

Morphological Properties of Stevia (*Stevia Rebaudiana Bert.*) Affected by Foliar Application of Iron, Zinc, and Salicylic Acid under Drought Stress

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Abstract

Background: Stevia (*Stevia rebaudiana* Bert.) is an important source of natural sugar, for the use of people, especially those with diabetes. However, there is no data on the growth of stevia under drought conditions affected by plant growth regulators (PGR).

Methods: A two-year experiment using 5 kg pots was conducted under field conditions. The objective was to determine the morphological properties of stevia affected by the foliar use of iron (FeSO₄, 4 mg/L), zinc (ZnSO₄, 3 mg/L), and salicylic acid (1 mM) under different levels of drought stress including control (field capacity, 0.5 atm), 3.5, 6.5, and 10 Atm. Soil water potential was investigated during the experiment using an eco-tensiometer, set with a computer. Different plant morphological properties including plant height (PH), number of leaf per plant (NLP), number of stem per plant (NSP), aerial part fresh weight (AFW), aerial part dry weight or biological yield (ADW), leaf dry weight (LDW), stem dry weight (SDW), and the ratio of LDW/ SDW were determined.

Results: Stress, PGR, and their interactions significantly affected the measured parameters. Accordingly, drought stress significantly decreased stevia growth, and there was not much of difference among different growth-related factors, under the stress, as they followed a similar trend of growth. The reduction of different growth parameters at the highest level of stress was in the range of 57-76%, related to the control. However, the use of PGR, especially the combined treatments, significantly increased the growth of the stressed plants related to the control treatment, with an increase range of 9.14-22.61% by the triple treatment.

Conclusion: Among the single treatments, the iron treatment, and among the combined treatments, the combination of the three PGR, followed by Fe+Zn was the most effective ones. It is possible to increase stevia growth under drought stress using the PGR including Zn, Fe and salicylic acid tested in this research.

Keywords: Combined PGR treatments, Eco-tensiometer, Field conditions, Natural sugar, Soil water potential.

Introduction

Stevia (*Stevia rebaudiana* Bert.) is a perennial shrub, originally from Paraguay, South America. It is an important medicinal plant containing different bio-chemicals including sweeteners, which are especially suitable for people with diabetes. Such sweeteners are of economic significance because they are non-carcinogenic and low in calories [1]. A range of different properties including being anti-carcinogenic [2], antimicrobial [3], anti-diabetic [4], anti-hyperglycemic, and antifungal have been indicated for stevia [1]. Such properties collectively indicate the suitable effects of sweeteners found in stevia for human health. Accordingly, different international food and pharmacy related organizations have authorized the use of steviol glycosides as natural sweetener products for use in food industry [5]. The most abundant diterpenoid glycosides found in stevia are the secondary metabolites including stevioside and rebaudioside, which are a few hundred times sweeter than conventional sugar (sucrose) without any side effects such as high blood pressure [6]. However, the growth and medicinal properties of stevia may be affected under stress.

Fallah *et al.* [7] investigated the effects of salt stress on the growth and the expression of the genes, which are responsible for the production of stevioside and rebaudioside in stevia. They found that under salinity stress, stevia growth and the expression of the genes, which result in the production of such steviol glycosides significantly decreased. The highest rate of stevioside and rebaudioside was produced under non-salinity stress. However, stevia was still able to produce stevioside and rebaudioside under salt stress.

Iron (Fe) and zinc are among the essential micronutrients for plant growth

and yield production as they participate in different plant functions including the process of photosynthesis as a part of chlorophyll structure, the activity of different enzymes, etc. [8]. However, under stress, and due to the decreased uptake of such micronutrients, plant growth and yield production decrease [9-11].

Salicylic acid (SA) is one of the plant hormones, regulating different plant activities including plant systematic resistance under different conditions including stress [12]. The alleviating effects of SA on plant growth have been indicated by research. For example, the SA effects on the alleviation of drought stress is by the production of different amino acids such as (proline) and sugar (sucrose), regulating plant osmotic potential [13,14]. With respect to the above-mentioned details, and since to our knowledge, there is not data on the use of Fe, Zn, and SA affecting stevia growth under drought stress, this study was conducted.

The objective was to investigate the alleviating effects of such PGR on stevia morphology under drought stress in a two-year experiment.

