Caryologia
International Journal of Cytology, Cytosystematics and Cytogenetics

Firenze University Press
www.fupress.com/caryologia

Citation: Basoz Sadiq Muhealdin, Sahar Hussein Hamarashid, Fairuz Ibrahim Ali, Nakhshin Omer Abdulla, Syamand Ahmad Qadir (2022). Studying some morphological responses of stevia (*Stevia rebaudiana* Bertoni) to some elicitors under water deficiency. *Caryologia* 75(4): 15-24. doi: 10.36253/caryologia-1922

Received: November 08, 2022

Accepted: December 14, 2022

Published: April 28, 2023

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Studying some morphological responses of stevia (*Stevia rebaudiana* Bertoni) to some elicitors under water deficiency

Basoz Sadiq Muhealdin[1], Sahar Hussein Hamarashid[1,*], Fairuz Ibrahim Ali[1], Nakhshin Omer Abdulla[1], Syamand Ahmad Qadir[2]

[1] *Agricultural Project Management Department, Technical College of Applied Science Halabja, Sulaimani Polytechnic University, Iraq*
[2] *Medical laboratory techniques department/Halabja Technical Institute, Research center/ Sulaimani Polytechnic University, Sulaymaniyah, Iraq*
*Corresponding author. E-mail: sahar.rashid@spu.edu.iq

Abstract. This research evaluated the effect of foliar spraying of different elicitors on modulating the effect of water stress on the stevia. The experimental design was the split-plot based on a randomized complete block design with three repetitions. Experimental treatments included different irrigation regimes (90% FC, 65% FC, and 40% FC) and foliar application of different elicitors (control, chitosan, salicylic acid, and melatonin). In this study, the content of chlorophyll A and b was reduced by intensifying the water deficit stress. Also, the highest content of the two pigments was allocated to the treatment of melatonin application. In the present study, melatonin foliar application under 90% FC irrigation conditions had the highest plant height, leaf area index, biomass, and carotenoid content, Moreover, the highest content of proline, phenol, DPPH, rebaudioside A, and steviosid was assigned to melatonin foliar application treatment under 40% FC irrigation conditions. Results revealed, although water stress reduced plant height, leaf area index, and plant biomass, the application of melatonin and salicylic acid under different irrigation conditions moderated the effect of water stress on these traits. Application of melatonin and salicylic acid under water deficit stress conditions also increased the content of proline, phenol, DPPH, rebaudioside, and steviosid.

Keywords: Drought stress, Enzyme activities, Foliar spraying, Pigment.

INTRODUCTION

About 230-220 species have been recorded for stevia, one of the most economically important species is S. rebaudian (Al Hassan *et al* 2017). The distinguishing feature of *S. rebaudiana (stevia*) from other species is the relatively high production of non-toxic and non-nutritive diterpenoid anticorn glycosides in the leaves of this species, the sweetness of which is 300 times of sucrose (Aghighi Shaverdi *et al.,* 2018). Consumers' desire for a natural zero-calorie sweetener has made the cultivation, production, and extraction of glycosides in this plant attractive (Aghighi Shaverdi *et al.,* 2018). Among the glycosides in stevia leaves and tissues, the most abundant glycosides are rebaudioside A (Reb-A) and steviosid (Arnon 1949)

Drought is known as one of the most destructive stresses that affect the growth, development, and reproduction of plants (Bi et al 2021). A typical effect of water deficit is to cause oxidative damage through the widespread accumulation of reactive oxygen species (ROS) (Duan *et al*., 2022). ROS overproduction leads to lipid peroxidation, enzyme inactivation, impaired protein structure / function, and nucleic acid damage (Duncan 1955). Plants, in turn, use enzymatic and non-enzymatic antioxidant systems to prevent the accumulation of ROS (Duncan 1955). Some stimulants of biological origin or eustress or with appropriate dosage and length, not only activate chemical defense in plants, but also increase plant productivity (Elizabeth Abreu and Munné-Bosch 2008). One of the purposes of plant physiologists is to identify and introduce substances that improve the resistance of plants to biotic and abiotic stresses. Salicylic acid is one of these substances. Salicylic acid treatment with appropriate dose has increased resistance to environmental stresses in different species. Regulation of tougher pressure and activation of the antioxidant system are the mechanisms of salicylic acid in improving water shortage stress resistance (Eraslan *et al*., 2007). Another substance that plays a role in modulating environmental stresses is chitosan. It is a natural polymer derived from chitin, which is used as a biological elicitor in agriculture (EFSA 2010).

Chitosan treatment enhances photosynthesis and stomatal closure by ABA synthesis (Guo et al., 2022; 2021). This substance increases antioxidant enzymes through the H_2O_2 and NO signaling pathways and induces the produces sugars, amino acids, organic acids, and other metabolites needed to signal stress, osmotic balance, and energy metabolism under unfavorable environmental conditions (Gao et al., 2016).

