Effects of Chemical Treatments (Iron, Zinc and Salicylic Acid) and Soil Water Potential on Steviol Glycosides of Stevia (*Stevia rebaudiana* Bertoni)

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ABSTRACT: *The most important characteristic of stevia is its high sweetness with zero calories which is due to the presence of Steviol glycosides (SVglys). This research aims to address the effect of Salicylic Acid (SA) and microelements viz. iron (Fe) and zinc (Zn) under different soil water potentials (-0.5, -3.5, -6.5 and -10 atm) on the production of SVglys and total sugar content in the leaves of stevia. The obtained results indicated that the soil water content and the exogenous application of SA and microelements significantly changed the accumulation of these sweet chemicals in the stevia leaves. The highest values of Stevioside (Stev), Rebaudioside C (Reb C), total SVglys and SVglys yield were obtained in SA + Fe + Zn treatment under the potential of -3.5 atm (76. 82, 2.82, 116.71 mg/g DW and 0.836 mg/g plant, respectively). Also, the HPLC results indicated that the highest rates of Rebaudioside A (Reb A) and the Reb A/Stev ratio (sweetness quality) belonged to SA + Zn treatment under the potential of -3.5 atm (28.63mg/g DW and 0.433). The application of SA + Fe + Zn was the most effective in terms of Rebaudioside B (Reb B), Dulcoside A (Dulc A), and total sugar (2.31, 5.73 and 335.8 mg/g DW, respectively). In general, our results suggest that it can be possible to improve the rate of secondary metabolites (SVglys) and hence the sweetness property in stevia leaves by applying SA, Fe, and Zn and particularly by the integrated application of these three agents.*

KEYWORDS: *Chemical treatments; Microelements; Soil water potential; Total sugar; Stevioside; Rebaudioside.*

INTRODUCTION

Stevia (*Stevia rebaudiana* Bert.), an anti-diabetic medicinal plant belonging to the Compositae (Asteraceae) family, is a multi-purpose plant and native to the northeast of Paraguay (South America) [1, 2]. Stevia is well-known for its sweet-tasting leaves and currently, the production of this herb has widely spread in many

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countries throughout the world such as Canada, Australia, China, Japan, Korea, Malaysia, and etc. [3]. The sweetness of stevia is related to diterpenoid glycosides of ent-kaurene type, which mainly present in the leaves of this plant [4]. Over 60 types of steviol glycosides (SVglys) have been identified in *S. rebaudiana* [5]. The SVglys mainly include Stev, Reb A, B, C, and Dul A [3]. SVglys found in stevia are a form of secondary metabolites [6] and all of them contain a similar chemical structure (steviol backbone), but differ by the number and type of carbohydrate residues at the C13 and C19 positions [7, 8]. Stev and Reb A are the most abundant SVglys in the leaves of stevia (5-10 % and 2-4 % of leaf dry weight, respectively) [9]. The concentration of these metabolites in stevia depends on the genotype and the environmental and growth conditions of the plant [10]. Stev and Reb A are differed only by the presence of one glucose moiety. Stev is formed by three molecules of glucose attached to an aglycone steviol ring, while Reb A has one additional glucose molecule [11]. These water-soluble glycosides are 200-300 times sweeter than sucrose [10] and despite strong sweet flavor, are not metabolized by the body (calorie-free) [12]. Hence, stevia is a suitable alternative to artificial sweeteners like saccharin, neotame, aspartame, and *acesulfame K* which is used as the food additive, a flavor enhancer, and a sweetener in beverages and food products in many countries [13, 14]. According to a report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA), SVglys are safe for human consumption and can be used as a proper sweetener for diabetics and weight watchers [15]. Besides SVglys, many other compounds, such as ascorbic acid, beta-carotene, riboflavin, labdanes, triterpenes, coumarins, flavonoids, sterols, indole-3-acetonitrile, organic acids, cinnamic acids, inorganic salts and some essentials oils have been also found in the leaves of stevia [16, 17].

The leaves are the economic part and a major source of high-potency sweetener in the stevia plant, thus the increase of leaf biomass along with the higher SVglys content is a critical issue for its production [14]. In addition to genetic factors, the growth and the accumulation patterns of secondary metabolites (SVglys) in stevia plants are considerably affected by the nutrient availability [10, 18]. Mineral nutrients based on the relative quantity needed for plants could be divided into two groups, macroelements and microelements [19]. Although microelements are used by plants in small quantities, these elements are needed to improve the physiological and biochemical activities and to ensure the optimum quality. Microelements, especially the elements like manganese (Mn), iron (Fe), copper (Cu), zinc (Zn) and molybdenum (Mo) are essential for the normal performance of all higher plants. Also, microelements are important components for enzymatic systems and catalyze the biological reactions [15, 20]. Moreover, it has been reported that the formation of secondary metabolites might be induced by applying the elicitors such as SA in medicinal plant species as well as in the species categorized as functional foods [21]. SA (or 2-hydroxybenzoic acid), is known as an endogenous phytohormone with phenolic nature that has a remarkable role in the growth and development of plants [22, 23]. It has been proven that SA is involved in the regulation of a wide range of diverse physiological and biochemical processes in plant cells like the uptake and transmission of ions, photosynthesis, nitrogen metabolism and proline metabolism [24].