Materials and Methods

Experimental Site

A two-year pot experiment was conducted in a research field located in the suburb (Kamalshahr) of Karaj, Iran, to determine the morphology of stevia (*Stevia rebaudiana* Bert.) affected by plant growth regulators (PGR) under drought stress. The experimental site is located in the northern latitude and eastern longitude of 35°50'08" and 51°00'37', respectively, 1312 m above the sea level (Figure 1). The meteorological data of the experimental 94 site is presented in Table 1.

Table 1 The temperature and rainfall data of the region in the two-year trial

Att. Month/Year	Temperature (°C)		Rainfall (mm)	
	2021	2022	2021	2022
March	12.9	13.1	46.2	97.1
April	19.7	20.6	29.6	35.2
May	23.9	24.9	0.0	0.0
June	25.9	27.5	0.0	0.6
July	26.3	26.4	0.8	0.0
August	23.3	23	0.6	0.0
September	15.5	16.2	9.6	12.2
October	6.8	10.1	4.4	3.2

**Figure 1** Different stages of the experiment

Experimental Treatments

The following experimental treatments were examined: (i) drought stress (on the basis of soil moisture potential) at control (D1, field capacity, 0.5 atm), mild stress (D2, 3.5 atm), relatively sever stress (D3, 6.5 atm), and sever stress (D4, 10 atm), and (ii) foliar use (single and combined) of PGR including iron (Fe), zinc (Zn), and salicylic acid (SA) including control (A1), Fe (A2), Zn (A3), SA (A4), Fe + Zn (A5), Fe + SA (A6), Zn + SA (A7), and Fe + Zn + SA (A8). Accordingly, the experiment was a factorial on the basis of a completely randomized block design with three replicates including total of 96 pots. Iron and zinc were supplied using FeSO₄ (4 mg/L) and ZnSO₄ (3 mg/L), respectively, and SA was used in the form of powder (1 mM). The treatments were prepared using distilled water, and used under clear sky in the evening (at 5-7 o'clock) to avoid sunlight and the evaporation of experimental treatments from the leaf surface. The solutions were applied on the plants using a handy pump at a constant pressure until the leaf surface became saturated and drops of the treatments appeared on the leaf surface.

A surfactant (tween 20), with the volume ratio of 0.5 % was used for a better absorption of the experimental treatments on the leaf surface. The pots were irrigated right after using the treatments to increase the utilization efficiency of the experimental treatments by plant.

The pot soil was collected from the field (0-30 cm), and its physical and chemical properties along with the irrigation water were determined using the standard methods (Table 2) [15].

The experimental soil was air-dried and mixed regularly on a piece of plastic for one week. The pots were made of PVC (polyvinyl chloride) with the diameter and height of 30 cm containing 4.5 kg of soil. The seedlings used for the experiment were produced by the method of tissue culture, and tested by the adaptation method. Accordingly, two seedlings were planted in each pot on the 10th of June. The experimental treatments were foliarly used at two different stages including 30 and 45 days after planting (V3 stage). During the use of the treatments the surface soil of each pot was covered with plastic to avoid soil absorption.

Table 2 Physical and chemical properties of the experimental soil and water

Soil									
Texture	Clay	Silt %	Sand	EC (ds/m)	Total N %	O.C. %	pH	K ppm	P
Loamy	16	16	68	1.2	0.08	0.64	8	149.34	3.74
CaCO ₃	Avail	Avail	Zn-DTPA	Cu-DTPA	Mn-DTPA	Fe-DTPA			
12.20	3.74	149.34	0.44	0.5	11	2.4			
Water									
SAR	Cations	Na ⁺	Ca ²⁺ +Mg ²⁺	Anions Meq/L	SO ₄ ²⁻	Cl ⁻	HCO ₃ ³⁻	pH	EC (ds/m)
1.2	8.3	16	6.1	8.5	2.3	1.5	4.7	7.6	0.69

EC: electrical conductivity, OC: organic carbon, and SAR: sodium adsorption

The drought stress was imposed at the time of using the second stage of the foliar treatments (V3 stage). The pots were uniformly irrigated before treating the pots with drought stress. Drought stress levels were determined using an eco-tensiometer (Germany, Model EQ15, SN: 02385), which was inserted to a depth of 30 cm (root depth). The soil was then completely saturated, and the trend of soil suction and soil moisture was investigated with a one-hour interval for a 60-d period. The collected data by the instrument sensor and microprocessor were analyzed by the computer, set with the tensiometer, and the related graphs were plotted to determine the irrigation time and frequency (drought stress), until harvest.

