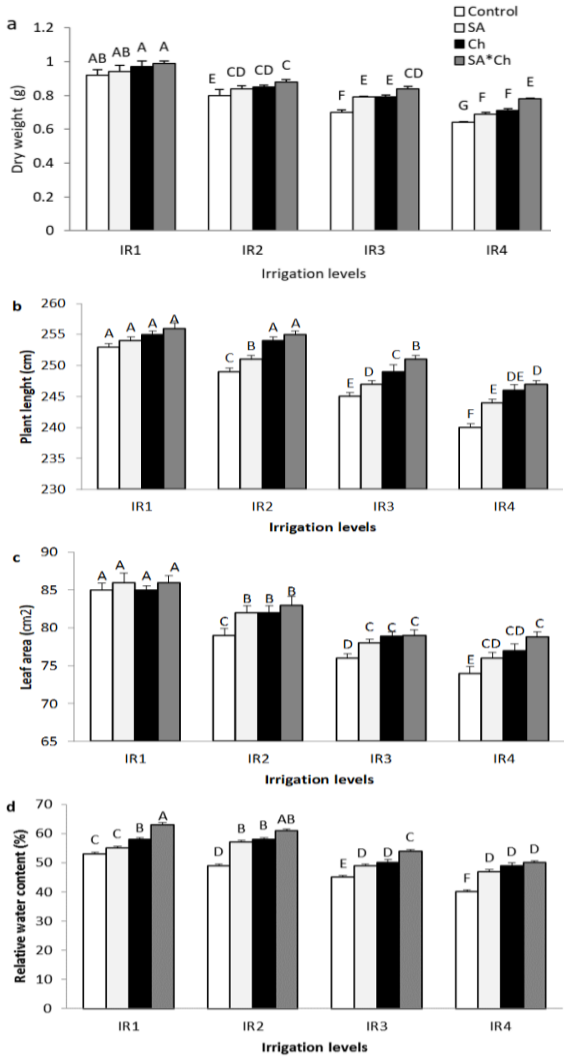

Fig. I. Effect of drought and its combination with salicylic acid (SA) (150 mg L^{-1}), chitosan (Ch) (150 mg L^{-1}), and their interaction (SA* Ch) on a) dry weight (g), b) plant length (cm), c) leaf area (cm^2), and d) relative water content (RWC) (%) in *Citrullus colocynthis*; data are given as means ± standard error, N = 3. IR1 (100% FC), IR2 (75% FC), IR3 (50% FC), and IR4 (25% FC). Bars with different letters denote significant differences at P≤0.05 based on a Dunkan test. Error bars indicate Standard Error (SE).