The biosynthesis of SVglys in stevia plants is also controlled by the environmental factors and agronomic management [18]. The reduction of soil moisture content is considered as one of the most major environmental factors, affecting many aspects of morphology, physiology, and biochemistry in plants [25-27]. *S. rebaudiana* originated from a semi-humid and subtropical region with an annual rainfall from 1500 to 1800 mm [28], so soil moisture is an important factor for its growth [26, 29]. The effect of drought stress on the accumulation of secondary metabolites has been frequently studied for many plants [30, 31], while this issue is not well-documented in stevia plant.

The growing demand for stevia sweeteners to use in food and pharmaceutical industries has led to its commercial production across the world. This plant has been recently introduced as a new commercial crop in Iran which is being cultivated in some regions of this country. The cultivation of stevia in Iran for the commercialization of its extract as a natural sweetener requires the comprehensive study of the effects of environmental and nutritional factors on the content of SVglys. According to our knowledge, although the effect

of drought stress on the content of SVglys has been investigated in some previous studies [9, 32, 33], the interaction between water stress and the application of SA along with Fe and Zn on the content of SVglys has not been evaluated so far. Consequently, the present research aimed to evaluate the role of foliar application with SA, Fe, and Zn (single or combined) on the SVglys accumulation and total sugar content in stevia leaves under different levels of soil water potential.

EXPERIMENTAL SECTION

Experimental site, Plant material, and growth conditions

A pot experiment was established under natural light (outdoor) at the Academic Center for Education, Culture and Research (ACECR), Kamalshahr (latitude 35° 54' 30" N and longitude 50° 52' 55" E and 1313 amsl), Alborz province, Iran, during 2016. This location is characterized by a warm Mediterranean climate with dry summer and total annual precipitation of 265 mm. Data on weather parameters recorded at Karaj meteorological station during the growing season are shown in Fig. 1.

The seedlings of stevia (*Stevia rebaudiana* var. Bertoni) used for this research, were obtained by tissue culture and after completing their acclimatization, two healthy seedlings (21-day old) with uniform size on 9 June 2016 were transplanted into plastic pots (30 cm diameter and 30 cm height). The pots were filled with air-dried soil up to 2 cm below its surface. In order to characterize soil properties, soil sampling was performed prior to planting. To do this, soil samples were air-dried, crushed and sifted through a 2 mm sieve and then submitted to the laboratory for measuring the soil chemical properties. Some physicochemical properties of used soil are listed in Table 1.

Chemical and drought stress treatments

The study was designed as a factorial experiment based on a Randomized Complete Block Design (RCBD) [34] with three replications. Experimental factors included four levels of soil water potential (field capacity (-0.5 atm) as control, moderate stress (-3.5 atm), relatively severe stress (-6.5 atm) and highly severe stress (-10 atm)) and the foliar application of SA, Fe and Zn applied in various combinations (SA, Fe, Zn, SA + Fe, SA + Zn, Fe + Zn and SA + Fe + Zn). Each pot

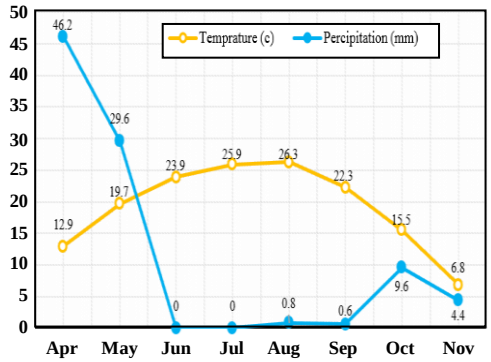

Fig. 1: Monthly average of precipitation and temperature during the growing season in 2016.

was considered as an experimental unit, which made up 32 pots for each replicate and 96 pots for the whole experiment.

In this study, Fe and Zn were supplied from sources of iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$, 21 ± 1%, Konjalesaz company, Iran) and zinc sulfate heptahydrate ($ZnSO_4.7H_2O$, 23 ± 1%, Konjalesaz company, Iran) respectively. SA (2-hydroxybenzoic acid, 90-94%, Sigma Aldridge Company Ltd.) in powder form with molecular weight 138.123 g/mol, was used in the experiment. The foliar application was done twice during the vegetative growth and branching of stevia (V3), 30 days after transplanting (DAT) and 45 DAT. The *Pedroza Carneiro* classification [35] was used to determine the stevia development stages for foliar spraying. Iron sulfate, zinc sulfate and SA (separately and combined) were dissolved in distilled water in the concentrations of 4 part per thousand (4 g/L), 3 part per thousand (3 g/L) and 1 mM, respectively. These concentrations were selected on the basis of some previous studies [36- 39]. Chemical compounds were sprayed on the plants existed in pots by a manual pump sprayer until run-off. Tween 20 (0.01 %, v/v) was added to the spray solutions as the surfactant to improve the foliar uptake [40]. No pests and diseases were observed during the experiment.