Another compound that increases plant resistance to biological and non-biological stresses is melatonin (N-acetyl-5-methoxytryptamine) (Janda et al., 1999; Meng et al., 2014; Ma et al., 2018; Porra et al., 1989; Peng et al., 2021; Su et al., 2019; Sun et al., 2021). A wide range of physiological reactions has been attributed to melatonin, which can moderate the adverse effects of environmental stresses such as drought and salinity on plants. Melatonin has an antioxidant activity and can detoxify ROS in cells (Karimi et al., 2015). Melatonin can increase photosynthetic capacity by increasing the absorption of water and nutrients and increasing the expression of genes associated with mitogen-activated protein kinases (LI et al., 2021). It has been reported that the use of melatonin under drought stress conditions prevents the accumulation of abscisic acid in the cell and has a synergistic effect with cytokinin (Lehmann et al., 2010; Liang et al., 2019; Mahajanab et al., 2021). Therefore, in the present study, we investigate the role of chitosan, salicylic acid, and melatonin on the physiological and biochemical responses of stevia under water deficit stress conditions.

MATERIALS AND METHODS

This experiment was carried out in the two cropping years of 2019 and 2020 as a split-plot based on a randomized complete block design with three replications in a research farm in the city of Sulaymaniyah located in the north of Iraq. The experimental area was located at 45°11' N; 45°58' E longitude and altitude, respectively, 690 m above sea level. The average annual rainfall in the experimental area was about 250 mm and the average maximum and minimum temperatures were 22 and 9 degrees, respectively. The soil properties of the test site are listed in Table 1.

Experimental treatments included different irrigation regimes (90% FC, 65% FC, and 40% FC) and foliar application of different elicitor (control, chitosan (2 gL^{-1}), salicylic acid (100 mg L^{-1}), and melatonin (100 μM), which were assigned to main and sub-plots, respectively.

Table 1. Soil physical and chemical properties of the experimental site.

Physical Properties	Value	Unit	Chemical properties	Value	Unit
sand	133.6	g kg^{-1}	Organic mater	20	g Kg^{-1}
silt	244.3	g kg^{-1}	pH	7.57	
clay	622.1	g kg^{-1}	ECe	1.4	dS m^{-1}
soil texture	Clay		Total nitrogen	20	mg Kg^{-1}
Bulk density	1200	Kg m^{-3}	Phosphors (soluble)	19	mg Kg^{-1}
Field capacity (33 k Pa)	320	g kg^{-1}	Potassium (soluble)	13.7	Meq Kg^{-1}
Wilting point (1500 k Pa)	188	g kg^{-1}	Calcium (soluble)	7.5	Meq Kg^{-1}

The seedlings obtained by tissue culture were initially cultivated in peat moss medium to select the well-established plantlets. After three weeks, the uniform seedlings were trans-planted into soil in May each year.

In this experiment, the dimensions of each plot were considered 2×2.5 m. Each plot also included four rows of crops. In this study, the distance between the rows was 50 and the distance between the plants was 30 cm.

The first two weeks the soil moisture was maintained within the range of field capacity and, then, the irrigation treatments were applied as 90, 65, and 40% of field capacity (FC). The soil moisture content was measured using the gravimetric method. Irrigations for each plat were conducted to replenish 100% of soil field capacity.

Chitosan, salicylic acid, and melatonin were sprayed three times after 30, 40, and 50 days from transplanting in both years.

Harvesting was done 62 days after transplanting the seedlings to the field. In the first stage, the leaves and stems were separated and then weighed.

Evaluating morphological characteristics

At the time of harvest, the plants in each plot were carefully removed from the soil and the roots of each sample were washed to remove soil residues. Stem length, leaf area index, and biological yield of each sample were measured.

Leaf area index was calculated according to Formula (1), in which LA leaf area and LG land area were occupied LAI = LA/LG photosynthetic pigments
80% of acetone extract with adsorption readings at 663, 646, and 440 nm was used to measure the concentration of photosynthetic pigments according to Arnon's (18) method. In addition to quantify the measurements, Porra et al. (19) method was used
Chlorophyll a = 12.25×A663−2.55×A646
Chlorophyll b = 20.31×A646−4.91×A663
Carotenoids = 4.69×A440−0.267× (A663×A646)
Pigment content was expressed as mg/gDW.

Proline content

To determined the proline content. 50 mg of leaf dry matter was homogenized in 5 ml of ethanol: Water mixture (60:40) and the homogenized solution was incubated for 24 h at +4 °C. After centrifugation at 10,000 rpm, the supernatant was collected. In the next step, 100 mL of ninhydrin acid 1% was mixed with 500 µL of supernatant of the extracted solution. The samples (reaction mixture) were placed in a water bath at 95 °C for 20 min and, then, cooled to the room temperature. After cooling, the reaction absorbance was measured at 520 nm and using proline as standard, the proline content of the samples was measured. The proline content of the samples was expressed based on mg proline / g fresh weight Zheng et al. (2018).

Secondary metabolite analysis

To extract phenol, plant samples were dried and, then, macerated in 80% methanol ((HPLC grade). In the next step, the samples were incubated for 24 h at 4 °C.

The incubated samples were centrifuged at 2000 rpm for 15 min and the supernatants of the extracts were collected for further analysis. Finally, Wolf et al.'s (20) method was used to analyze the phenol content. In this method, folin-ciocalteu reagent and gallic acid were used as the standard. The total antioxidant capacity was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay according to Karimi et al. (21).