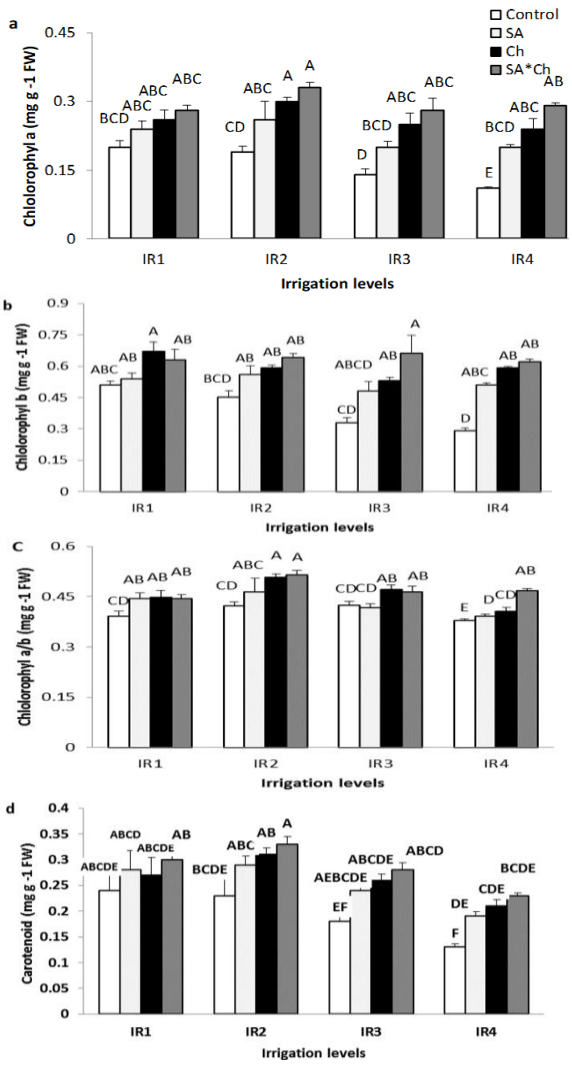

Fig. II. Effect of drought and its combination with salicylic acid (SA) (150 mg L^{-1}), chitosan (Ch) (150 mg L^{-1}), and their interaction (SA* Ch) on the contents of a) chlorophyll a, b) chlorophyll b, c) chlorophyll a/b ratio and d) carotenoid in *Citrullus colocynthis*. Data given as means ± standard error, N = 3. IR1 (100% FC), IR2 (75% FC), IR3 (50% FC) and IR4 (25% FC). Bars with different letters denote significant differences at P<0.05 based

chitosan and combined treatment significantly increased the RWC compared with the control. The highest RWC percent (63%) was obtained from foliar application of combined treatment in well-watered plants (irrigation equal to 100% field capacity).

Effects of SA, chitosan, and SA/chitosan combined ttreatment on photosynthetic pigments of C. colocynthis under ddrought stress

Results indicated that the content of chlorophyll a showed a significant decrease in controls at the severe drought stress (Fig. II. a). SA treatment alone elevated chlorophyll a content in severe drought stress compared to the respective controls. Chitosan treatment as well as combined treatment increased the amount of chlorophyll a in mild, moderate, and severe drought stress compared to controls.

Our findings indicated that the amount of chlorophyll b significantly decreased in severe

drought stress in controls (Fig. II. b). SA treatment could increase this pigment only in severe drought stress compared to the controls at this level of irrigation. Here, chitosan treatment as well as combined treatment had similar effects in increasing the amount of chlorophyll b in moderate and severe drought stress compared to the control. Also, the chlorophyll a/b ratio was affected by drought stress. In this regard, the ratio decreased significantly at the severe level of drought stress as shown in Fig. II. c. Furthermore, although SA was effective in increasing chlorophyll a/b ratio in some levels of drought stress, the best effect was achieved by chitosan and combined treatment of SA and chitosan at all levels of drought stress (Fig. II. c)

Figure II. d demonstrates that both SA and chitosan alone increased carotenoid content only at severe drought stress compared to the control. However, combined treatment elevated this parameter in mild, moderate, and severe drought stress.

Effects of SA, chitosan, and SA/chitosan combined treatment on biochemical traits of C. colocynthis under drought stress

Figure III. a indicates that the amount of protein decreased in control plants under moderate and severe drought stress. Both SA and chitosan treatments alone as well as combined treatment were effective in increasing protein in all levels of irrigation except the first level (irrigation equal to 100% field capacity).

The amount of sugar increased in control plants under severe drought stress as indicated in Fig. III. b. Neither single treatments nor combined treatment could alter sugar content at the first and second levels of irrigation (Fig. III. b). SA increased sugar content only at severe drought stress compared to the control. Chitosan only treatment and combined treatment had the maximum effect in increasing sugar in moderate and severe drought stress compared to the controls. Combined treatment could not induce a further increase in sugar content compared to chitosan treatment in these conditions.

