Evaluating the role of nutri-priming in improving PEG-induced drought stress tolerance of stevia (*Stevia rebuadiana* Bertoni)

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Abstract

Poor germination and low seedling growth of stevia are common problems in the cultivation of the plant. Nutri-priming is a new, safe, easy, and effective method to increase germination characteristics and seedling tolerance against stress. The objective of this study was to assess the effect of nutri-priming with nano potassium (nano-K), paclobutrazol (PBZ), and salicylic acid (SA) on stevia seed germination indices and physiological traits under drought stress conditions (0, -3, -6, and -9 bar) induced by polyethylene glycol 8000. Results indicated that germination indices (germination percentage and rate, mean germination time, seedling length, and seedling vigor index) and photosynthetic pigment contents (chlorophyll a, b, and total) were negatively affected by drought stress. While drought stress increased activity of antioxidant enzymes (peroxidase, catalase, and superoxide dismutase) and proline content. Seed nutri-priming with nano-K + SA + PBZ was the best treatment in improving germination traits and physiological characteristics. The highest germination percentage was related to seeds that were treated with nano-K + SA + PBZ under control and -3 bar drought treatments (62.4 and 64.0%, respectively). In all drought levels, the lowest amounts of germination percentage were found in unprimed seeds. The minimum germination percentage was obtained from unprimed seeds at -9 bar (29.05% reduction compared to unprimed seeds under the osmotic potential of 0 bar). As a result, this study indicated that the use of nano-K + SA + PBZ as a pre-sowing seed treatment can promote the poor germination performance of stevia and protect it from negative impacts of drought stress.

Keywords: *antioxidant enzymes; chlorophyll; paclobutrazol; proline; salicylic acid; seed germination*

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Introduction

Stevia (*Stevia rebaudiana* Bertoni), a member of the Asteraceae (Compositae) family, is an herbaceous perennial plant native to Paraguay (Gorzi et al., 2017). Steviol glycosides are around 200 times sweeter than sugar, yet much less calorific. Stevioside and Rebaudioside-A are dominant glycoside compositions of the plant which make the plant even 300 times sweeter than sucrose (Aghighi Shahverdi et al*.,* 2017). Although stevia is naturally propagated by seed, due to its poor germination rate, seed propagation is not widely used for commercial stevia production. Because of self-incompatible flowers, the crossing is usually carried out by wind

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and insects. Thus, the plant has a low fertility percentage and hence low seed formation which limits its large-scale cultivation. However, self-compatibility is also reported by some researchers (Aghighi Shahverdi et al*.,* 2017).

Drought is the most severe abiotic stress factor limiting plant growth and crop production. It is generally believed that abiotic stresses are considered to be the main source of germination and yield reduction (Pradhan et al*.,* 2020). Drought stress adversely affects crops throughout their lifecycle and causes yield losses, the extent of which varies significantly depending on the duration and intensity of the stress, the genetic background, and the developmental stage. The physiological processes primarily affected by drought or salinity stresses include ion toxicity, osmotic stress, nutrient deficiency, and oxidative stress (Boukari et al*.,* 2019). In general, seed germination and seedling emergence and establishment are less tolerant to abiotic stress as compared to adult plants; thus, water deficit stress during these early stages may result in high mortality rates and low crop performance (Tounekti et al*.,* 2020).

Zhang et al*.* (2015) reported that seed priming is the known method for rapid germination and sustained establishment of the plant in stress conditions and it is among the most influential factors on agricultural production. Mostly primed seeds demonstrate a faster and more harmonized germination and the emerged seedlings are more vigorous and tolerant to abiotic stresses than seedlings emerged from unprimed seeds (Tounekti et al*.,* 2020). Different priming methods include hydro-priming, osmo-priming, halo-priming, thermo-priming, and hormone-priming on which many studies are reported by researchers who worked on different plants (Aghighi Shahverdi and Omidi, 2015; Aghighi Shahverdi et al*.,* 2017). Nutri-priming is the newest priming method using micro or macronutrients and plant growth regulator for seed treatment before sowing (Aghighi Shahverdi et al*.,* 2017). Zahedifar (2013) reported that the seed priming with nano-potassium increased germination percentage and rate, plumule, and radical weight of corn plants significantly. The findings of Zahedifar (2013) indicated that seed priming is a useful way to increase seedling tolerance under salinity stress conditions. Ahmadvand et al*.* (2012) found that at the highest level of salinity stress, soybean radicle length of unprimed seeds was reduced by 65%, whereas, the decline was 26% in primed seeds with potassium nitrate. A few studies have demonstrated that nanomaterials have the potential to penetrate the seed coat and enhance the ability of absorption and utilization of water, which stimulates the enzymatic system and ultimately improves germination and seedling growth (Shang et al*.,* 2019). Nonetheless, the mechanism of nanomaterial-induced water uptake inside the seed is still largely unknown. The finding of Aghighi Shahverdi et al*.* (2017) leads to the conclusion that nutri-priming increases the antioxidant capacity of the plant to improve germination and seedling growth of stevia under salinity stress. Majda et al*.* (2019) reported that the Mo-priming increased means of germination, growth, and yield of bean. Many efficient priming compounds are used to broaden the application of this technique but the most studied is salicylic acid (SA). Pre-treatment of flax seeds with SA under stress conditions improved plant growth and alleviated the oxidative damage by increasing total lipid levels in addition to fatty acid unsaturation (Belkhadi et al*.,* 2010). Nazar et al*.* (2011) reported that the SA pre-treated mung bean plants exhibited changes in physiological processes to maximize the use of nitrogen and sulphur through the higher activity of nitrate reductase and ATP-sulfurylase and synthesis of glutathione.