Analysis of steviol glycosides

To estimate the glycoside content of the leaves, at the time of harvest, the leaves in each plot were randomly selected and dried at 40 °C in an air oven. Then, 100 g of the powdered leaf sample was placed in a 25 ml flask containing 10 ml of methanol and filtered for 24 hours. The reduced pressure filter was vacuum dried and, then, defatted with n-hexane. Afterwards, the moving phase was used to solve the samples (HPLC grade-acetonitrile: water in the ratio 1:1). Filters with pores of 0.22 µm were used to re-filter the samples. Filtered samples were used to determine the content of stevoside, Reb-A by liquid chromatography-mass spectrometry (LCMS-Shimadzu, 2020 system) (22). Standard curves with the samples were used to quantify glycoside content; these curves are used for standard stevoside and Reb-A samples (22).

Statistical analysis

Analysis of variance was performed using SAS software, also the comparison of the mean of treatments with the Duncan (1955) method was performed at a level of 5% probability.

RESULTS

Results showed the effect of irrigation regimes on all the traits was significant at the level of 1% probability.

A significant difference was detected among the different treatments in terms of the effect on plant height, leaf area index, biological yield, proline, chlorophyll a, chlorophyll b, phenol, rebaudioside A, and steviosid at the probability level of 1% and in terms of carotenoid content at the level of 5% probability. There was a significant interaction effect of irrigation and allicator treatments on plant height, biological yield, proline, phenol content, DPPH, rebaudioside A, and steviosid at 1% probability and on leaf area index and carotenoid content at 5% probability level was observed (Table 2).

Plant Height

In this study, the highest plant height with the average of 37.17 cm was allocated to normal irrigation conditions (90% FC) and the foliar application of salicylic acid. With the intensification of water stress, the plant height significantly decreased; but, in treatment of the 65% FC, application of salicylic acid could significantly increase plant height compared to the control treatment. There was no significant difference between the control treatment and other treatments using growth stimulants (Table 4). The results also showed that under irrigation conditions of the 40% FC, there was no considerable difference among the control treatment and the application of elicitors. Results revealed, the lowest plant height was allocated to irrigation treatment of the 40% FC in all the four growth elicitor treatments (Table 4).

Leaf Area Index

The results of mean comparisons showed that melatonin application under normal irrigation conditions (90% FC) had the highest leaf area index, while the control and chitosan treatment in the treatment of 40% FC showed the lowest amount. In this study, although in the irrigation treatment of 65% FC, there was no considerable difference between the control treatment and the other elicitor treatments, in the irrigation treatment of 40% FC, the use of salicylic acid increased the leaf area index significantly compared with the control and other treatments (Table 4).

Biological Yield

Among the interactions of irrigation and plant growth stimulant treatments, the use of melatonin in 90% FC treatment with the average of 49.34 g/plant showed the maximum biological yield. The lowest biological yield with the average of 12.14 and 10.97 g/plant was allocated to the control treatment and application of chitosan under irrigation conditions of 40% FC. Results showed that although biological yield decreased with exacerbation of water deficit, the melatonin spraying was able to improve this trait in the treatment of the 65% FC by 45.61% compared with the control treatment, In addition there was no remarkable difference among control treatment and elicitors application treatments in the irrigation treatment of the 40% FC (Table 4).

Table 2. Combine analysis of variance of irrigation and spraying elicitors on studied traits in stevia (*Stevia rebaudiana Bertoni*).

SOV	Df	Mean square										
		Plant Height	LAI	Biomass	Proline	Chlorophyll a	Chlorophyll b	Carotenoid	Phenol	DPPH	Rebaudioside A	Steviosid
Year (Y)	1	39.01^{ns}	1.25^{ns}	0.60^{ns}	0.0004^{ns}	0.0004^{ns}	0.008ns	0.00021^{ns}	0.027^{ns}	0.056^{ns}	0.50^{ns}	2.72^{ns}
Year (Replication)	4	13.08	0.85	0.21	0.0005	0.0002	0.004	0.0006	0.018	0.021	0.64	1.24
Irrigation levels (I)	2	1606.57**	305.92**	4182.82**	0.0029**	0.0057**	28.32**	0.0031**	0.065**	42.81**	27.73**	98.38**
Y× I	2	7.76^{ns}	17.36^{ns}	10.06^{ns}	0.0001^{ns}	0.0004^{ns}	0.0024ns	0.00007^{ns}	0.017^{ns}	0.18^{ns}	5.50^{ns}	4.05^{ns}
Error 1	8	13.56	5.88	60.04	0.0006	0.0007	4.33	0.000012	0.011	3.71	5.91	11.73
Elicitors (E)	3	37.29**	31.62**	289.38**	0.0075**	0.0147**	29.94**	0.0013*	0.040**	1.95^{ns}	13.47**	216.91**
Y×E	3	19.94^{ns}	0.45^{ns}	0.06^{ns}	0.0074^{ns}	0.0004^{ns}	0.052^{ns}	0.000025^{ns}	0.012^{ns}	0.092^{ns}	0.47^{ns}	7.16^{ns}
I × E	6	41.33**	8.88*	189.68**	0.0011**	0.0005^{ns}	5.54^{ns}	0.00040*	0.332**	3.98**	8.39**	46.95*
Y×E×I	6	10.36^{ns}	1.54^{ns}	11.16^{ns}	0.0004^{ns}	0.0004^{ns}	0.0011^{ns}	0.000032^{ns}	0.031**	0.05^{ns}	0.46^{ns}	6.27^{ns}
E2	36	11.12	3.41	48.21	0.0003	0.0006	2.12	0.00013	0.011	1.05	0.98	14.34
CV%	-	12.56	14.20	24.78	15.16	19.01	16.96	20.57	10.52	9.67	13.51	15.55

ns, *, and ** were significant at levels 1 and 5% respectively.