Sampling

The plant samples were collected from each pot, at the end of the vegetative growth (V3), after a 30-d stress period, 75 days after planting the seedlings in the pots. The following morphological traits were investigated during the two-year trial: 1) plant height (PH), 2) number of leaf per plant (NLP), 3) number of stem per plant (NSP), 4) aerial part fresh weight (AFW), 5) aerial part dry weight or biological yield (ADW), 6) leaf dry weight (LDW), 7) stem dry weight (SDW), and 8) the ratio of LDW/SDW. The dry samples were prepared using oven at 65°C for 48 h.

Statistical Analysis

Data were subjected to analysis of variance using SAS. The homogeneity of variances was determined using Bartlett's test. Means were compared using Least Significant Difference (LSD) at $P = 0.05$.

Results

According to the analysis of variance drought stress and PGR significantly affected all the measured growth parameters. The interaction effects of year and drought stress was not significant on any of the measured parameters. However, the interaction of year and PGR significantly affected only NLP, and the interaction of PGR and drought stress significantly affected all the parameters, except PH. The interaction of year, PGR and drought was significant on none of the parameters (Table 3).

The stress significantly affected NSP and the ratio of LDW/SDW in both years; however, the use of PGR just was significant on the ratio of LDW/SDW in the first year. The interaction of drought and PGR was significant on none of the two parameters (data not shown). The highest and the least PH values were resulted by D1 (60.62 cm) and D4 (25.89 cm), significantly different from each other. Similarly, the significantly different corresponding values for NLP (375.15 and 120.2), LDW (7.5 and 2.08 g), SDW (6 and 2.28 g), AFW (49.96 and 11.62 g), and ADW (13.23 and 4.46 g), and were resulted by D1 and D4 (Figure 2A-E).

The combined use of PGR resulted in the highest PH values ranging from 44.42 to 45.54 cm significantly higher than the values resulted by control and the single use of PGR. The highest NLP (351.46 and 376.2), LDW (6.03 and 6.27 g), SDW (5.10 and 5.13 g), and ADW (11.13 and 11.40 g) values were related to treatments A7 and A8 significantly higher than the other treatments.

Table 3 Analysis of variance indicating the effects of experimental treatments and their interactions on stevia plant morphological properties

S.V.	d.f.	pH	LDW	NLP	SDW	AFW	ADW
Y	1	0.617	6.50	27912.6	5.798**	86.8	14.69
Block	2	130.81	4.1	30246.1	1092.234	110.97	10.19
D	3	11501.96**	220.30**	56457.3**	4.684**	13386.08**	681.32**
Y*D	3	27.77	0.896	2294.2	51452.848	10.44	2.47
A	7	284.6**	38.33**	185912.4**	2.534**	1605.73**	38.33**
Y*A	7	43.76	0.094	5910.6*	5.071	5.06	0.488
A*D	21	27.65	2.66**	12235.5**	108424.972**	144.67**	6.78**
D*Y*A	21	11.5	0.063	838.14	6268.017**	2.60	0.211
Error	124	22.45	0.521	2435.9	4.145	12.69	1.29
C.V.	-	11.22	15.85	20.57	1.44	12.55	13.42

S.V.: Source of variation, d.f.: degree of freedom, D: drought stress, Y: year, A: foliar use, PH: Plant Height, NLP: Number of Leaves per Plant, LDW: Leaf Dry Weight, SDW: Stem Dry Weight, AFW: Aerial part Fresh Weight, and ADW: Aerial per Dry Weight

Each column with the different letters indicates significant differences by Duncan multiple range test at ($p < 0.05$)

*, **significant at $p \leq 0.05$ and $p \leq 0.01$, respectively

Treatment A8 (40.25 g) and A1 (17.64 g) resulted in the highest and least AFW values significantly different from each other (Figure 2A-E). The stress significantly decreased NSP and the ratio of LDW/SDW in both years as the highest and the least ones were resulted by D1 and D4, respectively. The PGR treatments, except the single use of SA and Zn, significantly increased the ratio of LDW/SDW only in the first year, compared with control (data not shown).

Discussion

Investigating the effects of drought stress [16] on the growth of stevia, as an important source of sweeteners, is of significance, especially in the semi-arid and arid areas of the world. There is not much data on the growth of stevia under drought conditions, and to our knowledge, there is not any data on the use of PGR used for the alleviation of drought stress on stevia growth under drought stress, which was investigated in this study.