Paclobutrazol (PBZ) [(2RS, 3RS)-1-(4-chlorophenyl)- 4, 4-dimethyl-2-(1H-1, 2, 4-trizol-1-yl)-pentan-3-ol], is one of the members of triazole family having growth-regulating property. The growth regulating properties of paclobutrazol is mediated by changes in the levels of important plant hormones including the gibberellins, abscisic acid, and cytokinins (Soumya et al*.,* 2017). Roles of PBZ in plants are altering growth regulation, improving plant water relation, improving membrane stability index, enhancing plant photosynthetic pigments, altering the level of plant growth hormones, inducing antioxidant activities, and increasing the level of proline (Hajihashemi and Ehsanpour, 2013; Soumya et al*.,* 2017). Gorzi et al*.* (2017) 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. Due to the seed germination problem in stevia and on the other hand, the lack of sufficient information about the role of SA, nano-K, and PBZ on the improvement of germination behavior and early seedling growth of stevia, the present study was conducted to survey the effect of nutri-priming with SA, nano-K, and PBZ on germination indices and physiological characteristics of stevia under drought stress.

Materials and Methods

Experimental design and seed material

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 8000 (0, -3, -6, and -9 bar) and seven combinations of priming (SA, PBZ, nano-K, SA + PBZ, SA + nano-K, PBZ + nano-K, and nano-K + SA + PBZ), and unprimed dry seeds were considered as control. The experiment was conducted at the Laboratory of Seed Science and Technology of, Islamic Azad University (Birjand Branch), Iran in 2018. Freshly matured seeds of stevia (*var*. Bertoni.) were collected in June 2018 from seed production Zargiyah Farms in Firozabad (34° 37'_N, 54° 45'_ E, 1790 m ASL). The mean seed dry weight per 100 seeds was 27.1 ± 0.5 mg and seed moisture was around 8.35%.

Stevia seeds were sterilized with sodium hypochlorite (5%) for 30 seconds and then washed with distilled water. According to the pilot experiments (three separate experiments), the best duration and concentration of seed priming with SA, PBZ, and nano-K were 24 hours at the concentration of 1 mM, 0.5 mM, and 0.5%, respectively. These data were used in the experiment (data not shown). Stevia seeds were entirely immersed in the determined concentrations of priming media (1 mM SA, 0.5 mM PBZ, 0.5% nano-K) at 15 °C in darkness. At the end of nutri-priming, the seeds were washed with distilled water and air-dried for 24 hours. In each Petri dish, 50 seeds were put on Whatman paper in Petri dishes and based on various treatments 7 mL of distilled water or PEG solution was added to each dish. The osmotic potentials of -3, -6, and -9 bars were obtained by adding 74.0, 109.7, and 137.3 g of PEG 8000 in 500 ml of distilled water, respectively. The required amount of PEG 8000 was calculated by Michel and Kaufmann (1973) formula:

$$\Psi s = -(1.18 \times 10\text{-}2) \times C - (1.18 \times 10\text{-}4) \times C2 + (2.67 \times 10\text{-}4) \times CT + (8.39 \times 10\text{-}7) \times C2T$$

where Ψs, C, and T are osmotic potential (bars), the concentration of PEG (g/L of distilled water), and temperature (°C), respectively. The distilled water potential is zero, so it was used as the control treatment (without drought stress).

The germination period was conducted in a growth chamber under controlled conditions with a temperature of 23 ± 2 °C, 16/8 h light/darkness, and 70% relative humidity. Germinated seeds count was done daily on the second day (Aghighi Shahverdi et al*.,* 2017) and finally at the end of the testing period (11 days) germination percentage, germination rate, mean germination time, mean daily germination, and seedling vigor index were calculated according to formulas in Table 1 (Aghighi Shahverdi et al*.,* 2017).

Photosynthetic pigments

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 physiological studies. Based on the method, 0.25 g of fresh tissue was extracted by using 5 ml 80% acetone.

Table 1
Equations used for various indexes of germination in the study

Germination percentage	GP = (N×100) / M
Germination rate	GR =∑Ni / Ti
Mean germination time	MGT =∑(DN) / ∑n
Mean daily germination	MDG = ∑Ni / ∑Ti
Seed vigor index	SVI= GP× Mean (SL)

N: sum of germinated seeds at the end of the experiment, M: total planted seeds, Ti: number of days after germination, Di: the number of day from the start of the test to the enumeration of nth, SL: seedling length

Then, the extract was centrifuged at 11000 rpm for 10 min. Besides, the optical density of the extract was measured at the wavelengths of 646.8, 663.2, and 470 nm (Aghighi Shahverdi et al*.,* 2017).

Peroxidase activity (POD)

To measure POD activity (EC 1.11.1.7), 0.2 g of fresh seedling was pulverized in a mortar using liquid nitrogen and then one ml of buffer Tris-HCl (0.05 M, pH = 7.5) was added. The obtained mixture was centrifuged for 21 min at 13,000 rpm, at 4 °C and the supernatant was used for enzyme activity measurements. The POD activity was determined in a reaction of the mixture which consisted of a suitable amount of 28 mM guaiacol, 5 mM H_2O_2, and 25 mM Na-phosphate buffer (pH 6.8) and enzyme (MacAdam et al*.,* 1992).

Superoxide dismutase activity (SOD)

For SOD activity (EC 1.15.1.1) measurement, the method of Beauchamp and Fridovich (1971) was used which is briefly described here. About 3 ml of the reaction mixture, containing 0.1 ml of 200 mM methionine, 0.01 ml of 2.25 mM nitro blue tetrazolium (NBT), 0.1 ml of 3 mM EDTA, 1.5 ml of 100 mM potassium phosphate buffer, 1 ml distilled water, and 0.05 ml of enzyme extraction were taken in test tubes in duplicate from each enzyme sample. The reaction was stopped by switching off the light and covering the tubes with black cloth. Absorbance was recorded at 560 nm.