The intermittent drought treatments (-0.5, -3.5, -6.5 and -10 atm) were imposed on the plants 45 days after transplanting (viz, simultaneously with the second foliar application) by withholding irrigation. Prior to applying the drought treatments, the soil moisture content was maintained near the Field Capacity (FC). The above-

Table 1: Some physicochemical properties of the used soil as potting media.

Characteristic	Test level
Soil pH (H2O 1:2.5 soil: water suspension, McLean method)	8.00
EC (ds m^{-1})	1.2
Organic matter content (% OM)	0.64
Total nitrogen (%)	0.08
Available- phosphorus (ppm)	3.74
Available- potassium (ppm)	149.34
Available-iron (mg kg^{-1})	2.4
Available-manganese (mg kg^{-1})	5.4
Available-zinc (mg kg^{-1})	0.44
Available-copper (mg kg^{-1})	0.36
Soil texture	Sandy loam

The soil samples were taken from 0-30 cm deep

mentioned drought treatments were measured using an EQ15 equitensiometer (Ecomatic instruments, Germany, SN: 02385). Potted plants were irrigated to the level of field capacity after each cycle of drought stress. The whole plants in all experimental units were harvested on 23 August 2016 (i.e., 75 DAT); leaves and stems were separated and stored in the freezer for further analysis.

Extraction and determination of diterpene glycosides

The content of SVglys was measured in the leaves of stevia from treated plants with different compositions of SA, Fe, and Zn under different levels of soil water potential according to the methods described in previous papers [2, 9]. Dried leaf samples were used for the extraction of the SVglys. For this purpose, first, the collected leaves from the middle part of the plants in each experimental unit (pot) were washed using running tap water. After removal of water from the surface of the leaves, the samples were dried in a circulating air oven at 65 ± 2 °C for 48 h and then finely powdered [9]. To prepare the plant extract, the powdered samples (0.1 g) were transferred to tubes (15 ml) containing 3 mL of distilled water and put in a water bath for 30 min at 80 °C. The resulting solution was cooled, then it was centrifuged at 25°C and at 12,000 rpm for 5 minutes. The supernatant recovered to new tubes. Afterward, 3 mL of distilled water was added to the remaining solid phase in the pellets and then the centrifugation was repeated as described above (12,000 rpm for 5 minutes). This procedure was replicated three times and the resulting supernatants from each process of the extraction were pooled. The pooled supernatant was anew centrifuged (12,000 rpm for 5 minutes) and the obtained supernatant was transferred to new tubes. In the next step, 1 mL of distilled water was added to the remaining volume, and the centrifugation was repeated (12,000 rpm for 5 minutes). The final supernatant of each treatment was diluted to the volume of 10 mL by distilled water, and filtrated by 0.45 µm nylon filter [2, 9]. A C18 cartridge was used for the purification of stevia extracts. To do this, first, the C18 cartridge was washed with 3 mL of methanol and then was conditioned with 3 mL of distilled water. In order to the purification of SVglys, 0.5 mL of filtered solution was passed through the cartridge and then to remove the waste material and plant pigments, the cartridge was washed with acetonitrile (ACN) /water mixture (20:80, v/v). Eventually, the SVglys were eluted from the cartridge using 1 ml of ACN/H_2O (80:20, v/v), and stored in 1.5 mL tubes at -20 °C for the chromatographic analysis.

High-performance liquid chromatography (HPLC)

Two reverse-phase C18 columns as series and a UV–vis detector at 202 nm were used for the chromatographic analysis of SVglys. The mobile phase consisted of ACN and H_2O (50-80 %, v/v) at a flow rate of 0.5 mL/min.

To determine the content of SVglys, the purified extract of stevia (at the volume of 40 µL) was injected into the HPLC pump. In this method, five types of SVglys including Stev, Reb A, Reb B, Reb C, and Dulc-A were detected using HPLC (Unican-crystal-20, England). In order to quantify the SVglys, pure Stev and Reb A (purity > 99 %; Sigma-Aldrich; US) were utilized as the external standards. The molecular weight ratio of Reb B, Reb C and Dulc A to Reb A were used for their quantification, because formerly, it has been proven that all SVglys have similar molar extinction coefficients [41]. Peak area for each of the SVglys was determined by Chromstar 7.0 software. The results were represented as milligram of SVglys in the leaf dry matter, using the calibration curves gained from the relationship between external standards (ppm) and their relative HPLC peak area. Also, the SVglys yield was calculated through multiplying the total SVglys content (the sum of Stev, Reb A, Reb B, Reb C, and Dulc A) and leaf dry weight, which expressed as gr per plant [9].