According to our results, drought stress significantly decreased stevia growth especially at the relatively sever

(6.5 atm) and sever (10 atm) stresses indicating that the plant is not tolerant under drought stress. The important aspect of plant physiology, which is affected by drought stress, and affects plant morphology, is the uptake of essential micronutrients (Fe and Zn), and the functioning of plant hormones including SA. The micronutrients and SA can affect plant growth and physiology under different conditions including stress [11,17]. Accordingly, it was proposed that if stevia is supplied by such PGR, it may be possible to alleviate the effects of drought stress on plant growth.

The authors [18] just recently indicated the significant effects of priming with the PGR tested in this research on the seed germination and seedling growth of stevia under drought stress. They found that although drought stress significantly decreased seed germination and seedling growth of stevia, the use of PGR, especially Zn+Fe+SA, alleviated the stress by increasing seed germination and seedling growth.

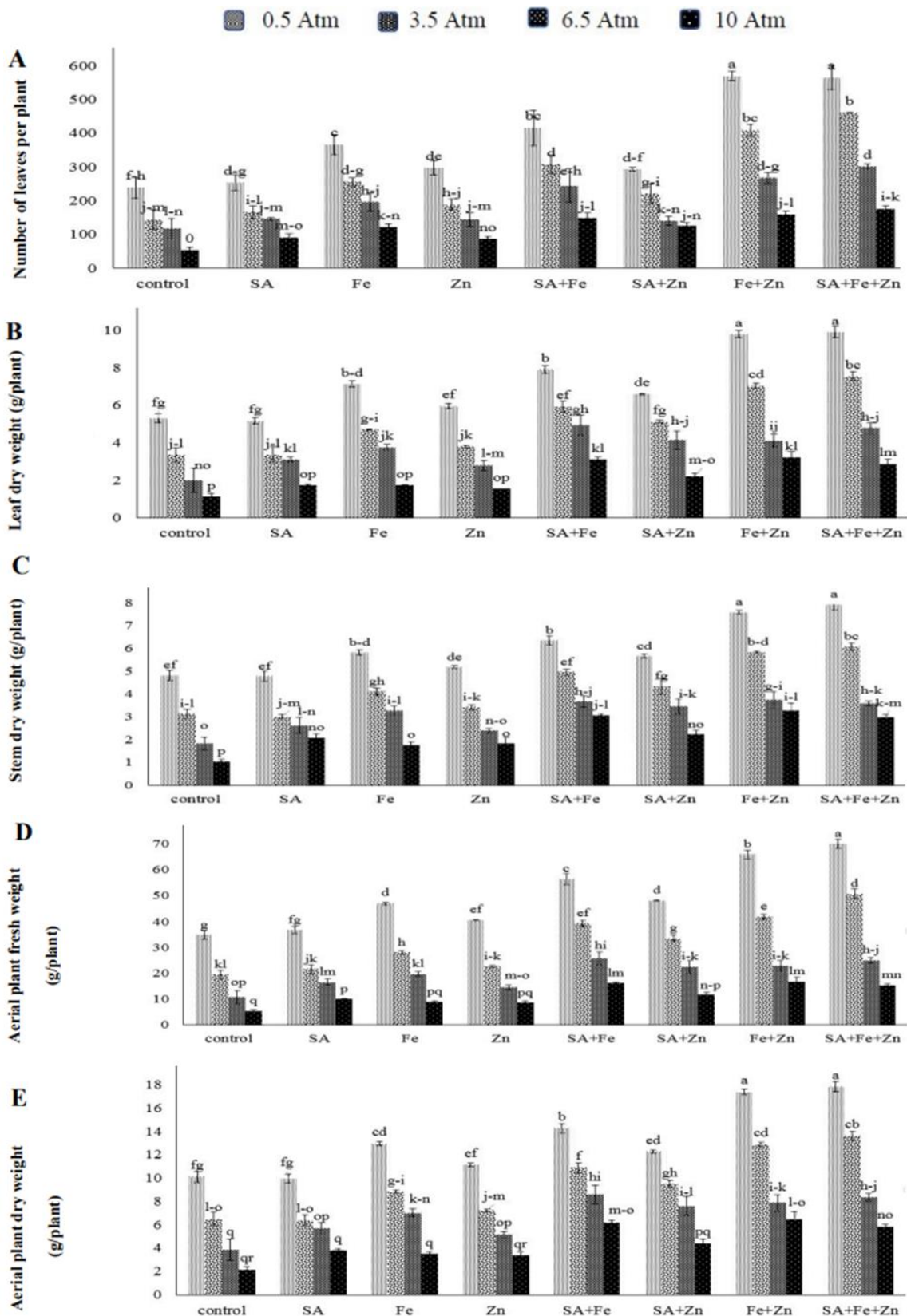


Figure 2 The interactive effects of drought stress and plant growth regulators (salicylic acid, Fe and Zn) on: A) number of leaves per plant (NLP), B) leaf dry weight (LDW), C) stem dry weight (SDW), D) aerial part fresh weight (AFW), and E) aerial part dry weight (ADW) in the two-year trial being presented with the standard error of the values

The main causes for such higher seed germination and seedling growth were due to the increased activities of antioxidant enzymes and seedling chlorophyll.

Yousefzadeh Najafabadi & Ehsanzadeh [19] examined the foliar application of SA (0 and 0.6 mM) on the growth of different genotypes of (*Sesamum indicum* L.) under field drought stress (60 and 80% reduction of soil available water). Although stress significantly decreased plant physiology and growth (leaf area index and plant dry matter), SA was able to alleviate the stress on plant physiology and growth.

In another research, Benhmimou *et al.* [20] investigated the effects of drought stress (irrigation practices at 100, 80, and 50% of the mean maximum evapotranspiration) on the growth and yield of stevia in a pot experiment. Similar to our results, plant growth parameters including number of leaves and leaf area per plant, plant height, stem diameter, plant fresh biomass, and leaf dry yield were significantly affected by drought stress indicating that stevia is not a tolerant plant under drought stress, however the plant may use some strategies to alleviate the stress.