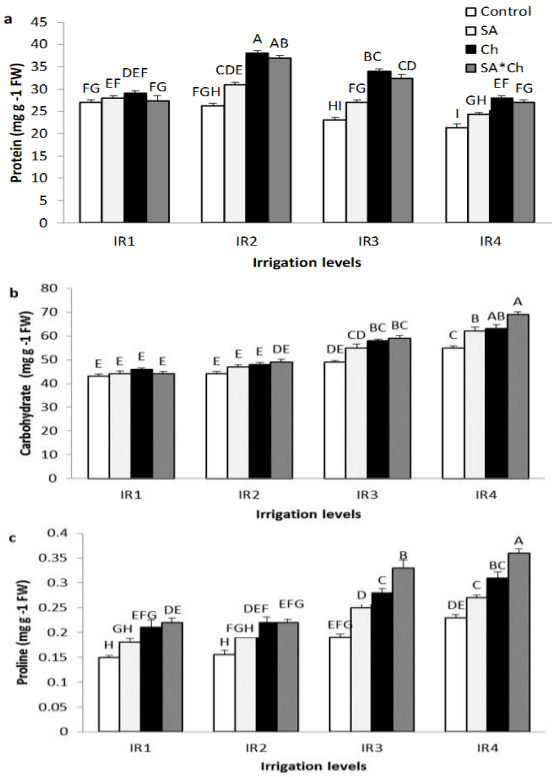

Fig. III. Effect of drought and its combination with salicylic acid (SA) (150 mg L^{-1}), chitosan (Ch) (150 mg L^{-1}), and their interaction (SA* Ch) on the contents of a) protein, b) sugar, and c) proline in *Citrullus colocynthis*; data are given as means ± standard error, N = 3. IR1 (100% FC), IR2 (75% FC), IR3 (50% FC), and IR4 (25% FC). Bars with different letters denote significant differences at P≤0.05 based on a Dunkan test. Error bars indicate Standard Error (SE).

The proline contents of controls increased in moderate and severe drought stress (Fig. III. c). While SA increased proline only in moderate and severe drought stress, chitosan increased it at all levels of irrigation compared to the respective controls. Combined treatment was similar to chitosan treatment in changing proline content under normal irrigation level and mild drought stress but was more than chitosan treatment in moderate and severe levels of drought stress. The maximum content of proline was recorded under combined treatment in the severe drought stress.

Effects of SA, chitosan, and SA/chitosan combined treatments on antioxidant enzymes in C. colocynthis under drought stress

The data indicated that severe drought induced an increase in the amount of MDA in controls as shown in Fig. IV. a. While chitosan treatment alone

reduced MDA at any level of irrigation, SA could diminish MDA concentration in moderate and severe drought stress. Furthermore, our results demonstrated that although combined treatment reduced MDA at any irrigation levels compared to the respective controls, it did not induce a further decrease in comparison with the single SA or chitosan treatments (Fig. IV. a).

Results showed that CAT and SOD antioxidant enzymes activities in *C. colocynthis* were significantly affected under drought stress and different treatment. Both CAT and SOD activities were increased under irrigation by 50% field capacity compared to the respective controls (Figs. IV. b and IV. c). While only chitosan could increase CAT activity under the first level of irrigation (without drought stress), both SA and chitosan treatments elevated CAT activity in all drought stress conditions (Fig. IV. b). However, there were no significant differences between SA and chitosan effectiveness in changing CAT activity under mild and moderated drought stress (irrigation levels 2 and 3) (P≤0.05). SA/chitosan combined treatment induced a more increase in CAT activity compared to each single treatment under the second level of irrigation. Although the maximum effect of combined treatment was observed at irrigation levels 2 and 3, no further increases in CAT activity was observed under these two conditions.

Results indicated that the effect of SA, chitosan, and the interaction between them was significant on SOD activity under different drought stress levels (Fig. IV. c). Although SA treatment alone could not increase SOD activity at any irrigation levels (P≤0.05), chitosan elevated SOD in mild and moderated drought stress. Here, combined treatment was effective in increasing SOD at the third level of irrigation; however, there was no significant difference between SOD activity under combined treatments in moderate and severe drought stress (P≤0.05).

Effects of SA, chitosan, and SA/chitosan combined treatments on cucurbitacin contents of C. colocynthis under drought stress

The data indicated that all levels of drought stress increased cucurbitacin-B content in control as