Total sugar

In order to determine the concentration of total sugar in stevia leaves, the described method by *McCready* [42] was followed. Briefly, 40 mg of leaf samples was mixed with 5 mL of 80 % ethanol. Thereafter, samples were transferred to a water bath for 10 minutes at 70 ºC. The alcoholic extract obtained was centrifuged at 1000 rpm for 15 min. The above process was repeated 4 times on the plant remaining remnants. Then, the supernatant was concentrated to one fifth and was used for the measurement of total sugar [43]. An aliquot of 0.2 mL of the concentrated extract was added to 3 mL of cold anthrone reagent and boiled in a water bath for 20 minutes at 100 ºC. The mixture was immediately cooled down in an ice bath. After cooled, the absorbance was spectrophotometrically read at 620 nm (Model UV-120-20, Japan). The content of total sugar was calculated using a glucose standard curve. The standard curve of spectrophotometer was drawn with different concentrations of glucose. A glucose-free solution was used as the blank wells (control).

Statistical analysis

After checking the data distribution normality assumption, the analysis of variance (ANOVA) test was performed by the Statistical Analysis System software (SAS Institute, Cary, NC, USA, Version 9.4) to test the significance of main effects and interaction effects. 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 AND DISCUSSION

Effect of soil water potential and chemical treatments on key metabolites

Stevia is known to produce the sweet terpenoids. Since these non-volatile compounds are the most important from a commercial view [44], we focused on these chemicals and quantified them. One of the purposes of this research was to evaluate the effect of soil moisture depletion on the accumulation of SVglys in the leaves of stevia. The significance levels for the SVglys compositions are shown in Table 2. The analysis of variance revealed that all of the SVglys were significantly affected by Soil Water Potential (SWP) ($p \leq 0.05$, F test), although the individual compounds presented the different behavior (Tables 2). Mean comparison of different levels of drought stress showed that the status of the total SVglys content (Stev + Reb A + Reb B + Reb C + Dulc A) changed with the decrease of soil moisture. Considering the main effect of Soil Water Potential (SWP), the highest total SVglys (95.25 mg/g DW) was observed in plants grown under moderate stress (-3.5 atm), which was 36.18 % higher in comparison with the control treatment (-0.5 atm). Thereafter, it was significantly decreased along with the further reduction of soil moisture ($p \leq 0.05$, LSD), so that the lowest amounts of the total SVglys were obtained under soil water potential of -0.5 and -10 atm (60. 78 and 61.36 mg/g DW, respectively) (Table 2).

Regardless of the spray treatments, the analysis of SVglys compositions revealed that the reduction of soil moisture at all examined levels caused a significant increase in the Stev content than control (-0.5 atm) ($p \leq 0.05$, LSD). Nevertheless, when soil water potential was dropped to below -3.5 atm, a significant reduction occurred in the Stev content relative to the moderate stress (-3.5 atm). In fact, the maximum (62.57 mg/g DW) and minimum (37.77 mg/g DW) content of Stev was obtained in the leave of plants grown under soil

Table 2: Effect of soil water potential and different compounds Fe, Zn and SA on the content of SVglys (total SVglys, Stev, Reb A, Reb B, and Reb C) in stevia leaves.

Treatments	Total SVglys (mg/g DW)	Stev (mg/g DW)	Reb A (mg/g DW)	Dulc A (mg/g DW)	Reb B (mg/g DW)	Reb C (mg/g DW)
SWP (atm)						
-0.5 atm (control)	60.78 ± 1.92c	37.77 ± 1.33d	14.71 ± 0.52 c	4.94 ± 0.17a	1.72 ± 0.09 b	1.62 ± 0.06b
-3.5 atm	95.25 ± 3.17a	62.57 ± 2.06a	22.46 ± 1.03a	5.41 ± 0.21a	2.61 ± 0.11a	2.19 ± 0.07a
-6.5 atm	78.96 ± 2.10b	54.09 ±1.41b	15.82 ± 0.59b	4.86 ± 0.21a	2.43 ± 0.11a	1.73 ± 0.08b
-10 atm	61.36 ± 2.30c	41.07 ± 1.70c	12.65 ± 0.57d	4.20 ± 0.20b	1.80 ± 0.12b	1.62 ± 0.07b
LSD (p = 0.05)	2.30	1.75	1.11	0.569	0.241	0.120
Foliar Application (FA)						
Control (non-FA)	53.36 ± 3.35e	35.42 ± 2.45e	11.08 ± 0.68f	3.80 ± 0.20c	1.52 ± 0.13d	1.52 ± 0.07de
SA	65.56 ± 3.28d	42.58 ± 2.64d	14.47 ± 0.67e	4.64 ± 0.28b	2.03 ± 0.16bc	1.84 ± 0.08bc
Fe	72.87 ± 4.07c	47.98 ± 3.08c	16.21 ± 0.91cd	5.06 ± 0.30ab	2.17 ± 0.16b	1.42 ± 0.09e
Zn	71.61 ± 5.24c	47.59 ± 3.72c	15.73 ± 1.45de	4.81 ± 0.25b	1.78 ± 0.21c	1.68 ± 0.14cd
SA + Fe	78.03 ± 5.02b	51.64 ± 3.49b	17.40 ± 1.49bc	4.93 ± 0.26ab	2.24 ± 0.16b	1.80 ± 0.13bc
SA + Zn	79.43 ± 5.16b	51.93 ± 3.25b	18.62 ± 1.89b	4.76 ± 0.26b	2.24 ± 0.17b	1.86 ± 0.09b
Fe + Zn	80.35 ± 4.96b	53.67 ± 3.72b	17.36 ± 1.14bc	5.08 ± 0.40ab	2.31 ± 0.16b	1.91 ± 0.07b
SA + Fe + Zn	91.47 ± 4.66a	60.2 ± 3.09a	20.40 ± 1.48a	5.73 ± 0.27a	2.83 ± 0.12a	2.29 ± 0.11a
LSD (p = 0.05)	3.26	2.48	1.57	0.805	0.342	0.170
Interaction effect						
LSD of SWP × FA	6.52	4.97	3.14	NS	NS	0.341