The results indicated that although the single treatments were also able to partially increase stevia growth under the stress, the most effective treatments were the combined ones including the double and the triple PGR [18]. This indicates that such PGR are not antagonistic and can be combined to treat stevia under the stress. This may be due to the role of SA in the transport of ions in plant under stress. Research has indicated the positive role of SA on the uptake of different nutrients including N, Mg, Fe, Mn, and Cu by salt stressed maize [21,22].

In addition to play an important role in the induction of plant systemic resistance, SA may alleviate stress by 1)

increasing H⁺-ATPase activity in plant roots, 2) mitigating oxidative stress (scavenging reactive oxygen species), 3) increased sugar and protein contents, 4) increased leaf relative water content, 5) reduction of leaf rolling, and 6) increased plant dry biomass and yield [23,24].

According to the results, Fe and Zn also significantly alleviated the stress on stevia growth. The most important effects of Fe and Zn on plant growth under stress, rather than their presence in the cellular molecules such as chlorophyll is their presence in the antioxidant enzymes, which play important roles in plant tolerance under stress. If plant is under Fe and Zn deficiency, plant tolerance under drought stress decreases, which is due to the decreased activity of antioxidant enzymes [25, 26].

In a field research, Sajedi *et al.* [26] investigated the single and the combined effects of selenium and other micronutrients including Fe and Zn on the growth and yield of corn under drought stress conditions. The experiment was a split plot on the basis of a completely randomized block design. The main and subplots were devoted to the stress and micronutrients treatment, respectively. Although salinity significantly increased the activities of antioxidant enzymes, the use of micronutrients [27] increased such activities further. The results indicated that the single and not the combined use of selenium or micronutrients including Fe and Zn can alleviate drought stress on corn growth and yield under field conditions mainly by affecting plant metabolism, specifically the acidity of antioxidant enzymes.

Conclusion

The growth of stevia, as a healthy source of natural sweeteners, affected by drought stress and treated by different

PGR including Fe, Zn and SA, was investigated in a two-year pot experiment under field conditions. The results indicated that stevia is not tolerant under drought stress, and its growth significantly decreases under the stress.

However, interestingly the proposed PGR treatments were able to alleviate the stress with a different intensity as the most effective treatments was the triple one including Fe+Zn+SA followed by the double and the single ones. Therefore, these PGR are not antagonistic to each other and can be used to alleviate drought stress in stevia.

Conflict of Interest

The authors declare they do not have any conflict of interest in this study.

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