

# MORPHO-PHYSIOLOGICAL RESPONSES OF STEVIA (*STEVIA REBAUDIANA BERTONI*) TO VARIOUS PRIMING TREATMENTS UNDER DROUGHT STRESS

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**Abstract.** Poor germination capacity of stevia is a major problem in its cultivation. Moreover, drought stress is one of the most major environmental constraints, which influences seed germination and early seedling growth of many crops. The aim of this research was to evaluate the impact of seed priming with salicylic acid (SA), zinc (Zn) and iron (Fe) on some germination parameters and physiological attributes of stevia seedlings under drought stress induced by polyethylene glycol (PEG) 6000 (0, – 3, – 6 and – 9 bars). The results revealed that germination traits (germination percentage, germination rate, mean germination time, germination value, seedling length, and seedling vigor index) and chlorophyll (Chl) content were negatively affected by drought stress. However, the reduction of germination parameters in seedlings exposed to drought stress in most cases was moderated by seed priming, which also increased the Chl content at all levels of drought stress as compared with the control. Drought stress also increased the proline accumulation and the enzymatic activity of catalase (CAT) and peroxidase (POD) in all priming treatments, but these enhancements were significantly higher in primed seedlings than those in unprimed ones. Among all priming treatments, priming with SA + Fe + Zn was found to be more effective than other treatments to improve growth and physiological characteristics under normal and drought stress conditions. Thus, we suggest that seed priming with SA, Fe, Zn and particularly the integrated application of these three agents at a suitable concentration can promote the poor germination performance of stevia and improve the seedling growth by increasing the antioxidant capacity under drought conditions.

**Keywords:** *antioxidant enzymes, chlorophyll, proline, salicylic acid, seed germination*

## Introduction

Stevia (*Stevia rebaudiana* Bert.), a member of the Asteraceae (Compositae) family, is an herbaceous perennial and short-day species (Yadav et al., 2011; Ucar et al., 2016). Naturally, this plant grows between 22–24° south and 53–56° west in South America (Paraguay and Brazil), where it is called ka'ah'e'ê (or sweet herb) (Lemus-Mondaca et al., 2012). Stevia is widely grown for its non-caloric sweet compounds. The sweetness of the stevia plant is related to the presence of *ent*-kaurene-type diterpene glycosides (stevioside and rebaudiosides), which can be 300 times sweeter than sucrose (Hajihashemi and Ehsanpour, 2014; Aghighi Shaverdi et al., 2018). Since the sweet herb has self-incompatible flowers, its pollination is probably carried out by insects and wind (Ucar et al., 2016; Shahverdi et al., 2017). As a result, stevia produces often few pollinated flowers which have low germination percentage and poor establishment (Yadav et al., 2011; Lemus-Mondaca et al., 2012). In general, the poor germination

capacity of stevia continues to be a major impediment for the plantation of this plant on a large-scale (Abdullateef et al., 2015).

Plants are frequently subjected to a lot of abiotic environmental stresses such as drought, high salinity, heavy metals, high/low temperatures and, or heat (Brito et al., 2016). Drought stress is considered as the most major environmental problem that severely affects crop growth and development worldwide, especially in arid and semi-arid regions (Askari et al., 2018). Among various stages of the plant's life cycle, seed germination and seedling emergence are the most sensitive stages to environmental stresses (Patade et al., 2011). Because of the reduction in water potential and water absorption during drought stress, seed germination may be limited (Farooq et al., 2009) and with increasing intensity of water deficit, the complete inhibition of seedling growth can occur (Kaya et al., 2006). Moreover, some investigations have pointed out that drought stress can accelerate the generation of reactive oxygen species (ROS) in plants (Zhang et al., 2015), resulting in reduced growth, cellular damage and eventually plant death (Al Hassan et al., 2017). To improve drought tolerance in plants, one of the most effective methods is the adoption of physiological improvement approaches like seed priming (Zheng et al., 2016). It has been reported that different priming treatments have a good potential to enhance germination uniformity, germination rate, seed vigor, as well as stronger seedling growth under stressed conditions (Patade et al., 2011; Paparella et al., 2015). The higher tolerance of primed seeds against environmental stresses can be occurred due to the activation of free radical scavenging enzymes such as SOD, POD, CAT, and the accumulation of osmoprotectants (e.g. proline) (Rouhi et al., 2012).

Under environmental stresses, seed priming with various plant growth regulators (such as SA) is a common and effective technique for modulating the destructive effects of stress (Ashraf and Foolad, 2005; Hussain et al., 2016). SA (2-hydroxybenzoic acid), a phenolic compound, is known as an endogenous signal hormone that activates a wide range of diverse physiological and biochemical processes in plant cells in response to stressful conditions (Kabiri et al., 2014; Najafabadi and Ehsanzadeh, 2017). In this regard, SA has been successfully applied to alleviate the adverse effects of drought stress on wheat germination parameters (Movaghatian and Khorsandi, 2013).

Micronutrients are essential for normal growth and development of plants, and most of the arable soils in Iran have the severe deficiency of micronutrients, especially Zn and Fe (Mirshekari et al., 2012). Among various priming techniques, seed priming with micronutrients (nutri-priming) has been reported to be a physiological beneficial method for overcoming the micronutrient deficiency in seeds which improves seedling emergence (Farooq et al., 2012). Various studies have revealed that this technique significantly improves germination rate, seed quality, early seedling growth and stress tolerance in different plants (Mirshekari et al., 2012; Imran et al., 2017; Shahverdi et al., 2017; Reis et al., 2018).

According to the seed germination problem in stevia and on the other hand the lack of sufficient information about the role of SA and micronutrients on the improvement of germination behavior and early seedling growth of stevia, the present study was conducted to examine the effect of seed priming with SA and micronutrients (Fe and Zn) on germination indices and biochemical characteristics of stevia under control and drought stress conditions.