The data are means ± standard error (n = 8 for soil water potential; n =4 for the foliar application). Means in each column with the same alphabetical letter (s) are not significantly different at 0.05 probability level according to LSD test. Abbreviations: LSD: Least significant difference; NS: non-significant; SVglys: Steviol glycosides; Stev: Stevioside; Reb A: Rebaudioside-A; Reb B: Rebaudioside-B; Reb C: Rebaudioside-C; SWP: Soil Water Potential; SA: Salicylic acid; Fe: iron; Zn: zinc.

water potential of -3.5 and -0.5 atm (control), respectively (Table 2). Similarly, the soil water potential of -3.5 atm in terms of the Reb A content was also superior (22. 46 mg/g DW) relative to the other treatments ($p \leq 0.05$, LSD). In contrast to the Stev content, the lowest value of Reb A content with the average of 12.65 mg/g DW was achieved in plants irrigated at -10 atm. Although the Dulc-A content showed a slight increase under moderate drought stress (-3.5 atm), no significant difference was observed between plants grown under soil potentials of -0.5, -3.5 and -6.5 atm. At the soil water potential of -10 atm, the Dulc A content was detected in the lowest amount (4.20 mg g^{-1} DW). Regarding the Reb B content, the maximum mean was recorded at -3.5 atm, followed by -6.5 atm (2.61 and 2.43 mg g^{-1} DW, respectively). The lowest amount of Reb B content (1.72 mg/g DW) was observed in plants grown under well-watered conditions (-0.5 atm). This treatment (-0.5 atm) was statistically at par with the soil water potential of -10 atm (1.8 mg/g DW) ($p \leq 0.05$, LSD). The accumulation of Reb C in the leaves of stevia was significantly increased by the soil water potential of -3.5 atm (2.19 mg/g DW), and thereafter decreased when the stress became more severe. No significant difference was found between the treatments of -0.5, -6.5 and -10 atm in terms of the Reb C content ($p \leq 0.05$, LSD).

These findings are consistent with some of the results obtained by *Badran et al.* [45] who reported an increased accumulation of Stev in the leaves of stevia plants under drought stress conditions. Similarly, *Benhmimou et al.* [26]

reported that drought stress caused a significant increase in the content of SVglys. They obtained the maximum value of Stev (62.6 mg/g DW) under severe drought stress, while the Reb A content was significantly increased under moderate drought stress (50 mg/g DW). *Hajihashemi and Geuns* [33] reported that the accumulation of SVglys in stevia leaves was negatively influenced by polyethylene glycol. In another study, the highest amounts of Stev (11.69%) and Reb-A (5.79%) were achieved in irrigation with low frequency [46]. According to the report of *Karimi et al.* [9], the highest value of total SVglys (55.2 mg/g DW) was obtained in plant irrigated at 60% FC (moderate drought stress). The major role of secondary metabolites is the adaptation of plants to biotic and abiotic stresses. When plants are exposed to environmental stresses, the content of secondary metabolites may increase because plant growth is often inhibited more than photosynthesis, and so the carbon fixed is mainly allocated to the synthesis of the secondary metabolites [47]. Accordingly, in this study, the increasing of SVglys content in the leaves of stevia is probably a mechanism to overcome drought stress which could result in enhanced tolerance to stress in the stevia plants. *Ceunen* and *Geuns* [48] stated that these secondary metabolites (SVglys) act as osmoprotectant molecules and lead to an increase in the growth of plants in environmental stresses. However, the response of plants to stressful conditions is related to the time and intensity of stress, plant species, genotypes, and growth conditions [9].