Catalase activity (CAT)

The CAT activity (EC.1.11.1.6) was performed using Chance and Maehly (1995) method. Three ml reaction mixture containing 2.5 ml 0.05 mM sodium phosphate buffer (pH 7) and 30 µg protein solution was added to glass bottle and at the time of measurement, 30µl 30% H_2O_2 was added to the reaction mixture and the absorbance at 240 nm, at 60 seconds, and at 25 °C was recorded spectrophotometrically. The control contained 2.5 ml of sodium phosphate buffer and 30 µg protein. Catalase activity was reported based on absorption alternations per mg protein per min.

Proline content

Proline content was determined according to the method described by Bates et al*.* (1973). Approximately 0.25 g of fresh seedling material was homogenized in 10 ml of 3% aqueous sulfosalicylic acid. Finally, 2 ml of filtrated solution was mixed with 2 ml acid-ninhydrin and 2 ml of glacial acetic acid in a test tube. The mixture was placed in a water bath for 1 h at 100 °C. The reaction mixture was extracted with 4 ml toluene, cooled to room temperature, and the absorbance was measured at 520 nm with a spectrometer.

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 the least significant difference test (LSD) at 0.05 statistical probability level and the graphs were drawn by MS–Excel.

Results

Germination indices

The effects of drought stress due to PEG, nutri-priming treatments and the interplay between them were significant on germination percentage and rate and mean germination time parameters. Also, drought stress and nutri-priming had highly significant effects on mean daily germination, seedling length, and seedling vigor index (Table. 2). Results showed that the germination percentage of stevia seeds significantly decreased by increasing drought stress levels. However, the amount of this trait was higher in the primed seeds than in unprimed treatment under normal and drought stress conditions. The highest germination percentage was related to seeds that were treated with nano-K + SA + PBZ under control and -3 bar drought treatments (62.4 and 64.0%, respectively). In all drought levels, the lowest amounts of germination percentage were found in unprimed seeds. The minimum germination percentage was obtained from unprimed seeds at -9 bar (29.05% reduction

Table 2
Effects of different levels of drought stress (control, -3, -6, and -9 bar) and nutri-priming treatments on germination indices of stevia (Stevia rebaudiana Bertoni)

Treatments	Germination percentage (%)	Germination rate (seed/day)	Mean germination time (day)	Mean daily germination (number)	Seedling length (cm)	Seedling vigo index
Drought stress (bar)						
Control (0)	50.3±1.9 a	3.0±0.1 a	4.7±0.1 c	4.6±0.2 a	12.3±0.6 a	631.4±49.2 a
-3	48.2±1.9 a	2.7±0.1 b	4.9±0.1 b	4.4±0.2 a	10.6±0.6 b	520.2±40.2 b
-6	39.8±1.6 b	2.0±0.1 c	5.5±0.1 a	3.6±0.1 b	7.7±0.4 c	307.0±21.7 c
-9	35.0±1.2 c	1.7±0.1 d	5.5±0.1 a	3.2±0.1 c	6.0±0.5 d	212.3±19.0 d
LSD (p≤0.05)	3.50	0.21	0.19	0.31	1.34	58.85
Nutri-priming treatments						
Nano-K	40.3±2.6 de	2.0±0.2 de	5.4±0.1 ab	3.7±0.2 de	8.5±1.1 b	348.1±55.8 c
SA	37.7±1.9 ef	1.9±0.1 ef	5.2±0.2 bc	3.4±0.2 ef	9±0.9 b	343.7±44.6 c
PBZ	48±2.9 b	2.7±0.2 b	4.9±0.1 cd	4.4±0.3 b	9.6±1.1 b	481.7±75.8 b
Nano-K+SA	42.7±2.9 cd	2.3±0.2 cd	5.1±0.1 cd	3.9±0.3 cd	9.2±0.8 b	405.3±51.9 bc
Nano-K +PBZ	45.3±2.2 bc	2.5±0.2 bc	4.9±0.2 cd	4.1±0.2 bc	9.3±1.1 b	443±75 bc
SA+PBZ	44±2.6 bcd	2.5±0.2 bc	4.8±0.1cd	4.0±0.2 bcd	9.8±0.9 ab	439.7±51.9 bc
Nano-K+SA+PBZ	53.3±3.3 a	3.1±0.3 a	4.9±0.2 d	4.8±0.3 a	11.7±0.9 a	652.3±86.2 a
Unprimed as control	35.3±2.1 f	1.7±0.1 f	5.6±0.2 a	3.2±0.2 f	6.2±0.8 c	228.0±38.4 d
LSD (p≤0.05)	4.96	0.30	0.27	0.45	1.90	105.9
Drought × Nutri-priming						
	*	*	**	NS	NS	NS

Means followed by the same letter in each column are not significantly different according to LSD test at 5 % level
NS: non-significant; * and **: significant at 0.05 and 0.01, respectively.

compared to unprimed seeds under the osmotic potential of 0 bar) (Fig. I).

Results indicated that the drought stress was decreased the germination rate while nutri-priming increased this index (Table 2). The unprimed seeds in all levels of drought stress showed the lowest mean of this trait whereas the highest germination rate (4.2 seed per day) was related to 3-X nutri-priming (integrated application) under non-drought stress.

Results indicated that the integrated application of nutri-priming agents was more effective than their separate application to alleviate the drought-induced damaging effects on the germination rate.

In high levels of osmotic potential, integrated nutri-priming showed a 50.0% increase compared to the un-primed seeds (Fig. I). As shown in Table 2, in response to drought stress, mean germination time significantly increased while nutri-priming decreased.