## Materials and methods

The present experiment was carried out at the Seed Science and Technology Laboratory of Agricultural College, Shahed University of Tehran, Iran in 2017.

### *Seed material and storage*

New mature seeds of stevia (Var. Bertoni) used in the experiment were purchased in late November 2017 from stevia production fields located in Fars province, Firoozabad city, Iran (34° 37' N, 54° 45' E and 1790 m ASL). The average of 100-seed weight was  $27.7 \pm 0.5$  mg and seed moisture content was around 8.64% (on a dry weight basis). To keep the seed vigor during the course of experimentation, the seed samples were stored in paper bags at  $4 \pm 1^\circ\text{C}$  and 20% relative humidity before being utilized for experiments. The 1.5-month seed samples were used in this research.

### *Priming and drought stress treatments*

The study was a factorial experiment based on a completely randomized design (CRD) with three replications in which the experiment factors included four levels of drought stress induced by PEG 6000 (0, -3, -6 and -9 bar) and seven combinations of priming (SA, Fe, Zn, SA + Fe, SA + Zn, Fe + Zn, SA + Fe + Zn), and unprimed dry seeds were considered as control. In this experiment, Fe and Zn were supplied from sources of iron (II) sulfate heptahydrate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 26% Fe) and Zinc sulfate heptahydrate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 21% Zn) respectively, as well as SA (2-hydroxybenzoic acid, Sigma Aldridge Company Ltd.) in powder form, was used.

### *Germination process*

Prior to conducting the experiment and based on three separate tests, the best duration and concentration of pretreatment for each of the priming agents (Fe, Zn and SA) were optimized. The factors evaluated in these experiments were five priming durations (0, 6, 12, 18 and 24 hours) and six concentrations of micronutrients (0, 0.25%, 0.5%, 1%, 1.5% and 2%) and SA (0, 0.25, 0.5, 1, 1.5 and 2 mM), which were tested separately. According to the results, the best duration and concentration of seed priming with micronutrients (Fe, Zn) and SA were 24 hours at the concentration of 0.5% and 24 hours at the concentration of 1 mM, respectively. These data were used in the experiment (data not shown).

To conduct the germination test, first, all used equipment and seeds were thoroughly disinfected. Stevia seeds were sterilized in 70% (v/v) ethanol for 1 min and 20% (v/v) sodium hypochlorite solution for 15 min and then were rinsed three times with sterile distilled water (Hajihashemi and Ehsanpour, 2013). The priming treatments were prepared in distilled water. Stevia seeds were entirely immersed in determined concentrations of priming media (1 mM SA, 0.5% Fe, 0.5% Zn, 1 mM SA + 0.5% Fe, 1 mM SA + 0.5% Zn, 0.5% Fe + 0.5% Zn and 1 mM SA + 0.5% Fe + 0.5% Zn) at 15 °C in darkness (Bradford, 1986). After the end of the priming process, the treated seeds were surface washed with distilled water and then dried at room temperature (about 21 °C) for 24 h back to the initial moisture content. After that, 100 primed and unprimed (control) seeds were counted and placed between double layers of sterilized Whatman paper (No. 2) in 12-cm-diameter Petri dishes and based on various levels of drought stress, 7 mL of PEG solution was added to each Petri dish. The osmotic

potentials of – 3, – 6 and – 9 bars were obtained by adding 148.01, 219.54 and 274.65 g of PEG 6000 in 1000 mL of distilled water, respectively. The required amount of PEG 6000 was calculated by Michel and Kaufmann's (1973) formula (Eq. 1):

$$\Psi_s = - (1.18 \times 10^{-2}) \times C - (1.18 \times 10^{-4}) \times C^2 + (2.67 \times 10^{-4}) \times CT + (8.39 \times 10^{-7}) \times C^2T \quad (\text{Eq. 1})$$

$\Psi_s$ , C, and T are osmotic potential (bars), the concentration of PEG (g L<sup>-1</sup> of distilled water) and temperature (°C), respectively. The distilled water potential is zero, so it was used as the control treatment (without drought stress). Petri dishes were wrapped with impermeable parafilm to avoid the loss of moisture and evaporation. Subsequently, all dishes were transferred in a programmed germination chamber at 23 ± 2 °C, 16/8 h light/darkness and 75% relative humidity (Liopa-Tsakalidi et al., 2012). The germinated seeds were daily counted, and the germination test was ended when the germination was not observed for three consecutive days. The seeds which had radicles with 2 mm long or more were considered as germinated seeds (ISTA, 2010). Eventually, at the end of the germination period (14 days), germination percentage, germination rate, mean germination time, seedling vigor index and germination value were calculated based on the relationships presented in Table 1.

**Table 1.** The computing relations of the parameters studied in the experiment

Parameters	Formula	Reference
Germination percentage	$GP = (N \times 100)/M$	Liopa-Tsakalidi et al., 2012
Germination rate	$GR = \sum_{i=1}^n \frac{S_i}{D_i}$	Maguire, 1962
Mean germination time	$MGT = \frac{\sum (Dn)}{\sum n}$	Salehzade et al., 2009
Germination value	$GV = GP \times MDG$	Czabator, 1962
Seedling vigor index	$SVI = GP \times \text{Mean (SL)} / 100$	Abdul-Baki and Anderson, 1973

N: the sum of germinated seeds at the end of the experiment, M: the total number of planted seeds, S<sub>i</sub>: the number of germinated seeds in each enumeration, D<sub>i</sub>: the number of day from the start of the test to the enumeration of n<sup>th</sup>, D: the time from the start of the experiment to the i<sup>th</sup> observation, n: the number of germinated seeds at time D, MDG: mean daily germination, SL: mean of seedling (root + shoot) length (mm)

After two weeks of growth, the seedlings from each replicate were collected and immediately frozen in liquid nitrogen and stored in the ultra-low freezer at –80 °C for biochemical studies.