In our study, the comparative performance of different compounds of Fe, Zn, and SA for improving the quantity and quality of the SVglys was also evaluated. Based on the results in Table 2, the effect of Foliar Application (FA) was appreciable on all of the measured SVglys ($p \leq 0.05$, F test). This indicates the importance of Fe, Zn, and SA in the biosynthesis of secondary metabolites and, consequently, in determining the leaf extract quality in stevia. The results also exhibited that, all measured SVglys except Reb B and Dulc-A were significantly affected by the mutual interaction between the soil water potential and the foliar application of Fe, Zn and SA (SWP × FA) ($p \leq 0.05$, F test) (Table 2). The results from Table 2 show that all single and combined treatments significantly increased the accumulation of Reb B and Dulc A in the leaves of stevia compared to the control treatment (non-foliar application) ($p \leq 0.05$, LSD). The Reb B concentration varied from 1.52-2.83 mg/g DW, and the highest value belonged to plants sprayed with the combination of treatments (SA + Fe +Zn) (Table 2). This treatment increased the accumulation of Reb B in the leaves of stevia by 46.28 % as compared with the control treatment (non-foliar application). The concentration of Dulc-A varied from 3.80-5.73 mg/g DW. As observed in Table 2, SA + Fe + Zn, Fe + Zn, Fe and SA + Fe treatments were the most productive in terms of Dulc A (5.73, 5.08. 5.06 and 4.93 mg g^{-1} DW). The listed treatments improved the concentration of Dulc A by 33.68 %, 25.19%, 24.90% and 22.9 % as compared to the control treatment, respectively.

Data related to SWP × FA effect on the content of SVglys from HPLC analysis is presented in Fig. 2. Taking into consideration the interaction effect, the maximum values of Stev content were recorded under the soil water potential of -3.5 atm in plants sprayed with SA + Fe + Zn and Fe + Zn (76.82 and 72.14 mg g^{-1} DW, respectively). Food industry prefers Reb A over other SVglys for its superior flavor profile [49]. According to the data presented in Fig. 2, the highest content of Reb A (28.63 mg/g DW) belonged to plants treated with SA + Zn at -3.5 atm which did not differ significantly than that obtained from SA + Fe + Zn at -3.5 atm (27. 45 mg/g DW) ($p \leq 0.05$, LSD). In addition, the minimum values of Stev and Reb A (26.5 and 8.65 mg g^{-1} DW) were observed under the soil water potential of -10 atm in the absence of foliar application (control treatment). Irrespective of the soil water potential (SWP), the accumulation of Stev and Reb A in SA + Fe + Zn treatment was increased by 41.16% and 45.68% relative to the control treatment (non-foliar application) (Table 2). The content of total SVglys in the stevia leaves changed from 40.85-116.71 mg/g DW. The highest and lowest of mean belonged to SA + Fe + Zn treatment at the soil water potential of -3.5 atm and control treatment at the soil water potential of -10 atm, respectively (Fig. 2). Considering the main effect of foliar application (FA), SA + Fe + Zn treatment increased the amount of Reb C and total SVglys in comparison with the control by 33.62% and 41.6 % (Table 2). The chemical composition of plants significantly depends on the mineral fertilization [50]. Generally, in the present study, the production of SVglys in the stevia leaves was improved by the foliar