Table 3. Mean comparison of main effects of irrigation and spraying elicitors treatments of on studied traits in Stevia (*Stevia rebaudiana Bertoni*).

Irrigation	Plant Height (cm)	LAI	Biomass (g plant-1)	Proline (Mg gFW-1)	Chlorophyll a (Mg gFW-1)	Chlorophyll b (Mg gFW-1)	Carotenoid (Mg gFW-1)	phenol mgGAE gDW-1	DPPH (%)	Rebaudioside A (%)	Steviosid (%)
90% FC	34.50a	16.71a	41.13a	0.108b	0.149a	0.096a	0.069a	0.961b	9.36c	6.48b	22.34b
65% FC	26.99b	12.75b	28.19b	0.120a	0.133b	0.086b	0.052b	1.026a	10.45b	7.03b	24.36ab
40% FC	18.16c	9.58c	14.73c	0.130a	0.118c	0.074c	0.047b	1.067a	12.00a	8.55a	26.39a
Elicitors											
Control	24.41b	11.49c	23.08b	0.148a	0.094c	0.068c	0.046c	0.822d	10.49a	6.63b	21.63b
Chitosan	27.42a	12.64bc	27.56ab	0.119b	0.141b	0.087b	0.056b	1.174a	10.52a	6.43b	21.09b
Salicylic acid	26.99ab	14.68a	28.57ab	0.108bc	0.136b	0.089ab	0.055b	0.993c	10.31a	8.11a	27.33a
Melatonin	27.38ab	13.23ab	32.85a	0.102c	0.163a	0.098a	0.067a	1.087b	11.07a	8.24a	27.39a

Data in columns followed by different letters are significantly different ($p \leq 0.01$) by Duncan's multiple range test.

Table 4. Mean comparison of irrigation and spraying elicitors interaction treatments of on studied traits in Stevia (*Stevia rebaudiana Bertoni*).

Irrigation	Elicitors	Plant Height (cm)	LAI	Biomass (g plant-1)	Proline (Mg gFW-1)	Carotenoid (Mg gFW-1)	Chlorophyll a (Mg gFW-1)	Chlorophyll b (Mg gFW-1)	Phenol mgGAE gDW-1	DPPH (%)	Rebaudioside A (%)	Steviosid (%)
90% FC	Control	30.35bcd	14.73bcd	32.88bcd	0.0937d	0.062bc	0.109a	0.081a	1.1197bc	8.11f	5.887ghi	18.76f
	Chitosan	33.95abc	15.94abc	38.84abc	0.0957cd	0.058b-e	0.147a	0.091a	1.1017bc	9.29ef	6.65f-h	20.81ef
	Salicylic acid	37.17a	17.65ab	43.45ab	0.1207bcd	0.07b	0.164a	0.102a	0.6317d	10.03de	5.302i	26.65bc
	Melatonin	36.53ab	18.51a	49.34a	0.123bcd	0.087a	0.176a	0.068a	0.674d	9.94de	8.088cd	23.15c-f
65% FC	Control	23.08ef	11.36def	20.61def	0.1033cd	0.047de	0.086a	0.111a	0.6747d	11.12bcd	6.328f-i	22.49c-f
	Chitosan	26.57de	13.69cd	28.26cde	0.1237bcd	0.056cde	0.149a	0.082a	1.1567ab	10.37de	5.649h-i	20.40ef
	Salicylic acid	30.02cd	14.03bcd	26.82cde	0.1063cd	0.045e	0.134a	0.089a	1.1683ab	9.92de	8.883bc	29.10b
	Melatonin	28.89cde	11.9def	37.07abc	0.1498ab	0.059bcd	0.164a	0.105a	1.108bc	10.4de	7.277def	25.43bcd
40% FC	Control	19.8f	8.39f	12.14f	0.1113cd	0.031f	0.088a	0.055a	1.0033c	12.24ab	7.7de	23.66cde
	Chitosan	18.9f	8.3f	10.97f	0.1057cd	0.055cde	0.126a	0.094a	1.2647a	11.91abc	7.018d-g	22.06def
	Salicylic acid	17.22f	12.36cde	20.05def	0.1313bc	0.048cde	0.11a	0.07a	1.18ab	10.98cd	10.15a	26.25bcd
	Melatonin	16.71f	9.28ef	15.75ef	0.1733a	0.055cde	0.148a	0.079a	1.151ab	12.88a	9.362ab	33.60a

Data in columns followed by different letters are significantly different ($p \leq 0.01$) by Duncan's multiple range test.