### **Antioxidant enzymes assay**

To evaluate the activity of antioxidant enzymes, 0.5 g of frozen samples were homogenized in 5 mL of cool extraction buffer (50 mM potassium phosphate buffer (pH = 7.5)), containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT) and 2% (w/v) polyvinylpyrrolidone (PVP)). The homogenate was centrifuged at 15,000 × g for 25 min and obtained supernatants were used as the enzyme source for CAT and POD assays. The whole extraction process was done at 4 °C.

### ***Peroxidase assay***

The POD activity (EC 1.11.1.7) was determined following the method of MacAdam et al. (1992) with some modification. In this method, The enzymatic activity was assayed by adding 50  $\mu$ L of enzyme extract to the reaction mixture (3 mL), containing 0.1 M potassium phosphate buffer (pH 6.0), 50  $\mu$ L guaiacol and 50  $\mu$ L H<sub>2</sub>O<sub>2</sub> (3%), and absorption alterations were immediately recorded at 436 nm for 3 min per 15 s by spectrophotometer. The control contained 3 mL of 0.1 M potassium phosphate buffer, 50  $\mu$ L guaiacol and 50  $\mu$ L H<sub>2</sub>O<sub>2</sub>.

### ***Catalase assay***

The CAT activity (EC.1.11.1.6) was assayed according to the method of Chance and Maehly (1955) which is briefly described here. The reaction mixture consisted of 50 mM sodium phosphate buffer (pH = 7.0), 15 mM H<sub>2</sub>O<sub>2</sub>, and 25  $\mu$ L of the enzyme extract in a total volume of 3 mL. The absorbance at 240 nm for 1 min at 25 °C was recorded spectrophotometrically. One unit of CAT was defined as the amount of enzyme that decomposes 1  $\mu$ mol of H<sub>2</sub>O<sub>2</sub> per min.

### ***Proline determination***

The free proline content was measured using the method described by Bates et al. (1973). Frozen samples (0.5 g) were homogenized in 10 mL of 3% (w/v) sulpho salicylic acid and centrifuged at 4000  $\times$  g for 10 min. After centrifugation, this homogenized solution was filtered with Whatman's paper (No. 2) and then 2 mL of filtrated solution was mixed with acid-ninhydrin (2 mL) and glacial acetic acid (2 mL) in a test tube. The mixture was placed at 100 °C for 1 h in a water bath and immediately transferred to an ice bath for a few minutes. After cooling, the reaction mixture was thoroughly mixed with toluene (4 mL), and then the absorbance was measured at 520 nm.

### ***Total chlorophyll content***

The Chl content was measured using the methods of Lichtenthaler (1987). In this method, chlorophyll was extracted in chilled 80% acetone in dark. After centrifugation at 5000  $\times$  g for 10 min, the total chlorophyll content was determined at 663.2 and 646.8 nm with a spectrophotometer. The total chlorophyll content was calculated according to *Equation 2*:

$$\text{Total Chl} = 7.15 (A_{663.2}) + 18.71 (A_{646.8}) \quad (\text{Eq.2})$$

where A is absorbance at the specific wavelength.

### ***Statistical analysis***

After checking the data distribution normality (Kolmogorov-Smirnov and Shapiro-Wilk test) assumption, the studied traits were statistically analyzed by the Statistical Analysis System software (SAS Institute, Cary, NC, USA, Version 9.4). The differences among means were separated using least significant difference test (LSD) at 0.05 statistical probability level and the graphs were drawn by MS–Excel.

## Results

### Germination percentage (GP)

The effects of drought stress due to PEG, priming treatments and the interplay between them were significant ( $P \leq 0.01$ ) on GP parameter (*Table 2*). The results of stevia seed germination under drought stress after various priming treatments are shown in *Table 3*. The highest GP was observed in seeds primed with SA + Fe + Zn and Fe + Zn under control conditions (without drought stress), as well as seeds primed with SA + Fe + Zn at  $-3$  bar (66.66%, 60.33% and 60.33%, respectively). GP was drastically affected by drought stress, so that the minimum GP was obtained from unprimed seeds at  $-9$  bar (72.4% reduction compared to unprimed seeds under the osmotic potential of 0 bar). The priming treatments improved seed germination of stevia under normal and drought stress conditions. At the highest level of drought stress ( $-9$  bar), the maximum GP (33.6 and 32.33%) was achieved from primed seeds by Fe + Zn and SA + Fe + Zn, respectively (*Table 3*). The listed treatments improved the GP parameter by 57.4% and 55.7% as compared to the control seeds (without priming) at this level.

### Germination rate (GR)

Drought stress, priming and the interaction of these two factors had highly significant effects ( $P \leq 0.01$ ) on GR trait (*Table 2*). The results showed that the GR of stevia seeds was significantly decreased with increasing drought stress levels (*Table 3*). Nevertheless, the amount of this trait was higher in the primed seeds than in unprimed ones under normal and drought stress conditions. The most value of GR (18.52 seed per day) belonged to seeds which were treated by SA + Fe + Zn under normal conditions. The lowest amounts of GR were found in unprimed seeds and primed seeds by SA, Fe, and Zn under the osmotic potential of  $-9$  bar (2.11, 2.52, 3.02 and 3.01 seed per day, respectively). The results clearly indicated that the integrated application of priming agents was more effective than their separate application to alleviate the drought-induced damaging effects on GR (*Table 3*).