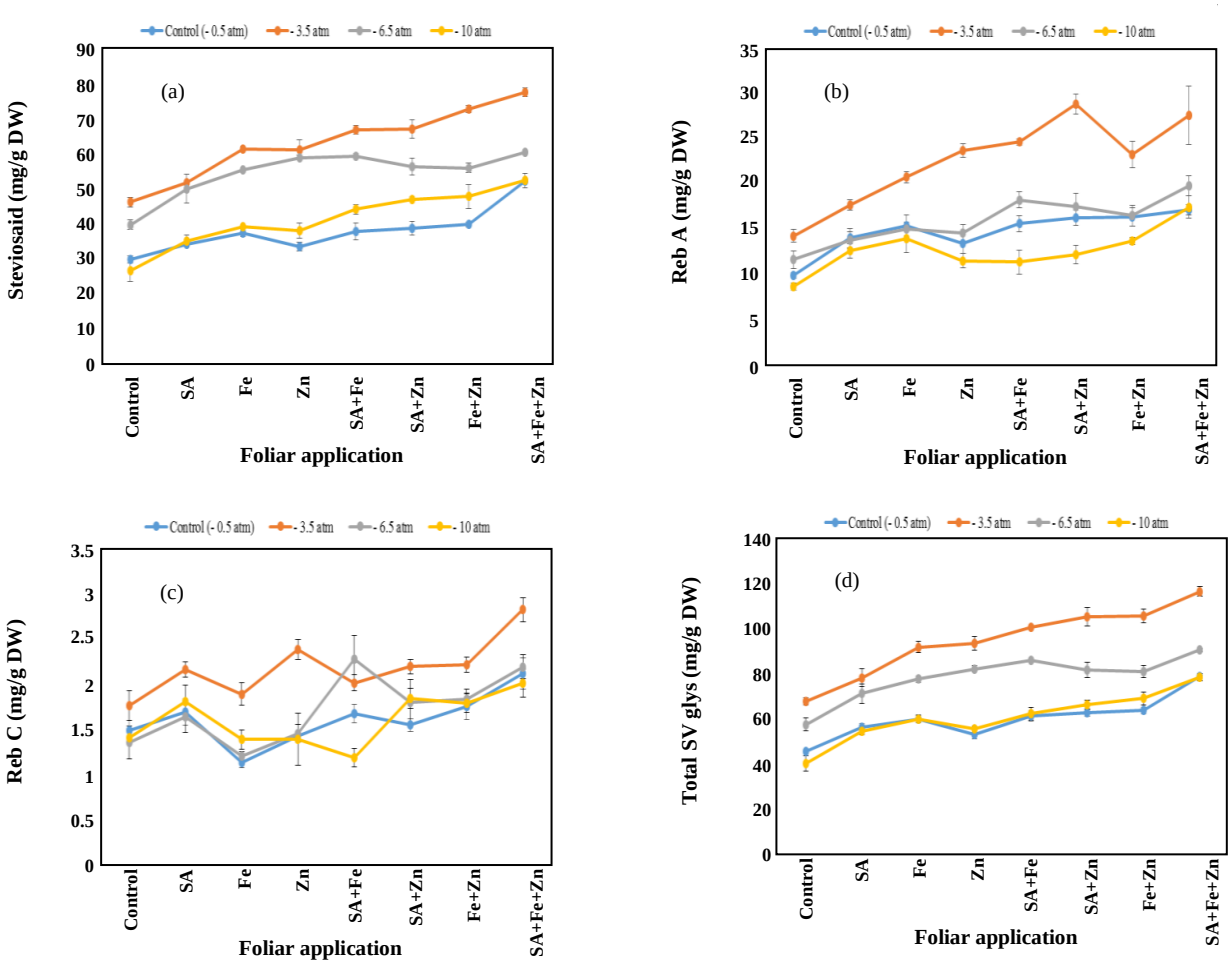

Fig. 2: Interaction effect of foliar application and different levels of the soil moisture on the content of Stev, Reb A, Reb C, and Total SVglys. Vertical bars indicate a mean standard error (±).

application of micronutrients, which may be related to increased levels of photosynthetic pigments [51]. Also, it is well known that the biosynthesis of secondary metabolites and the variation in the quantity of SVglys in the stevia leaves are strongly affected by genotype, phonological stage as well as environmental conditions [51]. Although some studies have been carried out to explore the effect of macronutrients on the production of secondary metabolites (SVglys) in the stevia leaves, reports on the possible positive effects of micronutrient (especially in the combination with SA) on the SVglys content are scarce. Both macro- and micro- nutrients may be essential for the synthesis of SVglys [3]. It has been reported that micro-nutrients such as Fe, Zn, Al, Cu, and B are key ingredients in many biological compounds, and play an important role in the production of secondary metabolites [53]. *Javed et al.* [1] reported the positive impact of ZnO nanoparticles on the production of Stev and Reb A under in-vitro conditions. In their study, the amount of Reb A was significantly increased from 2.07 % in control treatment to 3.65 % in the treatment supplemented with 1 mg/L ZnO nanoparticles. Similarly, the amount of Stev changed between 0.73 % in the control treatment and 1.17 % in treatments having 1 mg/L ZnO nanoparticles. In another study [51], the highest rates of Reb A, Stev, Reb B and Reb C were obtained in 30 mM salinity level and the integrated application of three microelements, iron (Skustren138), boron (boric acid) and selenium (sodium selenite). *Soufi et al.* [6] evaluated the effects of chilling stress and different signaling molecules (SA, hydrogen peroxide (H_2O_2), 6-benzylaminopurine (BAP)) on the content of SVglys in aerial parts of stevia. In their study, all foliar spray treatments were effective to increase the production

of SVglys. The Reb A content changed between 2.34 mg/g dried plant (control) and 6.16 mg/g dried plant (0.5 mM SA). The content of Reb B and Reb C were determined between 0.01 mg/g dried plant (control) to 1.36 mg/g dried plant (30 μM BAP) and 2.65 mg/g dried plant (control) to 9.72 mg/g dried plant (30 μM BAP), respectively. The content of Dulc A varied between 0.0001 mg/g dried plant (control) and 0.109 mg/g dried plant (10 mM H_2O_2), while the Stev content changed between 1.14 mg/g dried plant (control) and 4.21 mg/g dried plant (0.5 mM SA).