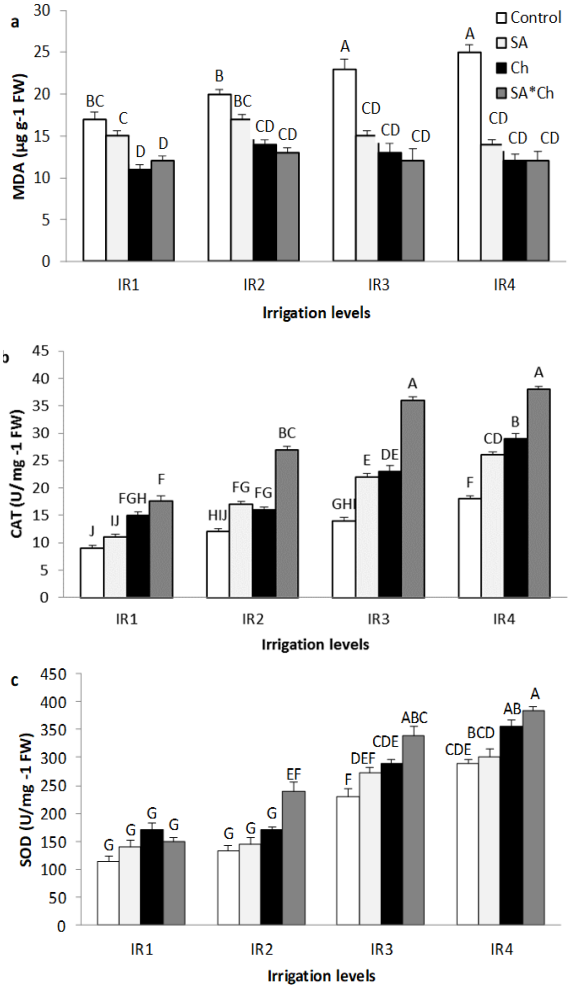

Fig. IV. Effect of drought and its combination with salicylic acid (SA) (150 mg L^{-1}), chitosan (Ch) (150 mg L^{-1}), and their interaction (SA* Ch) on a) malondialdehyde (MDA) content, b) catalase (CAT) activity and c) superoxide dismutase (SOD) activity in *Citrullus colocynthis*; data are given as means ± standard error, N = 3. IR1 (100% FC), IR2 (75% FC), IR3 (50% FC), and IR4 (25% FC). Bars with different letters denote significant differences at P≤0.05 based on a Dunkan test. Error bars indicate Standard Error (SE).

shown in Fig. V. a. Single treatments as well as combined treatment increased cucurbitacin-B content under moderate and severe drought stress as shown in Fig. V. a. There were no significant differences in its content between single and combined treatments.

Fig. V. b shows that the content of cucurbitacin-C increased in controls under moderate and severe levels of drought stress. Furthermore, both SA and chitosan treatment as well as combined treatment increased cucurbitacin-C content at any levels of drought stress as depicted in Fig. V. b.

The content of cucurbitacin-E did not change under any level of drought stress in controls as shown in Fig. V. c. Statistical analysis showed that neither SA nor chitosan only treatments changed the content of cucurbitacin-E at any level of irrigation as indicated in Fig. V. c. However, combined treatment could increase its content at severe drought stress as compared to the respective controls.

The changes in contents of cucurbitacin-L are demonstrated in Fig. V. d under different levels of irrigation. Although chitosan and combined treatment increased cucurbitacin-L content at any level of drought stress, SA increased its content at moderate and severe drought stress.

Discussion

Drought causes adverse effects on plant growth and physiological properties (Pirzad et al., 2011). Environmental stresses such as drought result in the generation of ROS in plants due to disruption of cellular homeostasis (Mishra et al., 2011; Srivastava and Dubey, 2011). Plants possess complex antioxidative defense system comprising of scavenging enzymes and metabolites to scavenge ROS (Sharma et al., 2012). Increased activity of antioxidative enzymes has been reported under drought stress in several plant species (Sayfzadeh and Rashidi, 2011; Sharma et al., 2012). Various chemicals such as growth regulators and stress signaling molecules are being successfully used to protect plants against oxidative stress (Farooq et al. 2010). SA is a hormone-like substance which plays an important role in plants' defense mechanism against biotic and abiotic stresses (Arfan et al., 2007). Chitosan is a cationic marine polysaccharide with unique bioactive properties, used to protect plants against oxidative stress (Guan et al., 2009). In this experiment, we demonstrated that chitosan and SA foliar spray mitigated the effects of drought stress by reducing the extent of lipid peroxidation and increasing the antioxidant enzymes activity (SOD and CAT), RWC, sugar, proline, and cucurbitacin contents.

Water stress resulted in significant decreases in vegetative growth, leaf area, and RWC. Furthermore, our results showed that exogenous