**Table 2.** Analysis of variance for the effect of priming treatments and drought stress on stevia seed germination indices

Sources of variance	df	Mean squares and significance					
		GP	GR	MGT	GV	SVI	SL
Drought (D)	3	5489.5**	576.08**	29.1**	18.04**	808.3**	1420.8**
Priming (P)	7	561.6**	25.1**	0.24 <sup>ns</sup>	1.77**	162.9**	334.1**
D × P	21	33.9**	1.9**	0.33**	0.08 <sup>ns</sup>	10.11**	13.31**
Experimental error	64	15.1	0.81	0.11	0.057	2.91	4.67
Coefficient variation (%)	-	9.18	10	5.99	16.52	18.16	11.11

ns, \* and \*\*: non-significant, significant at 5% and 1%, respectively. df: degrees of freedom, GP: germination percentage, GR: germination rate, MGT: mean germination time, GV: germination value, SL: seedling length, SVI: seedling vigor index

### Mean germination time (MGT)

As shown in *Table 2*, drought stress and the interaction of drought stress and priming treatments had highly significant effects on MGT parameter ( $p \leq 0.01$ ). In response to

drought stress, MGT was significantly increased so that the maximum values of MGT were observed at the highest level of drought stress (– 9 bar) while the lowest required times for germination (4.12, 4.15, 4.18, 4.56 and 4.65 days) were recorded in the absence of stress (0 bar) in seeds treated with Fe, SA, SA + Fe + Zn, SA + Fe and Fe + Zn, respectively. At – 9 bar, those seeds primed with SA + Fe + Zn had the shortest required time for germination (6.68 days), which was significantly lower than unprimed seeds at this level.

**Table 3.** Mean comparison of the interaction between different priming treatments and drought stress for stevia seed germination indices

Treatments		GP (%)	GR (seed/day)	MGT (day)	SL (mm)	SVI
Drought (bars)	Priming					
0	Control	52c..f	13.44cd	4.71i..l	20.5fg	10.82ef
	SA	51.33c..f	14.12bc	4.15m	25.9c.e	13.32de
	Fe	56.66bc	15.38b	4.12m	24.8c.e	14.09b.d
	Zn	54.66b..e	13.96b.d	4.71i..l	19.3gh	10.56ef
	SA + Fe	58.66b	15.04b	4.56k.m	26.8b.d	15.79b.d
	SA + Zn	59.00b	14.51bc	4.89h..k	27.3bc	16.18bc
	Fe + Zn	60.33ab	15.32b	4.65j..m	27.8bc	16.8b
	SA + Fe + Zn	66.66a	18.52a	4.18lm	37.4a	25.01a
–3	Control	43.33h.j	7.43ij	6.46cd	16.86h.j	7.34g..j
	SA	43.66g..j	8.53hi	5.58e.g	24.4c.e	10.67ef
	Fe	50d..g	10.05fg	5.46fg	20.8fg	10.42f
	Zn	48f..i	9.48gh	5.45fg	15.4i.k	7.4g..j
	SA + Fe	56b.d	11.88e	5.14f..j	23.7d.f	13.3de
	SA + Zn	55.33b..e	11.25ef	5.42f.g	24.8c.e	13.77cd
	Fe + Zn	55.33b..e	11.8e	5.13f..j	27.9bc	15.5b.d
	SA + Fe + Zn	60.33ab	12.57de	5.24f..i	38.2a	23.11a
–6	Control	15.66op	3.06no	5.46fg	12.4k.m	1.98l..o
	SA	28.66lm	5.92kl	5.06g...k	18.6g.i	5.29i.k
	Fe	38jk	7.27i.k	5.56e.g	16.1h.j	6.24h..k
	Zn	37.33jk	7.03jk	5.63ef	11.6l.n	4.32k.m
	SA + Fe	43.33h.j	8.22h.j	5.62ef	19.03gh	8.28f.h
	SA + Zn	42.66ij	8.67g.i	5.34f.h	23.1ef	9.99fg
	Fe + Zn	42.66ij	8.10h.j	5.68ef	17.4g..j	7.48h.j
	SA + Fe + Zn	49.33e..h	8.41h.j	6.09de	30.1b	14.97b.d
–9	Control	14.33p	2.11o	7.52a	5.76p	0.85o
	SA	17.33op	2.52no	7.23ab	8.14n.p	1.43no
	Fe	21.66no	3.02no	7.32a	7.76op	1.7m.o
	Zn	20n.p	3.01no	7.04ab	5.8op	1.19no
	SA + Fe	25.33mn	3.66mn	7.19ab	9.07m..p	2.3l..o
	SA + Zn	24.66mn	3.67mn	7.12ab	9.36m.o	2.23l..o
	Fe + Zn	33.66kl	5.00lm	7.18ab	11.49l.n	3.83k..n
	SA + Fe + Zn	32.33kl	5.18l	6.68bc	14.4j.l	4.67j.l

Means in each column with the same alphabetical letter (s) are not significantly different at 0.05 probability level according to LSD test. GP: germination percentage, GR: germination rate, MGT: mean germination time, SL: seedling length, SVI: seedling vigor index

### ***Seedling length (SL)***

Based on the analysis of variance, drought stress, priming treatments and the interaction among them had highly significant effects ( $P \leq 0.01$ ) on SL trait (*Table 2*). Drought stress was found to severely limit the seedling growth of stevia. The longest SL pertained to the seeds primed by SA + Fe + Zn at  $-3$  and  $0$  bar (control) levels of drought stress (38.2 and 37.4 mm, respectively), and the shortest SL was observed at the highest level of drought stress ( $-9$  bar) in unprimed seeds (5.76 mm) (*Table 3*). Under severe drought stress, the SL in SA + Fe + Zn treatment was 60% higher compared to the control.