Different results were obtained by the interaction effects of drought stress and nutri-priming. In 0 and -9 bar osmotic potential, the highest mean germination time was related to unprimed seeds (5.3 and 6.6 days, respectively)

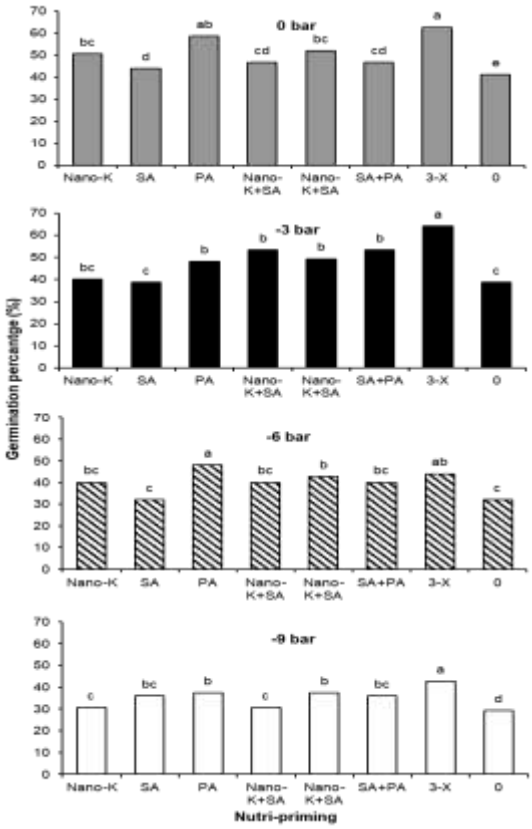

Fig. I. Germination percentage of stevia seeds under different drought levels (0, -3, -6, and -9 bar PEG) and nutri-priming treatments (different letters in each factor indicate significant differences at $p < 0.05$)

Table 3
Effects of different levels of drought stress (control, -3, -6, and -9 bar) and nutri-priming treatments on physiological traits of stevia (*Stevia rebaudiana* Bertoni)

Treatments	Total chlorophyll content (µg/g FW)	Chlorophyll a content (µg/g FW)	Chlorophyll b content (µg/g FW)	Carotenoids content (µg/g FW)	Proline content (µmol/g FW)	Peroxidase activity (U/mg protein.min)	Catalase activity (U/mg protein.min)	Superoxide dismutase activity (U/mg protein)
Drought stress (bar)								
Control (0)	17.5±1.1 a	12.5±0.7 a	5.0±0.4 a	2.2±0.2 a	115.6±9.1 d	2.8±0.2 b	0.83±0.1 d	1.5±0.1 c
-3	10.1±0.7 b	7.5±0.7 b	2.6±0.2 b	1.8±0.2 b	226.3±11.2 c	3.0±0.2 b	1.15±0.1 c	3.2±0.1 b
-6	5.4±0.3 c	4.0±0.3 c	1.4±0.1 c	1.3±0.1 c	319.9±15.5 b	3.0±0.1 b	1.44±0.3 b	3.1±0.1 b
-9	6±0.5 c	4.3±0.3 c	1.7±0.2 c	0.9±0.1 d	353.6±16.1 a	8.0±0.5 a	1.53±0.1 a	6.1±0.5 a
LSD (p ≤0.05)	0.93	0.71	0.42	0.24	16.31	0.43	0.08	0.29
Nutri-priming treatments								
Nano-K	7.8±0.9 c	5.8±0.7 d	2.0±0.2 cd	1.3±0.1 cde	122.6±15.3 f	4.4±1.2 a	1.45±0.1 a	3.8±0.9 b
SA	8.2±1.4 c	5.7±1.0 d	2.4±0.4 c	1.2±0.2 de	225.2±30 e	4.3±0.8 ab	1.38±0.6 b	4.7±1.2 a
PBZ	9.8±1.7 b	7.2±1.2 c	2.6±0.5 bc	1.7±0.2 bc	256.1±29.6 cd	4.4±0.7 a	1.35±0.1 a	3.7±0.4 b
Nano-K+SA	10.8±1.7 b	8.3±1.3 b	2.5±0.4 c	2.0±0.3 ab	253.2±33.2 d	4.1±0.8 abc	1.12±0.1 b	2.7±0.3 e
Nano-K+SA +PBZ	10.7±1.9 b	7.3±1.4 bc	3.3±0.6 a	1.4±0.3 cde	277.9±27.5 bc	3.8±0.7 bc	1.12±0.1 b	3±0.4 de
SA+PBZ	10.2±1.9 b	7.0±1.3 c	3.2±0.6 ab	1.38±0.2 cd	297.6±32.6 ab	4.6±0.5 a	1.17±0.1 b	3.1±0.3 cde
Nano-K +SA+PBz	14.1±2.4 a	10.5±1.6 a	3.6±1.0 a	2.4±0.3 a	301.6±31.3 a	4.4±0.8 a	1.18±0.1 b	3.2±0.4 cd
Unprimed as control	6.4±0.6 d	4.8±0.5 d	1.6±0.2 d	1.0±0.2 e	296.7±32 ab	3.6±0.2 c	1.14±0.1 b	3.4±0.5 bc
LSD (p ≤0.05)	1.32	1.01	0.60	0.34	23.06	0.61	0.12	0.41
Drought × Nutri-priming	**	**	**	**	**	**	**	**

Means followed by the same letter in each column are not significantly different according to LSD test at 5 % level
NS: non-significant;* and **: significant at α=0.05 and 0.01%.

while in -6 bar treatment with SA no significant difference was observed . At - 9 bar, the seeds primed with 3-X had the shortest required time for germination (5.1 days), which was significantly lower than unprimed seeds at this level.