Proline

The results revealed that the leaf proline content increased with exacerbation of drought, so that the irrigation conditions of the 40% FC with spraying of melatonin with the average of 0.173 (Mg/gFW) had the maximum proline content. Results demonstrated that spraying of melatonin in both 65% FC and 40% FC conditions was able to improve the proline content compared with the control by 41.62 and 55.85%, respectively. The lowest proline content was assigned to 90% FC treatment with all four eliminator treatments (Table 4).

Photosynthetic Pigments

The results showed that irrigation conditions of 40% FC and 65% FC reduced the chlorophyll content by 10.85 and 20.91 percent, respectively, compared to 90% FC irrigation conditions (Table 3).

In the present study, the use of melatonin with the average of 0.163 (Mg/gFW) had the highest leaf chlorophyll a content and increased the amount of this trait compared to the use of chitosan, salicylic acid, and control by 15.60, 15.60, and 72.30 percent, respectively. In this study, the control treatment with the average of 0.094 (Mg/gFW) had the lowest chlorophyll a content (Table 3).

Chlorophyll b content decreased due to water shortage so that the supply of the 90% FC and 40% FC had the maximum and minimum chlorophyll b contents, respectively (Table 3).

Results revealed that melatonin application had the highest chlorophyll b content. The difference between melatonin and salicylic acid was not significant. The lowest chlorophyll content was recorded for control treatment of foliar application (Table 3).

In our experiment spraying of melatonin in irrigation treatments of 90% FC improved carotenoid content compared with the control treatment by 40.32 percent and had the maximum carotenoid content (Table 4). In our study, although in irrigation treatment of 60% FC, there was no notable difference among the control treatment and elicitors, in the irrigation treatment of 40% FC, foliar spraying of chitosan, salicylic acid, and melatonin increased the carotenoid content by 77.41, 54.88, and 77.10 percent compared to the control.

Phenol content

Based on the results, with the intensification of water deficit stress, the phenol content was increased, so that the foliar spraying of chitosan and salicylic acid in irrigation treatment of 60% FC and the use of chitosan, salicylic acid, and melatonin in irrigation treatment of 40% FC had the highest phenol content and enhanced the amount of this trait remarkable compared with the control. The lowest phenol content was allocated to irrigation treatment of 90% FC and the foliar spraying of salicylic acid and melatonin (Table 4).

DPPH

In our study water stress rose the amount of DPPH, so that the treatment of melatonin, chitosan, and control under irrigation treatment of 40% FC had the highest amount of DPPH activity. The minimum DPPH content was recorded in the control treatment under 90% FC irrigation conditions (Table 4). In our experiment, water shortage and foliar application of elicitors had a synergistic effect on DPPH activity.

Rebaudioside A

Results revealed that water deficit stress and foliar spraying of salicylic acid and melatonin had a synergistic effect on rebaudioside A content. Irrigation treatment with 40% FC with foliar spraying of salicylic acid and melatonin had the highest amount of glycoside rebaudioside A. It should be noted that the application of these two treatments in the irrigation regime of 65% FC significantly increased the amount of rebaudioside A in comparison to the control and foliar spraying chitosan treatments (Table 4). The lowest amount of rebaudioside A was recorded under normal irrigation (90% FC) and salicylic acid application.

Steviosid

In our study irrigation regime of 40% FC along with melatonin foliar spraying had the highest steviosid glycoside content, Furthermore foliar spraying of salicylic acid under 90% FC irrigation conditions, foliar spraying of salicylic acid and melatonin under 65% FC irrigation conditions, and application of melatonin under 40% FC irrigation conditions could significantly improved steviosid glycoside content compared with control and other treatments (Table 4).

DISCUSSION

Our research findings showed water deficit diminished plant height, but spraying of salicylic acid, especially in irrigation treatment of the 65% FC, was able to moderate the negative effect of water deficit on plant height. Under drought stress, cell elongation and cell differentiation are reduced due to decreased total water potential inside the plant (Xu et al., 2021). It seems that the application of salicylic acid can mitigate the adverse effect of drought stress on plant growth by preventing a reduction in cell divisions and cell size (Yan et al., 2018). In the study by Karimi et al. (Tardieu et al., 2000) on stevia, drought stress decreased plant height, while the use of external SVglys could not affect this trait.

In this study, foliar application of melatonin under normal irrigation conditions produced the maximum leaf area index. leaf area index decreased with the intensification of water shortage. Results revealed the use of salicylic acid in the irrigation treatment of 40% FC, increased the leaf area index significantly compared to the control and other treatments. Probability salicylic acid can improve nutrient uptake, especially under stress, which in turn can increase growth (25). It seems that salicylic acid can increase photosynthesis and, thus, increase growth by increasing the amount of chlorophyll in the leaves that are at the beginning of the aging process (26). Karimi et al. (21) showed that drought stress decreased leaf growth in stevia, but the external application of SVglys reduced the leaf losses.

In our research, the use of melatonin under normal irrigation had the highest biological yield, This trait was reduced by reducing the available water of the plant. The results also revealed that foliar application of melatonin under water shortage conditions increased biological yield compared to the non-foliar spraying treatment. Increased biological yield in the present study can be due to the positive effect of foliar application on leaf area index and photosynthetic pigments under different irrigation, leaf area development and photosynthetic pigments increased the rate of photosynthesis and accelerate plant vegetative growth. One of the adverse effects of drought stress is accelerating the production of reactive oxygen species (ROS) (Ucar et al., 2016), which leads to cell damage, reduced growth and biomass production, and ultimately cell death.