### ***Seedling vigor index (SVI)***

According to the results, the effect of drought stress, priming treatments and the interaction among them was highly significant ( $p \leq 0.01$ ) on SVI (*Table 2*). Depending on the reduction in SL and GP under drought stress, SVI was gradually reduced with increasing drought stress levels, so that the lowest means were recorded at  $-9$  bar (*Table 3*). On the other hand, priming treatments, especially the integrated application of SA, Fe, and Zn increased SVI under drought and normal conditions compared to unprimed seeds. The highest amount of this trait was detected in primed seeds with SA + Fe + Zn under the osmotic potentials of  $0$  and  $-3$  bar (25.01 and 23.11, respectively) (*Table 3*).

### ***Germination value (GV)***

Variance analysis of data revealed that the GV parameter was significantly affected by drought stress and priming agents ( $p \leq 0.01$ ), but the interplay of seed priming  $\times$  drought stress on this trait was not significant (*Table 2*). Comparison of mean values at different levels of drought stress showed that the increase of PEG concentration from the control to the highest level ( $-9$  bar) resulted in an 81.85% reduction in GV of stevia seeds. Presented results in *Table 4* showed that all the seed priming treatments except SA treatment had a significant positive effect on GV compared with unprimed seeds. In comparison with the control (without priming), various priming treatments enhanced the GV of stevia seeds between 10.78 – 56.03%. The best result in this respect (with an average of 2.07) was achieved by applying the composition of SA, Fe and Zn (*Table 4*).

### ***Free proline content***

The proline content was significantly affected by drought stress and tested priming compounds ( $p \leq 0.01$ ). Drought stress due to PEG at all the studied concentrations gradually increased the accumulation of free proline in stevia seedlings, so that the highest and the lowest content of proline were observed at the osmotic potentials of  $-9$  and  $0$  bar (control) ( $0.363$  and  $0.233 \mu\text{mol g}^{-1}$  FW respectively) (*Table 4*). Also, the proline content in primed seeds was significantly higher as compared to the control seeds (without priming). Among all studied priming treatments, the highest amount of the proline content belonged to seeds which were treated by SA + Fe + Zn, Fe + Zn and SA + Zn, respectively. The proline content in SA, Fe, Zn, SA + Fe, SA + Zn, Fe + Zn and SA + Fe + Zn treatments was increased by 48.56, 53.26, 53.72, 56.79, 59.02, 59.14 and 59.6% compared with the control (*Table 4*).



**Table 4.** Mean comparison of the effect of drought stress and various priming treatments on germination value (GV) and proline content

Drought stress (bars)	GV	Proline content ( $\mu\text{mol g}^{-1}$ FW)
0 (Control)	2.37a	0.233d
– 3	1.92b	0.282c
– 6	1.06c	0.327b
– 9	0.43d	0.363a
Priming treatments		
Control	0.91d	0.143f
SA	1.02d	0.278e
Fe	1.37c	0.306d
Zn	1.27c	0.309cd
SA + Fe	1.63b	0.331bc
SA + Zn	1.60b	0.349ab
Fe + Zn	1.72b	0.350ab
SA + Fe + Zn	2.07a	0.354a

Means in each column with the same alphabetical letter (s) are not significantly different at 0.05 probability level according to LSD test

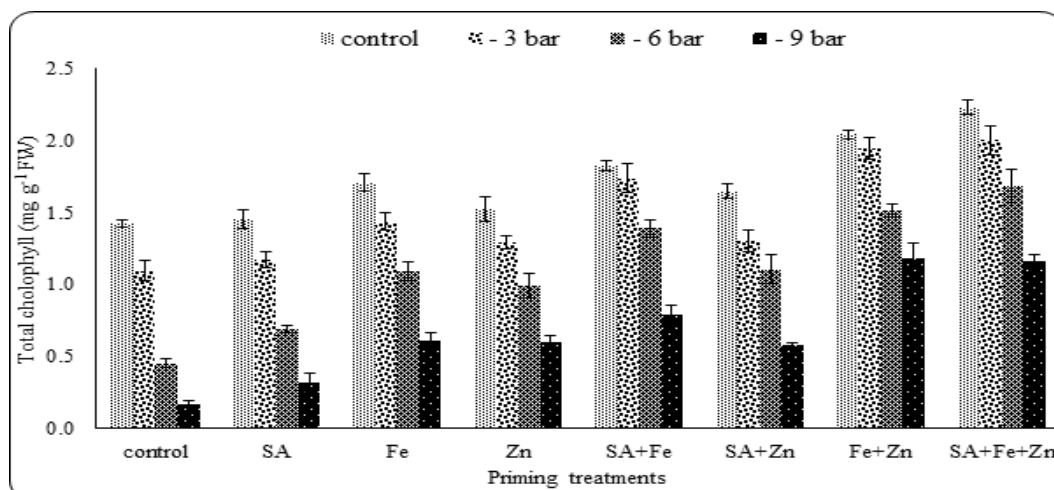
### Total chlorophyll content

Data recorded in *Table 5* shows that the total Chl content was significantly affected by drought stress, priming agents ( $p \leq 0.01$ ) and the interaction between them ( $p \leq 0.05$ ). The Chl content was significantly decreased with increasing PEG concentration in all studied treatments when compared with the control conditions (without drought stress). However, priming treatments alleviated effectively the damaging effect of drought stress on Chl content. The lowest amount of Chl content was recorded at the highest level of drought stress (– 9 bar) in unprimed seeds. The highest values of total Chl content were recorded in the combination of three priming agents (SA + Fe + Zn) and the combination of Fe and Zn under normal conditions (2.22 and 2.04 mg/g FW, respectively). At all levels of drought stress, the most effective priming treatments with the highest total Chl content were SA + Fe + Zn and Fe + Zn (*Fig. 1*).