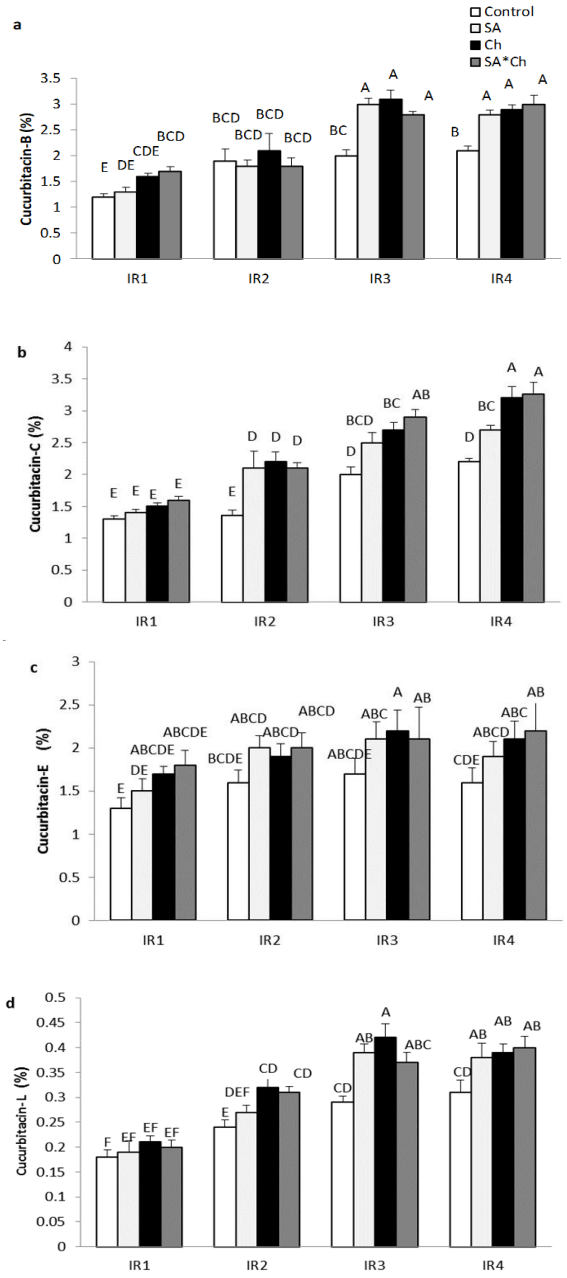

Fig. V. Effect of drought and its combination with salicylic acid (SA) (150 mg L^{-1}), chitosan (Ch) (150 mg L^{-1}), and their interaction (SA* Ch) on the contents of a) cucurbitacin-B, b) cucurbitacin-C, c) cucurbitacin-E and d) cucurbitacin-L in *Citrullus colocynthis*; data are given as means ± standard error, N = 3. IR1 (100% FC), IR2 (75% FC), IR3 (50% FC), and IR4 (25% FC). Bars with different letters denote significant differences at P≤0.05 based on a Dunkan test. Error bars indicate Standard Error (SE).

spraying of chitosan and SA significantly increased these parameters compared to the untreated plants under stress. These results are in accordance with previous findings showing that chitosan increases the vegetative growth indices

and RWC of sour orange seedlings under drought stress (Mohamed, 2018) . Chitosan may increase the uptake and transport of essential nutrients via adjusting cell osmotic pressure and thereby, improving plant growth and development (Malekpoor et al., 2016) . The significant effect of chitosan on plant growth may be attributed to an increase in the key enzyme activities of nitrogen metabolism and also photosynthesis which enhanced the plant growth (Górnik et al., 2008).

Alleviation of drought stress by SA through improved carbon assimilation and increased plant growth has been also reported (Kang et al., 2012; Sharma et al., 2017). Positive stimulating effects of SA foliar application on plant growth were discussed as it may regulate stomatal openings and reduce transpiration water loss under drought conditions, enabling the plants to maintain turgor, carry on photosynthesis, and become productive under water stress conditions (Rao et al., 2012). The increase in RWC may be related to the role of SA in accumulation of compatible osmolytes in plants subjected to drought stress (Kabiri and Naghizadeh, 2015).

Severe drought stress led to a significant decrease in the chlorophyll a, b, chlorophyll a/ b ratio, and carotenoid contents (Fig. II). The decrease in photosynthetic pigments under drought stress might be related to the reduced synthesis of the main chlorophyll pigment complexes, oxidative damage of chloroplast lipids, and proteins, destruction of the pigment protein complexes, inhibition of chlorophyll biosynthesis, and the increase of chlorophyllase activity (Lai et al., 2007). Reduction in chlorophyll a/b ratio observed in the severe drought stress is consistent with the previous reports (Shekari et al., 2016; Shobbar et al., 2012).