The result showed that under irrigation conditions of the 40% FC with the use of melatonin had the highest proline content. Results also revealed that the use of melatonin in both 65% FC and 40% FC conditions was able to raise the proline content compared to the control treatment. Proline acts as an osmolytic / sprotactant factor under water deficit stress (Zheng et al., 2018). The results of our study showed a significant increase in proline content under water deficit stress treatments. It has been reported that the use of melatonin under water deficit conditions induces drought resistance in plants by increasing proline biosynthesis (Zhang et al., 2015). Consistent with the results of the present study, Liang et al. (Zhao et al., 2017) in *Actinidia cinnis*, Campos et al. (2019) in *Brassica napus*, and Li et al. (2019) in *Cophea Arabica* have found that melatonin application significantly increases leaf proline content in these plants. In the study by Ghanbari et al., the use of chitosan and salicylic acid in milk thistle (*silybum marianum* L.) remarkably raised the proline content under drought stress.

Water deficit causes the ROS accumulation, resulting in the degradation of the molecular structure of chlorophyll, and finally declining plant photosynthesis. Under moisture shortage conditions, a decrease in Chl content was considered a typical sign of oxidative stress. The decrease in Chl content under drought stress is mainly due to degradation of Chl as the result of ROS activity (Zhang, et al ,2022). Reduction of chlorophyll content due to water deficiency, in chickpea, maize , and basil , has been documented in previous investigations. In the present study, the use of melatonin had the highest leaf chlorophyll a and b content. Similar to other photosynthetic pigments, water deficit decreases carotenoid content, but the use of all three elicitors increased carotenoid compared with non-foliar spraying treatment (control) under irrigation treatment of 40% FC. Under water deficit conditions stress, the use of elicitors can prevent the degradation of pigments molecules. Decreased pigments molecule degradation after melatonin treatment may be due to decreased down-regulation of genes of chlorophyll-degrading enzymes such as Chlase, PPH, and Chl-PRX.

The results showed that induction of water deficit stress and foliar application of elicitors increased leaf phenol content so that the use of chitosan and salicylic acid under irrigation treatment of 60% FC and the use of chitosan, salicylic acid, and melatonin in irrigation treatment of 40% FC showed the highest amount of phenol content. As mentioned, drought stress increases the accumulation of ROS in cells that can damage cellular structures. The use of elicitors can increase the activity of antioxidant enzymes to strengthen the plant's defense system and prevent accumulation of ROS in cells. Increased phenol content in response to melatonin treatment under drought stress had been reported in the study by Sharma et al. (2017) on Grafted (*Carya cathayensis*). In the study by Karimi et al. (2017), the highest values of antioxidant capacity in stevia were allocated to water deficit conditions and plants treated with SVglys, while the lowest values were reported under normal irrigation conditions and no use of SVglys. Application of salicylic acid by regulating the activity of antioxidant enzymes can improve plant tolerance to adverse conditions (Li, *et al*, 2021; Sun, *et al.* 2021; Xu, *et al*, 2021; Zhang, *et al.* 2022).

The results showed the spraying of melatonin and chitosan under irrigation treatment of 40% FC showed the maximum amount of DPPH activity. Consistent with our results, the use of melatonin had a protective role against water shortage in corn and oats . In the study by Ahmed et al. (42), water deficit increased rebaudioside A and stevioside content in steva. compatible solutes such as soluble sugars under melatonin treatment increase, These substances are responsible for maintaining the turgor and osmotic pressure of plant cells under water deficit conditions. In the study by Jalal et al. (2018), salicylic acid treatment increased sugar content on plant in both drought stress and control treatments. Salicylic acid increases plant resistance to drought stress by stimulating sugar production in the cell. Karimi et al. (2019) showed that drought stress increased rebaudioside A content in stevia (Bi, *et al.,* 2021; Duan, *et al.,* 2022; Guo, *et al*, 2021; Guo, *et al,* 2022).

The results showed that application of salicylic acid under 90% and 65% FC irrigation conditions and application of melatonin under 40% FC irrigation had a positive effect on increasing steviosid glycoside content, therefore, the content of this substance increased in all irrigation conditions in response to different elicitors. In a study on milk thistle (*silybum marianum* L.), the highest soluble sugars was reported in the application of chitosan and salicylic acid under water stress conditions. The positive effect of melatonin on increasing the amount of soluble sugars has also been reported in the studies by Liang et al. (2011), Campos et al. (2012), and Li et al. (2018).

CONCLUSION

To summarize, the findings of this research revealed that water deficit stress markedly decreased plant growth and increased the proline, phenol, DPPH, rebaudioside, and steviosid contents. However, exogenous application of salicylic acid and melatonin enhanced tolerance of the *stevia* to water deficit stress by increasing the growth, anti-oxidative enzyme activities, chlorophyll (Chlo) content, and synthesis of rebaudioside and steviosid. Therefore, salicylic acid and melatonin mitigated the adverse effect of drought stress in stevia and their spraying on the leaves was beneficial for plant recovery and growth under water deficit stress.