In response to drought stress, mean daily germination significantly decreased so that the lowest level of this trait was achieved in -9 bar (3.2 seeds) and the highest in 0 and -3 bar treatments (4.6 and 4.4 seeds, respectively). On the other hand, nutri-priming increased the mean of the trait so that the highest mean daily germination was found in the integrated application (3-X) which increasing by 33.3% compared to the unprimed seeds (Table. 2). Based on the comparison of the mean (Table. 2), drought stress was found to severely limit the seedling growth of stevia. The greatest seedling length belonged to the control treatment of drought stress (12.3 cm), and the lowest seedling length (6.0 cm) was observed at the highest level of drought stress (-9 bar). We observed that increasing in seedling length under influence of nutri-priming. Nutri-priming by 3-X caused the highest seedling length (11.7 cm) showing 47.0% increases compared to the unprimed seed. As shown in Table. 2, depending on the reduction in germination percentage and seedling length under drought stress, the seedling vigor index was gradually reduced with increasing drought stress levels, so that the lowest means were recorded at -9 bar (66.3% reduction compared to 0 bar osmotic potential). On the other hand, nutri-priming treatments, especially the integrated application of nano-K, SA, and PBZ increased seedling vigor index compared to unprimed seeds (65.0%).

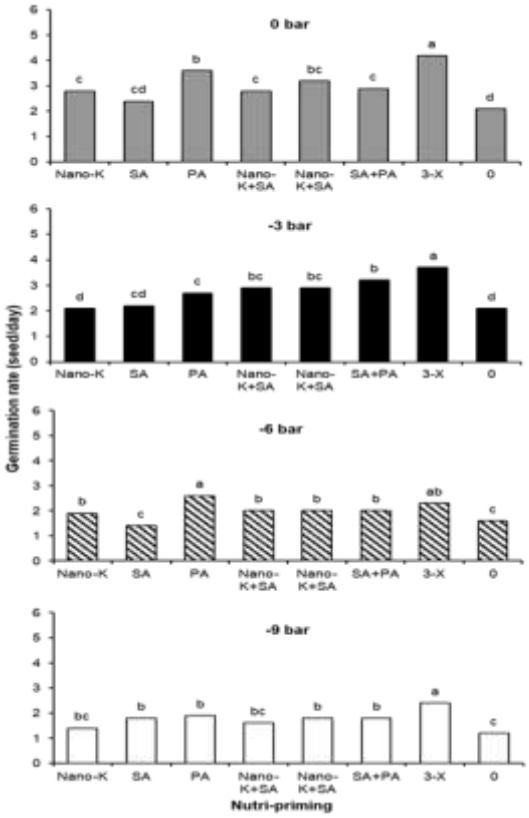

Fig. II. Germination rate of stevia seeds under different drought levels (0, -3, -6, and -9 bar PEG) and nutri-priming treatments; different letters in each factor indicate significant differences at $p<0.05$

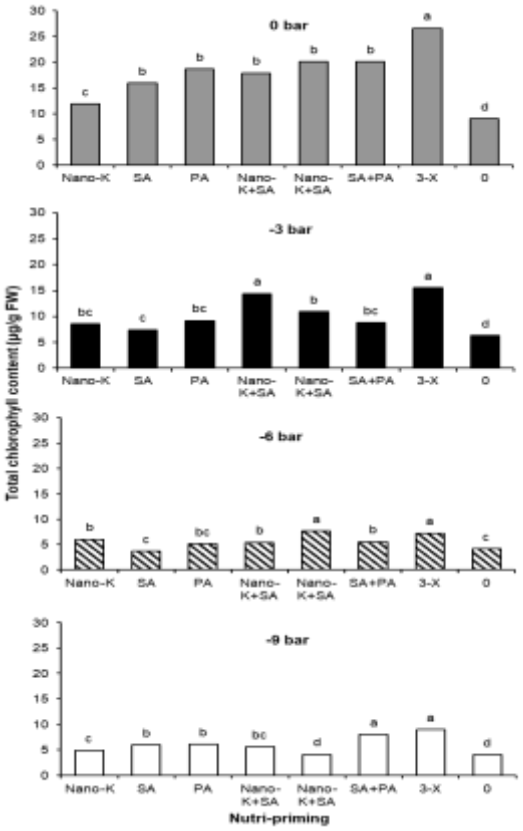

Fig. III. Total chlorophyll of stevia seeds under different drought levels (0, -3, -6, and -9 bar PEG) and nutri-priming treatments (different letters in each factor indicate significant differences at $p < 0.05$)

Physiological characteristics

According to the analysis of variance, interaction effects of drought stress and nutri-priming were significant on the content of total chlorophyll, chlorophyll a, chlorophyll b, carotenoids, and proline and activity of POD, CAT, and SOD enzymes (Table. 3). Results indicated that the drought stress caused a decrease in the photosynthetic pigments such as total chlorophyll, chlorophyll a and b, and carotenoids while nutri-priming increased these pigments (Table. 3). In interaction effects, the highest total chlorophyll and chlorophyll a and b contents (26.6, 17.7, and 8.9 µg/g FW) were related to the co-application of nano-K, SA, and PBZ under no stress conditions (0 bar). However, nutri-priming treatments alleviated effectively the damaging effect of drought stress on photosynthetic pigment contents.

In -9 bar osmotic potential, integrated application of 3-X increased photosynthetic pigment contents (55.5, 53.8, and 57.6%) compared to the unprimed seeds. Also, the co-application of SA + PBZ resulted in the highest means of total chlorophyll and chlorophyll a contents. Unprimed seeds under all drought levels showed the lowest means of these traits (Fig. III).