**Table 5.** Analysis of variance for the effect of priming treatments and drought stress on physiological traits of stevia seedling

Sources of variance	df	Mean squares and significance			
		Total Chl	Proline	CAT	POD
Drought (D)	3	5.13**	0.075**	6.16**	196.42**
Priming (P)	7	1.44**	0.063**	0.376**	14.73**
D × P	21	0.022*	0.0006 <sup>ns</sup>	0.085*	1.102*
Experimental error	64	0.012	0.0008	0.046	0.628
Coefficient variation (%)	-	9.05	9.40	17.56	9.76

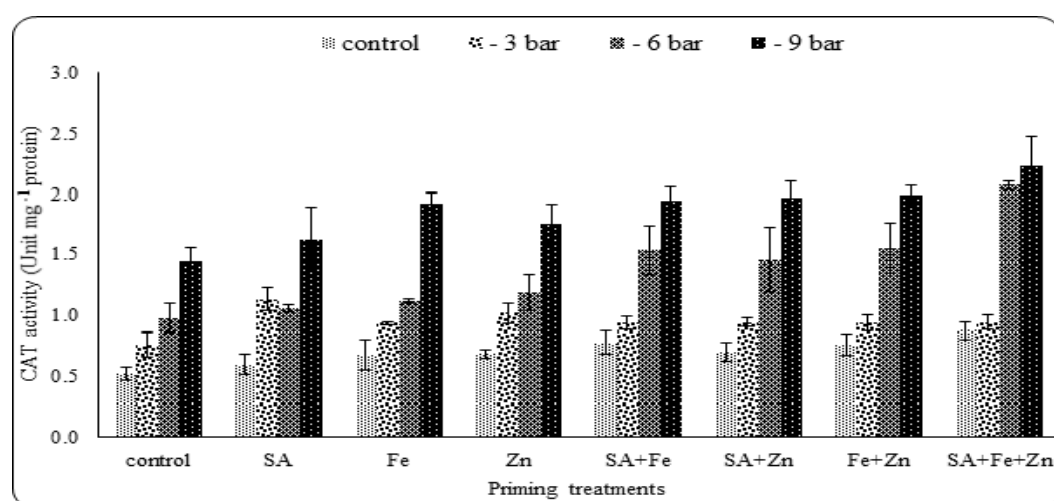
ns, \* and \*\*: non-significant, significant at 5% and 1%, respectively, df: degrees of freedom, Chl: chlorophyll content, CAT: catalase activity, POD: peroxidase activity



**Figure 1.** Total Chlorophyll content in primed and unprimed seedlings of stevia under normal and drought stress conditions (LSD 5%= 0.185). Error bars indicate standard error (SE)

### CAT activity

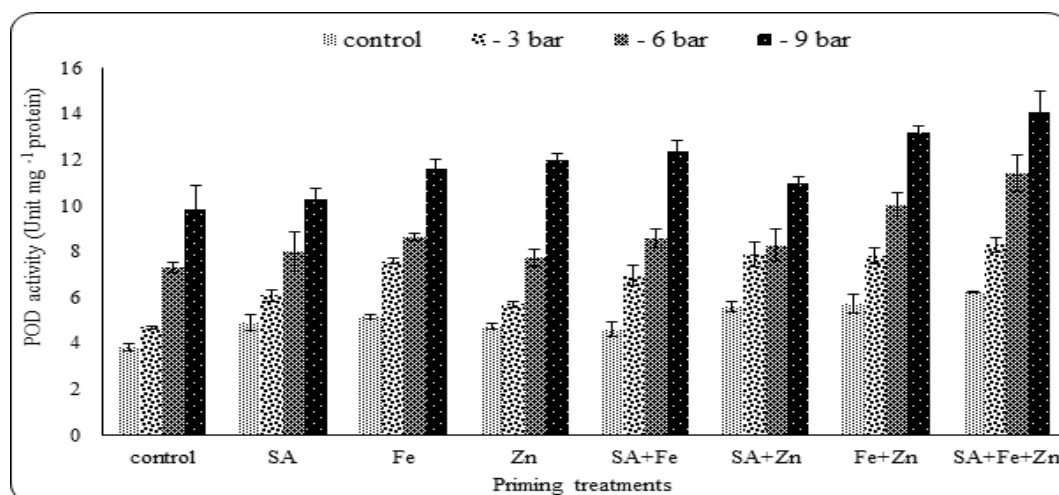
Statistical analysis demonstrated that the enzymatic activity of CAT varied significantly in response to drought stress, priming treatments ( $p \leq 0.01$ ) and the interaction between them ( $p \leq 0.05$ ) (Table 5). Data regarding the CAT activity in primed and unprimed seedlings of stevia under drought stress are shown in Figure 2. The CAT activity was significantly increased in primed and unprimed seedlings with increasing drought stress. On the other hand, seed priming promoted the CAT activity in the stevia seedlings under drought stress, so that the maximum values of enzymatic activity were obtained from primed seeds with SA + Fe + Zn at the osmotic potential of - 9 bar (2.23 Unit/mg protein.min, respectively). In comparison to the control, under severe drought stress (- 9 bar), the enzymatic activity of CAT was significantly increased in the seedlings which were raised from primed seeds by SA + Fe + Zn, Fe + Zn, SA + Zn, SA + Fe and Fe (Fig. 2).



**Figure 2.** The catalase (CAT) activity in primed and unprimed seedlings of stevia under normal and drought stress conditions (LSD 5%= 0.35). Error bars indicate standard error (SE)

### POD activity

According to the results, drought stress, priming treatments ( $p \leq 0.01$ ) and the interaction between them ( $p \leq 0.05$ ) had significant effects on the POD activity (Table 5). Results regarding the POD activity of stevia seedlings under the influence of drought stress and different priming treatments are presented in Figure 3. The enzymatic activity of POD was considerably enhanced with elevating of drought stress in all studied treatments, so that the lowest POD activity for all treatments was observed under the controlled conditions. The average values revealed that the priming treatments caused an increase in the POD activity of the stevia seedlings compared to the control seeds. The maximum activity of POD was recorded in seedlings primed with SA + Fe + Zn and Fe + Zn at the osmotic potential of  $-9$  bar (14.04 and 13.15 Unit/mg protein.min, respectively). Compared with the control seeds (without priming), the SA + Fe + Zn, Fe + Zn, SA+ Fe, Zn and Fe were the most effective treatments regarding the POD activity at the highest level of drought stress (Fig. 3).