REFERENCES

Aghighi Shaverdi M, Omidi H, Tabatabaei S. 2018 Morpho-physiological response of stevia (*stevia rebaudiana* bertoni) to salinity under hydroponic culture condition (a case study in Iran). Appl Ecol Environ Res. 16(1): 17-28. DOI:10.15666/aeer/…

Al Hassan M, Chaura J, Donat-Torres M P, Boscaiu M, Vicente O. 2017. Antioxidant responses under salinity and drought in three closely related wild monocots with different ecological optima. AoB Plants. 9(2): plx009. https://doi.org/10.1093/aobpla/plx009

Arnon DI. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiol . 24: 1–15.

Bi, D., C. Dan, M. Khayatnezhad, Z. Sayyah Hashjin, Z. Y. Ma 2021. Molecular Identification And Genetic Diversity In Hypericum L.: A High Value Medicinal Plant Using Rapd Markers Markers. Genetika *53*(1): 393-405.

Chen, Weimiao; Khayatnezhad, Majid; Sarhadi, Nima 2021. Protok gena i struktura populacije kod allochrusa (Caryophylloideae, Caryophyllaceae) pomocu molekularnih markera. Genetika 53(2): 799-812.

Duan, F., Fei Song, Sainan Chen, Majid Khayatnezhad, Noradin Ghadimi, 2022. Model parameters identification of the PEMFCs using an improved design of Crow Search Algorithm. International Journal of Hydrogen Energy, *47*(79): 33839-33849.

Duncan, D. B.1955. Multiple range and multiple 'F' tests. Biometrics., 11(1): 1-42. https://doi.org/10.2307/300147

Elizabeth Abreu M, Munné-Bosch S. 2008. Salicylic acid may be involved in the regulation of droughtinduced leaf senescence in perennials: A case study in field-grown Salvia officinalis L. plants. Environ Exp Bot, 64:105–112. https://doi.org/10.1016/j.envexpbot.2007.12.016

Eraslan F, Inal A, Gunes A, Alpaslan M. Impact of exogenous salicylic acid on growth, antioxidant activity and physiology of carrot plants subjected to combined salinity and boron toxicity. Scientia Horticulturae, 2007; 113: 120–128.

EFSA (European Food Safety Authority). , 2010. Panel on food additives and nutrient sources (ans): scientific opinion on safety of steviol glycosides for the proposed uses as a food additive. EFSA J. 8 (4): 1537

Guo, H., Wei Gu, Majid Khayatnezhad, Noradin Ghadimi, (2022): Parameter extraction of the SOFC mathematical model based on fractional order version of dragonfly algorithm. International Journal of Hydrogen Energy, **47**(57):24059-24068.

Guo, L.N., She, C., Kong, D.B., Yan, S.L., Xu, Y.P., Khayatnezhad, M. and Gholinia, F. 2021. Prediction of the effects of climate change on hydroelectric generation, electricity demand, and emissions of greenhouse gases under climatic scenarios and optimized ANN model. Energy Reports **7:** 5431-5445.

GaoW, Zhang Y, Feng Z, Bai Q, He J, Wang Y. 2016. Effects of melatonin on antioxidant capacity in naked oat seedlings under drought stress. Molecules, 23:1580. DOI: 10.3390/molecules23071580

Janda T, Szalai G, Tari I, Paldi E. 1999. Hydroponic treatment with salicylic acid decreases the effect of chilling injury in maize (*Zea mays* L.) plants. Planta. 208:175-180.

Jia, Y., M. Khayatnezhad, s. mehri (2020). Population differentiation and gene flow in Rrodium cicutarium: A potential medicinal plant. Genetika 52(3): 1127-1144.

Karimi M, Ahmadi A, Hashemi J, Abbasi A, Tavarini S, Pompeiano A, Guglielminetti L, Angelini L G. 2015. The positive role of steviol glycosides in stevia (*Stevia rebaudiana* Bertoni) under drought stress condition. Plant Biosystems. 150 (6): 1323-1331 . doi.org/10.1080/11263504.2015.1056857.

LI, ANG; MU, Xinyuan; Zhao, Xia; Xu, Jiamin; Khayatnezhad, Majid; Lalehzari, Reza; (2021):Developing the non-dimensional framework for water distribution formulation to evaluate sprinkler irrigation; Irrigation and Drainage, **70:** 659-667

Lehmann S, Funck D, Szabados L, Rentsch D. 2010. Proline metabolism and transport in plant development. J Amino Acids. 39(4): 949-962. DOI: 10.1007/s00726-010-0525-3

Liang D, Ni Z, Xia H, Xie Y, Lv X, Wang J, Lin L, Deng Q, Luo X. 2019. Exogenous melatonin promotes biomass accumulation and photosynthesis of kiwifruit seedlings under drought stress. Sci. Hortic. 246: 34–43. doi.org/10.1016/j.scienta.2018.10.058

Liu, S., Wang, Y., Song, Y., Khayatnezhad, M., & Minaeifar, A. A. 2021. Genetic variations and interspecific relationships in Salvia (Lamiaceae) using SCoT molecular markers. Caryologia, 74(3), 77-89.