As shown in Table. 3, the carotenoid content significantly decreased with increasing PEG concentration compared with the control conditions (without drought stress). However, nutri-priming treatments alleviated effectively the damaging effect of drought stress on carotenoid content. The lowest amount of carotenoid content was achieved in unprimed seeds. In the interaction of drought and nutri-priming, the highest values of carotenoid content were recorded in the combination of two nutri-priming agents (nano-K + SA) under no drought stress conditions. In drought stress conditions, the most effective priming treatments with the highest carotenoid

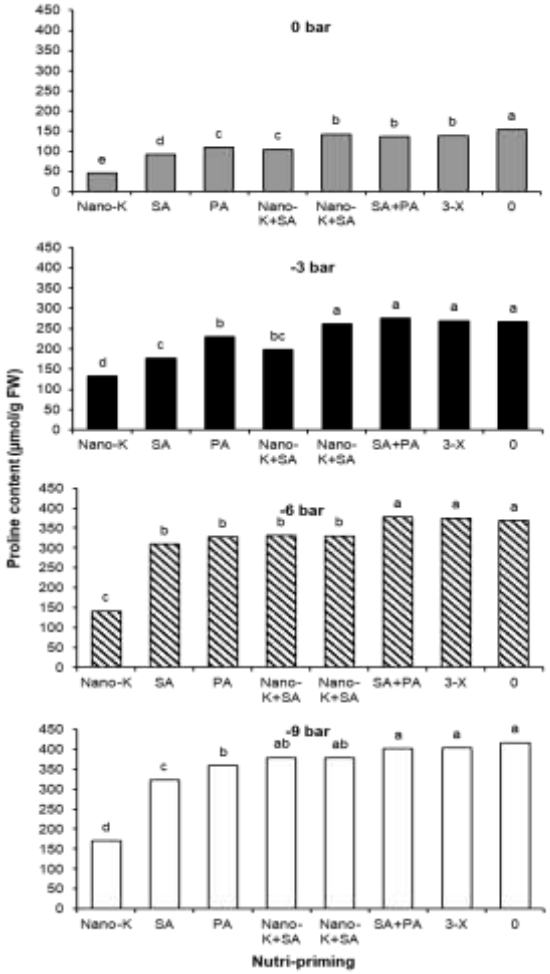

Fig. IV. Proline content of stevia seeds under different drought levels (0, -3, -6, and -9 bar PEG) and nutri-priming treatments; different letters in each factor indicate significant differences at p<0.05.

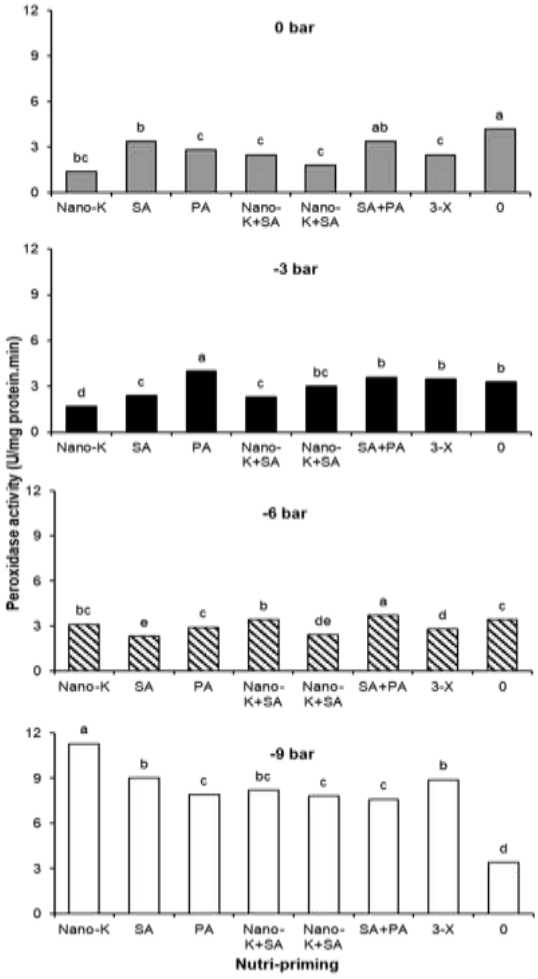

Fig. V. Peroxidase activity of stevia seeds under different drought levels (0, -3, -6, and -9 bar PEG) and nutri-priming treatments (different letters in each factor indicate significant differences at p < 0.05)

content was nano-K + SA + PBZ. Drought stress due to PEG at all the studied concentrations gradually increased the accumulation of free proline in stevia seedlings. The highest proline content was achieved in unprimed seed under no drought stress conditions (4.2 µmol/g FW) and SA + PBZ and 3-X treatments under drought stress levels (-3, -6, and -9 bar). Seeds primed with nano-K showed the lowest means of the traits in all drought levels (Fig. IV). As shown in Table 3, antioxidant activities significantly increased with increasing drought stress.

The activity of POD, CAT, and SOD enzymes significantly increased in primed and unprimed seedlings with increasing osmotic potential. On the other hand, seed nutri-priming promoted the activity of antioxidant enzymes in the stevia seedlings under drought stress, so that the maximum activity of POD (11.3 U/mg protein.min), CAT (2.0 and 2.1 U/mg protein.min, respectively), and SOD (11.4 U/mg protein) were obtained from primed seeds with nano-K, nano-K and SA, and SA treatments, respectively at the osmotic potential of - 9 bar.

Results indicated that the integrated application of nano-K, SA, and PBZ decreased the activity of antioxidant enzymes compared to the single application under a high level of drought. Compared with the control seeds (without priming), SA + PA, nano-K+ SA, and 3-X were the most effective treatments regarding the antioxidant enzyme activities at the highest level of drought stress (Figs. V and VI).

Discussion

The present study aimed to survey the effect of nutri-priming with SA, nano-K, and PBZ on germination indices and physiological characteristics of stevia under drought stress. 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 germination percentage, germination rate, mean germination time, seedling length, and seedling vigor index were significantly decreased along with the increase in PEG concentrations (Table. 3).