Our findings indicated that exogenous spraying of chitosan and SA successfully improved leaf chlorophyll and carotenoid contents in severe drought stress. Furthermore, SA and chitosan combination improved these parameters in all levels of drought stress. These results are in accordance with other findings (Fayez and Bazaid, 2014) showing that SA increases the contents of photosynthetic pigments in drought stress treated barley plants. There is also another work reporting that SA treatment increased chlorophyll and carotenoids contents in *Nigella sativa* plants under drought stress (Kabiri et al., 2014). Foliar application of chitosan could also enhance chlorophylls content in cucumber, radish, and cowpea (Farouk and Amany, 2012; Farouk et al., 2008; Farouk et al., 2011).

The results of our study demonstrated that by increasing drought stress, the amount of protein decreases but sugar and proline contents increase. Proline accumulation and decrease in protein content in plants under drought stress have been reported previously (Irigoyen et al., 1992). Here, we indicated that application of both chitosan and SA increased these traits and improved plant tolerance to drought stress. These findings are in accordance with the studies showing that proline and soluble sugars accumulate in plants for osmotic adjustment and protection of membrane integrity during drought stress (Juan et al., 2005; Vurayai et al., 2011).

Furthermore, It was reported that SA foliar spray alleviates harmful effects of drought stress by increasing sugar, protein, and proline content (Dianat et al., 2016). Chitosan could also increase protein and sugar content in common bean (Abu-Muriefah, 2013).

The level of MDA is often used as an indicator of the extent of damage occurring to plant in response to oxidative stress (Miller et al., 2010). In the current study, the drought stress treatment applied to *C. colocynthis* led to an induction of lipid peroxidation in leaves as indicated by the increases in MDA. Furthermore, chitosan and combination of SA and chitosan effectively decreased the lipid peroxidation in all levels of drought stress. In this regard, it has been reported previously that the drought stress induces lipid peroxidation (measured as MDA) in wheat (Fayez and Bazaid, 2014) and barley (Hameed et al. 2011). There is a report demonstrating that chitosan significantly decreased the production of MDA in the leaves of apple seedlings while increasing antioxidant enzyme activities (SOD, CAT) under drought stress condition (Yang et al., 2009). SA application could also decrease MDA content in *Satureja hortensis* under drought stress condition (Yazdanpanah et al., 2011).

SOD and CAT enzymes are the most important antioxidant enzymes in the scavenging system of ROS. SOD and CAT activities enhanced by chitosan and SA application in *C. colocynthis* leaves under drought stress (Fig. III). These results were consistent with a report that chitosan could promote SOD activity and decrease MDA content in *Hydrilla verticillata* (XU et al., 2007). The antioxidant properties of chitosan may be attributable to its abundant active hydroxyl and amino groups, which detoxify ROS by generating nontoxic macromolecular radicals (Xie et al., 2001). SA treatment has also been reported to enhance CAT and SOD activities in drought-stressed *Lycopersicon esculentum* plants (Hayat et al., 2008) and *Lippia citriodora* (Dianat et al., 2016).

Plant secondary metabolites can be changed by drought stress (Solinas et al., 1996). In the present study, cucurbitacin-C and L increased under moderate and severe levels of drought stress. Meantime, cucurbitacin-B content increased at all levels of drought stress. There are also previous studies showing that active compounds significantly increased in *Trachyspermum ammi* under drought stress (Azhar et al., 2011).

It is well established that elicitors such as chitosan induce defense mechanisms in plants including the accumulation of secondary metabolites under drought stress (Boonlertnirun et al., 2010). In this study, chitosan and combined treatment increased cucurbitacin-L and cucurbitacin-C contents at all levels of drought stress. It was reported that SA modified the bioactive compounds of *Salvia macrosiphon* (Rowshan et al., 2010) and *Amaranthus tricolor* L.(Khandaker et al., 2011). In our study SA increased cucurbitacin-B, C and L under drought stress.

Our findings indicated that there is no significant change in the content of cucurbitacin-E at any levels of irrigation. These findings are in accordance with this fact that the effect of drought stress on plant secondary metabolites is a species-specific phenomenon and depends on the severity of the stress (Azhar et al., 2011; Hendawy and Khalid, 2005).

Conclusion

Our results indicated that chitosan and SA may reduce the level of ROS and increase cell membrane stability under drought stress possibly through activation of antioxidant enzymes. Combination of SA and chitosan showed the major impact on improving physiological parameters and cucurbitacin contents of *C. colocynthis* and therefore, could be a potential candidate to protect the plant against adverse effects of drought stress.

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