Mahajanab M, Kumar B, Probir Kumar T. . 2021. Moisture stress and nitrogen availability modulate the secondary metabolite profiles, enzymatic activity, and physiological and agronomic traits of Stevia rebaudiana. Plant Physiol Biochem 162: 56-68. doi.org/10.1016/j.plaphy.2021.02.018

Meng JF, Xu TF, Wang Z Z, Fang Y L, Xi ZM, Zhang ZW. 2014. The ameliorative efects of exogenous melatonin on grape cuttings under water-deficient stress: Antioxidant metabolites, leaf anatomy, and chloroplast morphology. J. Pineal Res. 57: 200–212. DOI: 10.1111/jpi.12159

Ma, S., M. Khayatnezhad, A. A. Minaeifar. 2021. Genetic diversity and relationships among Hypericum L. species by ISSR Markers: A high value medicinal plant from Northern of Iran. Caryologia, 74(1): 97-107.

Mehri, H. Shirafkanajirlou, I. Kolbadi 2020. Genetic diversity, population structure and chromosome numbers in medicinal plant species *Stellaria media* L. VILL.. *Caryologia* 73(1): 57-65. doi: 10.13128/caryologia-680

Ma X, Zhang J, Burgess P, Rossi S, Huang B. 2018. Interactive efects of melatonin and cytokinin on alleviating drought-induced leaf senescence in creeping bentgrass (*Agrostis stolonifera*). Environ. Exp. Bot. 145: 1–11.

Porra RJ, Thompson WA, Kriedemann PE. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectrometry. Biochim Biophys Acta. 975: 384–394. doi.org/10.1016/S0005-2728(89)80347-0

Peng, X., Khayatnezhad, M. and Ghezeljehmeidan, L. 2021: Rapd profiling in detecting genetic variation in stellaria l. (caryophyllaceae). Genetika-Belgrade *53:* 349-362.

Shi, B., Khayatnezhad, M., Shakoor, A. 2021. The interacting effects of genetic variation in Geranium subg. Geranium (Geraniaceae) using scot molecular markers. Caryologia, 74(3), 141-150.

Su X, Fan X, Shao R. Guo J. Wang Y, Yang J, Yang Q, Guo L. 2019. Physiological and iTRAQ-based proteomic analyses reveal that melatonin alleviates oxidative damage in maize leaves exposed to drought stress. Plant Physiol Biochem. 142:263–274. DOI: 10.1016/j.plaphy.2019.07.012

Sun, Q., Deli Lin, Majid K., Mohammad T., 2021. Investigation of phosphoric acid fuel cell, linear Fresnel solar reflector and Organic Rankine Cycle polygeneration energy system in different climatic conditions. Process Safety and Environmental Protection, **147**:993-1008.

Xu, Y.-P., Ping Ouyang, Si-Ming Xing, Lu-Yu Qi, Majid khayatnezhad, Hasan Jafari,2021. Optimal structure design of a PV/FC HRES using amended Water Strider Algorithm. Energy Reports, **7:** 2057-2067.

Tardieu F, Reymond M, Hamard P, Granier C, Muller B. 2000. Spatial distributions of expansion rate, cell division rate and cell size in maize leaves: a synthesis of the effects of soil water status, evaporative demand and temperature. J. Exp. Bot. 51: 1505–1514.

Wang, J., Ye, Q., Zhang, T., Shi, X., Khayatnezhad, M., Shakoor, A. 2021. Palynological analysis of genus Geranium (Geraniaceae) and its systematic implications using scanning electron microscopy. Caryologia, 74(3), 31-43.

Yan Y, Pan C, Du Y, Li D, Liu W. 2018. Exogenous salicylic acid regulates reactive oxygen species metabolism and ascorbate–glutathione cycle in *Nitraria tangutorum* Bobr. under salinity stress. Physiol Mol Biol Plants. 24(4):577–589. DOI: 10.1007/s12298-018-0540-5

Ucar E, Ozyigit Y, Turgut K. 2016.The Effects of Light and Temperature on Germination of Stevia (*Stevia rebaudiana* BERT.) Seeds. Türkiye tarım araşt. Derg. 3(1): 37-40. DOI:10.19159/tutad.76528.

Zheng J, Ma X, Zhang X, Hu Q, Qian R. 2018. Salicylic acid promotes plant growth and salt-related gene expression in *Dianthus superbus* L. (*Caryophyllaceae*) grown under different salt stress conditions. Physiol Mol Biol Plants. 24(2):231–238. doi: 10.1007/s12298-017-0496-x

Zhang M, Jin ZQ, Zhao J, Zhang G, Wu F. 2015. Physiological and biochemical responses to drought stress in cultivated and Tibetan wild barley. Plant Growth Regul. 75(2): 567-574.

Zhao P, Lu GH, Yang YH. 2017. Salicylic acid signaling and its role in responses to stresses in plants. In: Pandey G (ed) Mechanism of plant hormone signaling under stress, 1st edn, vol 11.

Zhang, J., M. Khayatnezhad, and N. Ghadimi, 2022. Optimal model evaluation of the proton-exchange membrane fuel cells based on deep learning and modified African Vulture Optimization Algorithm. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects, *44*(1):287-305.