In the present study, it was found that the average germination percentage was dropped to below 30% 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 reactive oxygen species (ROS) levels under drought stress (Guo et al*.,* 2017). Muscolo et al*.* (2014) reported that the alteration of some enzymes and hormones found in the seed could lead to the reduction of final germination under drought stress conditions.

Also in this study, seedling length, and seedling vigor index (the product of seedling length and germination percentage) which are the important traits in the primary establishment of seedling (Gorzi et al*.,* 2017) reduced with increased drought stress. According to Toscano et al*.* (2017), this result could be ascribed to a lack of energy to start the germination process, as energy is generally obtained by an increase in the respiratory pathway after the imbibition, and in the presence of low levels of water potential water absorption proceeds slowly. The differences in mean germination time compared to the control became progressively wider with decreasing water potential. It has been reported that drought stress not only affects seed germination but also increases the mean germination time in canola plants (Willenborg et al*.,* 2004). According to Toscano et al*.* (2017), drought stress experienced by seeds during germination does not always indicate a depressed seedling growth, in terms of length, at favorable conditions. Indeed, in our study, the seedling length was significantly depressed only in seeds germinated at the lowest water potential (-9 bar).

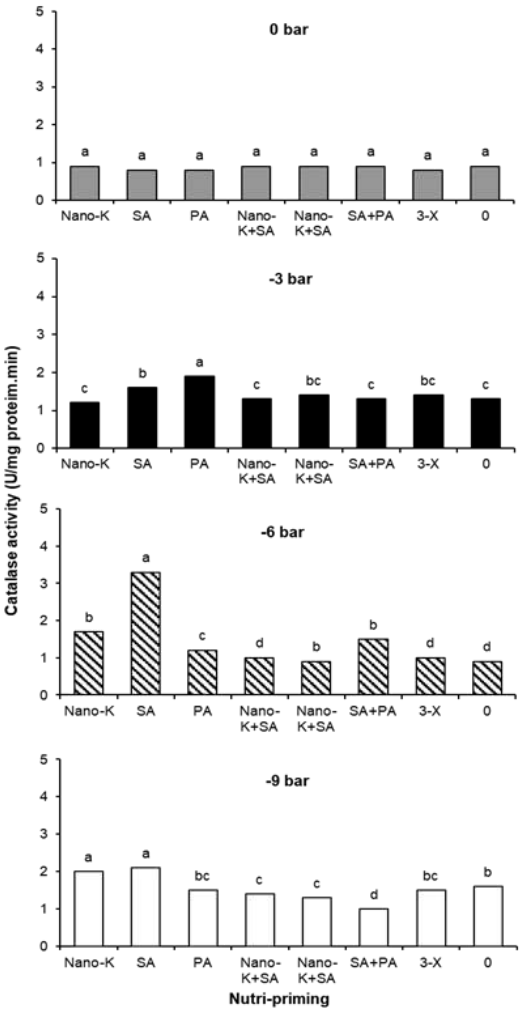

Fig. VI. Catalase activity of stevia seeds under different drought levels (0, -3, -6, and -9 bar PEG) and nutri-priming treatments; different letters in each factor indicate significant differences at p<0.05.

According to Maswada et al*.* (2018), seed germination entails three distinct phases: (i) imbibition, (ii) lag phase, and (iii) radicle growth and emergence. The purpose of priming is to prolong the lag phase, which allows some pregerminative physiological and biochemical processes to take place before radicle protrusion. Increased and synchronized germination of primed seeds occurs due to (i) reduction in the lag time of imbibition, (ii) activation of an enzyme involved in seed germination, (iii) build-up of germination-enhancing metabolites, (iv) metabolic repair during imbibition, and (v) osmotic adjustment (Aghighi Shahverdi et al*.,* 2017; Maswada et al*.,* 2018). In the present study, the comparative performance of different nutri-

priming treatments for improving drought tolerance at germination and early growth stages of stevia was also evaluated. Seed nutri-priming has two beneficial effects, firstly it enhances germination indices (such as germination percentage and rate and seed vigor index) and secondly, it moderates the adverse effects of drought stress due to PEG. Seed priming with nano-K, SA, and PBZ not only improved the measured germination indices and seedling growth of stevia in both control and stress conditions, but also alleviated drought stress damages. The enhanced germination of primed seeds under stress conditions is due to the increase in the activity of antioxidant enzymes, membrane-bound enzymes, and proline contents (Naguib and Abdalla, 2019). In this study, similar results were obtained and a positive and high correlation was observed between the activity of antioxidant enzymes and proline content, and these traits showed a negative and significant correlation with germination percentage. This eliminates the negative effects of the overproduction of reactive oxygen species (Mustafa et al*.,* 2017). Similar findings were also reported by Aghighi Shahverdi et al*.* (2017) and Gorzi et al*.* (2017) in stevia seed.

In this regard, it was observed that the integrated application of priming agents in most cases was more effective than their separate application. The beneficial effects of seed nutri-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 (Gorzi et al*.,* 2017). As Table. 2 suggests, at all drought stress levels, the highest values of germination percentage, germination rate as well as seedling length and seedling vigor index were noticed in seeds primed with nano-K + SA + PBZ. 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 expansion, cell division, and meristematic growth, which cause an increase in plant growth (Aghighi Shahverdi et al*.,* 2017; Gorzi et al*.,* 2017). Various ameliorating agents such as SA (Singh et al*.,* 2017) and PBZ (Hajihashemi and Ehsanpour, 2013) have been used for combating various abiotic stresses including drought stress. The increase in seedling vigor by 3-X over control was mainly due to the enhanced seedling biomass (Table. 2). The increased germination rate due to nanoparticles is related to their penetration into seeds (Maswada et al*.,* 2018). Furthermore, Alidoust and Isoda (2014) demonstrated that small particle size of nano-K may lead to a higher bioavailability of potassium to seeds and can increase the motion and uptake of water, nutrients, and oxygen via pores in the seed coat.