**Figure 3.** The peroxidase (POD) activity in primed and unprimed seedlings of stevia under normal and drought stress conditions (LSD 5%= 1.29). Error bars indicate standard error (SE)

### Discussion

Evaluation of drought stress effect on some seed germination and physiological characteristics of stevia seedlings was one of the objectives in the present investigation. In recent years, drought stress is known as an important environmental factor which seriously impacts the productivity of many crops and can retard growth and development of them (Lipiec et al., 2013). The results of our study showed that the seed germination indices of stevia were affected by the deleterious effects of drought stress induced by PEG, so that GP, GR, and GV were significantly decreased along with the increase of PEG concentration (Table 3). In the present study, it was found that the average of GP was dropped to below 24% at the highest PEG concentration ( $-9$  bar). Poor and erratic germination might be attributed to the lower water uptake by seeds and the elevation of ROS levels under water deficit (Kaya et al., 2006). It has been reported that under stress conditions, the alteration of some enzymes and hormones found in the seed could lead to the reduction of final germination (Botía et al., 1998). Also in this study, SL and SVI (the product of SL and GP) which are the important traits in the

primary establishment of seedling were obviously reduced with increased drought stress (*Table 3*). This might be due to the reduction of water content in plant tissues under water stress which declines turgor pressure in cells, and consequently inhibits cell enlargement, cell division and plant growth (Farooq et al., 2009). These results are consistent with the findings presented in some previous research in other plants (Patane et al., 2013; Zaher-Ara et al., 2016). Additionally, the decrease of water potential was accompanied by a significant increase of MGT (*Table 3*), because seeds under water deficit require more time to reach an adequate level of hydration to initiate germination (Steiner et al., 2017). The increase of required time for germination under drought stress has been reported in the research done by Steiner et al. (2017) in wheat and black oat.

In our study, the comparative performance of different priming treatments for improving drought tolerance at germination and early growth stages of stevia was also evaluated. The results showed that seed priming with SA and micronutrients (Fe and Zn) not only improved the measured germination parameters and seedling growth of stevia in both normal and stress conditions but also alleviated drought stress damages. In this regard, it was observed that the integrated application of priming agents in most of the cases was more effective than their separate application. The beneficial effects of seed priming on germination might be related to the stimulation of pre-germination metabolic procedures, the increment in protein synthesis, the repair and the build-up of nucleic acids, the repair of membranes and osmotic adjustment (Ibrahim et al., 2016). As can be seen in *Table 3*, at all drought stress levels, the highest values of GP, GV as well as SL and SVI were noticed in seeds primed by SA + Fe + Zn. The reason of the increment in seedling weight and length as a result of nutri-priming might be due to the role of these elements in increased cell division, cell expansion and meristematic growth, which caused an increase of plant growth (Shahverdi et al., 2017). It has been reported that in the presence of zinc, hormones such as auxin are increased. Therefore, it seems that the increase of auxin in seed due to the presence of zinc increases the growth of seedling (Laware and Raskar, 2014). In this regard, Munawar et al. (2013) reported that treating seeds of *Daucus carota* L. with Zn (1.5%) and Mn (1.5 and 2%) resulted in the highest mean values of the shoot and root length. Begum et al. (2014) concluded that canola seed priming with ZnSO<sub>4</sub> and CuSO<sub>4</sub> leads to higher GP, GR and SL under NaCl stress. Previously, various studies on dill (Mirshekari et al., 2012), maize (Imarn et al., 2017) and wheat (Reis et al., 2018) have reported the enhanced germination and yield after priming with micronutrients. On the other hand, several studies have revealed that pre-soaking of seeds in the optimal concentrations of plant growth regulators such as SA could improve the germination of crops, particularly under stress conditions (Ansari and Sharifzadeh, 2012; Arbaoui et al., 2015; Kumari et al., 2017). Sakhabutdinova et al. (2003) reported that SA prevents the reduction of Indole acetic acid (IAA) and cytokinin levels in plant tissues, and alleviates the inhibitory effects of water stress on plant growth. The findings of Wang et al. (2016) also indicated that seed priming with SA improved the seedling emergence and growth of rice.

Chl is one of the main components of chloroplast in photosynthesis. Under water stress, the reduction of Chl content has been considered a usual symptom of oxidative stress (Fathi and Tari, 2016). In our study, the total Chl content was significantly diminished with an increase in the level of applied PEG as compared to the control seedlings. The priming treatments (especially Fe + Zn, SA + Fe + Zn and SA + Fe) did not only moderate the adverse effect of drought stress on the Chl content, but also had a significant stimulatory effect on the biosynthesis of Chl, so that under severe drought

stress (– 9 bar), the maximum amount of Chl was recorded in the seedlings which were raised from primed seeds by Fe + Zn and SA + Fe + Zn (*Fig. 1*). The decrement of Chl content under drought stress is mainly due to damage to the chloroplast membrane and Chl degradation as result of the activity of ROS (Madany and Khalil, 2017). These findings are in agreement with previous results that drought stress reduced the total Chl content, whereas priming treatments minimized the deleterious effects of stress conditions (Hussain et al., 2017; Madany and Khalil, 2017). Zn is an essential element for Chl synthesis, pollen function, and germination (Cakmak, 2008). It has been reported that Zn element by the protection of the sulfhydryl group keeps the Chl content (Latef et al., 2017). Fe is an essential element required for the maintenance of chloroplast structure and is involved in Chl synthesis (Rout and Sahoo, 2015). The limitation of this element has a remarkable effect on the productivity of photosynthetic organisms (Tewari et al., 2013). Shahverdi et al. (2017) reported that stevia seed priming with Fe alone and the integrated application of selenium (Se) and boron (B) significantly increased the total Chl content under salinity stress. In another research, Li and Zhang (2012) concluded that rice seed priming with SA increases the photosynthetic activities in this plant.