Fathi and Tari (2016) reported that the reduction of chlorophyll content has been considered a usual symptom of oxidative stress under drought stress. In the current study, the photosynthetic pigment contents (chlorophyll a, b, and total) were significantly diminished with an increase in the level of applied PEG as compared to the control seedlings. Chlorophyll loss was shown to be accompanied by the damage of the mesophyll chloroplasts, which leads to a lower photosynthetic rate under stress (Wang and Blumwald, 2014). Also, the reduction in chlorophyll content under drought stress has been considered as a typical symptom of oxidative stress and may be the result of pigment photo-oxidation and chlorophyll degradation. Zafari et al*.* (2020) indicated that drought stress caused a reduction in photosynthetic pigments such as chlorophyll a, b, and total and carotenoids in safflower.

The chlorophyll content significantly increased in nano-K-treated seedlings as compared to the no priming treatment under the same concentration of drought. Based on these results, we conclude that the integrated application of 3-X nutrients is the best treatment for enhanced photosynthetic pigment contents under drought stress. Similar findings were also reported by Acharya et al*.* (2020). The nutri-priming treatments (especially nano-K + SA + PBZ) did not only moderate the adverse effect of drought stress on the chlorophyll content, but also had a significant stimulatory effect on the biosynthesis of chlorophyll, so that under severe drought stress, the highest amount of photosynthetic pigment contents were recorded in the seedlings which were raised from primed seeds by nano-K + SA + PBZ. The PBZ application was found to enhance photosynthetic pigment

contents in *Triticum aestivum* (Nouriyani et al*.,* 2012). Its treatment also increased the content of chlorophylls under drought stress in *Festuca arundinacea* and *Lolium perenne* (Shahrokhi et al*.,* 2011). Paclobutrazol significantly enhanced chlorophyll a, b, and carotenoids in wheat cultivars (Aly and Latif, 2011). Furthermore, PBZ application was found to increase carotenoid content in *Catharanthus roseus* (Jaleel Manivannan et al*.,* 2007) and *Sesamum indicum* (Abraham et al*.,* 2008). Carotenoids have additional roles in i) scavenging ROS, ii) stabilizing photosynthetic complexes, iii) participating in energy dissipation, and iv) helping the plants to alleviate the negative effects of drought stress (Zafari et al*.,* 2020).

According to Gorzi et al*.* (2017), ROS is generated in both normal and stress conditions in the plant cells, but in response to different environmental stresses such as drought stress, the production of ROS is significantly enhanced which could result in the progressive oxidative damages. 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 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 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 (Rouhi and Sepehri, 2020). 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 nano-K + SA + PBZ) significantly enhanced the accumulation of free proline in stevia seedlings (Table 3). In agreement with our results, Gorzi et al*.* (2017) and Aghighi Shahverdi et al*.* (2017) reported that the seedlings derived from primed seeds of stevia with nutrients had the higher values of the proline content than unprimed seeds under drought and salinity stresses. It has been reported that SA treatment enhances the proline accumulation with concomitant induction of different stress enzymes (Shaikh-Abol-Hasani and Roshandel, 2019).

Omidi et al*.* (2018) found that in drought-stressed plants the concentration of proline, protein, and lipid peroxidation as well as the activity of antioxidants including SOD, CAT, and ascorbate peroxidase increased. Drought stress-induced the expression of proline biosynthetic genes such as pyrroline-5-carboxylase synthase1, 1-pyrroline-5-carboxylase synthase 2, and 1-pyrroline-5-carboxylase reductase. However, under drought stress, the activity of proline catabolic genes including proline 5-carboxylate dehydrogenase and proline dehydrogenase1 decreased, which is a defensive strategy used by plants under stress. Similarly, our results indicated that the highest level of stress significantly increased the rate of SOD and CAT. In the current study, proline content significantly and positively correlated with POD, CAT, and SOD activities. Catalase 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*.,* 2019; Shams Peykani and Farzami Sepehr, 2018). These enzymes play an important role in the scavenging of H_2O_2 (Das and Roychoudhury, 2014). In the current study, drought stress activated the activity of SOD, CAT, and POD in stevia seedlings, so that the lowest activities were observed under the controlled conditions. The increased enzymatic activity, as seen in our study, has been considered as a part of seed strategy to overcome free radicals (Simioni et al*.,* 2018).

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 (Pouramir-Dashtmian et al*.,* 2014). In this regard, Gorzi et al*.* (2017) 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 with SA increased the antioxidant activity and led to the improvement of germination parameters in sorghum, which is coherent with the present study.

In the current study, the co-application of nano-K + SA + PBZ compounds resulted in the moderate activity of antioxidant enzymes under severe drought stress conditions (-9 bar). It seems that excessive activity of antioxidant enzymes

leads to loss of photosynthetic material and, conversely, less activity will not reduce oxidative stress.

It is concluded from findings of our research that PEG-induced drought stress had inhibitory effects on all germination indices studied in stevia; nevertheless, seed nutri-priming effectively promoted germination parameters, seedling growth, the photosynthetic pigment contents, 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, SOD, and CAT and the higher accumulation of free proline. Accordingly, our results suggest that seed nutri-priming with nano-K, SA, and PBZ, as well as the concurrent application of them at appropriate concentrations can improve the drought tolerance of stevia at germination stage. Therefore, for the development of stevia germination practice under drought stress, co-application of nano-K + SA + PBZ induced physiological activities may be utilized for enhancing the germination characteristics. However, further studies under field conditions are necessary to make a practical recommendation.

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