In plant cells, ROS are generated in both normal and stress conditions, but in response to different environmental stresses such as drought stress, the production of ROS is significantly increased which could result in the progressive oxidative damages (Sharma et al., 2012). Plants usually have several defensive mechanisms to overcome the oxidative stress (Lipiec et al., 2013), which include enzymatic and non-enzymatic (such as proline) antioxidants and as well as reparation systems that orchestrate stress signaling and block the adverse effects of ROS (Demidchik, 2015). Proline is known to act as an osmolyte /osmoprotectant agent under drought stress (Lehmann et al., 2010) and has the important role in the osmotic pressure adjustment, scavenging free radicals, stabilizing sub-cellular structures (e.g. membranes and proteins) and storing carbon and nitrogen (Bartels and Sunkar, 2005). The results of our study demonstrated a significant increase in the amount of proline content under drought stress. According to the results, all priming treatments (especially SA + Fe + Zn, Fe + Zn and SA + Zn) significantly enhanced (48.5-59.6%) the accumulation of free proline in stevia seedlings (*Table 4*). The better ability of primed seeds is attributed to a wide range of metabolic and physiological improvements (Shehab et al., 2010). In agreement with our results, Kabiri et al. (2014) reported that the seedlings derived from primed seeds of fennel with SA had the higher values of the proline content than unprimed seeds under drought stress. Fallah et al. (2018) have documented that the various priming treatments such as ZnSO<sub>4</sub> (0.5%) increase the proline content in *Nigella sativa* L. under drought stress. Also, it has been reported that seed priming by micronutrients increases the proline content under salinity stress (Shahverdi et al., 2017). Hayat et al. (2010) reported that SA treatment enhances the proline accumulation with a concomitant induction of different stress enzymes.

CAT and POD are described to be two of the most important antioxidant enzymes that protect plants against cell oxidative damages caused by drought and other environmental stresses (Huang et al., 2016). These enzymes play an important role in scavenging of H<sub>2</sub>O<sub>2</sub> (Hussain et al., 2016). In our study, drought stress activated the activity of CAT and POD in stevia seedlings, so that the lowest activities were observed under the controlled conditions. The increasing of the enzymatic activity, as seen in our study has been considered a part of seed strategy to overcome free radicals (Chiu et al.,

2005). Also, in primed seedlings, the activities of CAT and POD were higher than those in unprimed ones which eventually helped in alleviating the effect of oxidative stress on germination indices. The increased antioxidant activity in the primed seedlings, resulting in the better growth might be related to their higher ROS scavenging ability under stress conditions (Hussain et al., 2016). Generally, among studied priming treatments, SA + Fe + Zn had the most positive effect in increasing the activity of these enzymes (Figs. 2 and 3). Zn is used in numerous physiological and biochemical processes in plant cells, including protein synthesis, membrane function, and gene expression. It is a metal component of many important enzymes, acting as a regulatory cofactor for many enzymes, and has the major role in defense systems of plants against stress conditions (Cakmak, 2000; Reis et al., 2018). In this connection, Aboutalebian and Nazari (2017) mentioned that osmopriming with ZnSO<sub>4</sub> increased the activity of SOD, POD and CAT enzymes in canola under chilling stress. Fe element also is well known as an important co-factor for many antioxidant enzymes (such as CAT and POD) (Kusvuran et al., 2016). Ruiz et al. (2000) reported that the activities of the CAT and POD enzymes were correlated with the Fe content in leaves. SA has been found to act as the endogenous signal molecule which participates in the regulation of many physiological processes in plants in response to stressful conditions (Kabiri et al., 2014). Pouramir-Dashtman et al. (2014) stated that seed priming with SA could ameliorate the destructive effects of oxidative stress caused by the generation of ROS by increasing the antioxidant defense system in rice. In this regard, Hara et al. (2012) mentioned that SA in low concentration could increase the oxidative capacity in plants. Similar findings were also reported by Sheykhbaglou et al (2014), who claimed that seed priming by SA increased the antioxidant activity and led to the improvement of germination parameters in sorghum, which coherent with the present study.

## Conclusion

It is concluded from findings of our research that PEG-induced drought stress had inhibitory effects on all germination parameters studied in stevia, nevertheless, seed priming effectively promoted germination characteristics, seedling growth, the total Chl content and the antioxidant capacity under different levels of drought stress as compared to the unprimed ones. The better germination performance and vigorous seedling growth in stevia through seed priming treatments under drought stress could be related to the enhanced enzymatic activities of POD and CAT and the higher accumulation of free proline. Accordingly, our results suggest that seed priming with SA and micronutrients (Fe and Zn) particularly the concurrent application of them at the appropriate concentration can improve drought tolerance of stevia in germination stage. Nevertheless, confirmatory trials under natural field conditions over several years are needed to ensure whether studied priming treatments would result in the improvement of seedling emergence and vegetative growth of stevia as well as its resistance to drought stress on the farm. Also, it is recommended to develop more comprehensive results and evaluate the effect of seed priming with other micronutrients in combination with different plant hormones on germination behavior and the physiological and biochemical changes of stevia during unfavorable conditions. Besides, further researches at transcriptomic and proteomic levels are required to decipher the molecular mechanisms of seed priming-induced drought tolerance in stevia plants.

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