Effect of soil water potential and chemical treatments on SVglys yield

Dry leaf yield and the content of SVglys found in leaves are the two most important traits of stevia. Hence, finding an optimal balance between these two traits in order to optimize SVglys yield especially under environmental stresses, is so important [9]. The data presented in Table 3 showed that leaf dry weight and SVglys yield (SVglys production by the leaves of each plant) were significantly affected by different levels of soil water potential ($p \leq 0.05$, F test). Irrespective of foliar spray treatments, the optimum SVglys yield (0.483 g $plant^{-1}$) was obtained under the soil water potential of -3.5 atm, which was significantly more than that of the other treatments ($p \leq 0.05$, LSD). Similar results were also observed by *Karimi et al.* [9], who found that mild drought stress could improve the SVglys yield by increasing the SVglys compositions. Nevertheless, with reducing the soil moisture, a significant reduction in leaf dry yield and SVglys yield occurred, so that the minimum values of leaf dry yield (2.14 g/plant) and SVglys yield (0.138 g $plant^{-1}$) were observed in the presence of soil water potential of -10 atm. In addition, it was clear that all foliar spray treatments significantly increased the SVglys yield relative to the control ($p \leq 0.05$, LSD) (Table 3). As observed for SVglys compositions (Table 2 and Fig. 2), the most effective treatment for improving the SVglys yield was the combined application of Fe, Zn, and SA (Table 3). This treatment was 73.59 % superior compared to the control plants (non-foliar application). The interaction effect of SWP × FA for the leaf dry weight and SVglys yield are illustrated in Fig. 3 (a, b). The integrated application of SA + Fe +Zn and Fe + Zn in the control level of soil water potential (0.5 atm) had the highest leaf yield. The highest values of SVglys yield (0.836 and 0.758 g/plant) were recorded in SA + Fe + Zn treatment at soil water potentials of -3.5 atm and -0.5 atm, respectively. The lowest amount of leaf yield (0.936 g/plant) and the SVglys yield (0.039 g/plant) was achieved in the absence of the foliar application (control treatment) and the soil water potential of -10 atm (Fig. 3a and b). *Kafle et al*. [49] mentioned that avoidance of micronutrient deficiencies (especially Cu and Fe) is essentials for optimum SVglys yield in stevia plants.

Effect of soil water potential and chemical treatments on Reb A: Stev ratio

The ratio of Reb A to Stev is considered as the accepted measure of sweetness quality of the stevia extract [16]. Although Stev makes up the majority of the sweetener in stevia plants, Reb A due to its desirable flavor profile has a more important role in the quality of stevia extract. Stevioside is responsible for the bitter aftertaste while the Reb A has no bitter taste [9, 16]. Due to the changes in the two main SVglys (Stev and Reb A), the Reb A: Stev ratio also varied in response to the different levels of soil water potential (SWP) and SWP × FA interaction ($p \leq 0.05$, F test) (Table 3). Data presented in Table 3 revealed that the reduction of soil water potential significantly reduced the Reb A: Stev ratio compared to the control (-0.5 atm) ($p \leq 0.05$, LSD). Regardless of the effect of foliar application (FA), the maximum ratio (0.391) was recorded in plants grown under well-watered conditions (-0.5 atm) (Table 3), while the minimum rates were recorded in plants grown under soil water potential of -6.5 atm and -10 atm (0.292 and 0.312 respectively). *Karimi et al*. [9] reported that Reb A/ Stev ratio (sweetness quality) was significantly influenced by soil water depletion and the highest ratio belonged to 60 % FC. The lowest ratio was observed in 45 % FC (severe stress).

The results of the mean comparison of drought stress and the foliar application (interaction effects) are presented in Fig. 3c. Based on the results, some treatments were effective in the improvement of the sweetness quality of stevia extract. The highest ratio was recorded in plants treated by SA + Zn at the soil water potential of -3.5 atm, followed by SA + Zn, SA + Fe, and Fe + Zn at the soil water potential of -0.5 atm (0.433, 0.423, 0.413 and 0.413 respectively). These results

Table 3: Effect of soil water potential and different compounds Fe, Zn, and SA on leaf dry weight, SVglys yield, Reb A/ Stev ratio and total sugar in the stevia leaves

Treatments	Leaf dry weight (g/ plant)	SVglys yield (g/plant)	RebA: Stev ratio	Total sugar (mg/g DW)
SWP (atm)				
-0.5 (control)	6.86 ± 0.35a	0.429 ± 0.03b	0.391 ± 0.01a	316.71 ± 5.07b
-3.5	4.88 ± 0.31b	0.483 ± 0.04a	0.358 ± 0.009b	333.61 ± 6.57a
-6.5	3.58 ± 0.27c	0.290 ± 0.02c	0.292 ± 0.007c	296.42 ± 3.48c
-10	2.14 ± 0.19d	0.138 ± 0.01d	0.312 ± 0.01c	301.87 ± 8.08bc
LSD (p= 0.05)	0.403	0.033	0.024	15.78
Foliar Application (FA)				
Control (non-FA)	2.80 ± 0.52d	0.150 ± 0.02f	0.316 ± 0.009b	282.93 ± 7.94d
SA	3.16 ± 0.38d	0.206 ± 0.02e	0.347 ± 0.01ab	319.60 ± 12.25a-c
Fe	4.14 ± 0.55c	0.303 ± 0.03d	0.345 ± 0.01ab	299.30 ± 7.11cd
Zn	3.36 ± 0.48d	0.237 ± 0.03e	0.337 ± 0.02ab	302.98 ± 7.75b-d
SA + Fe	5.24 ± 0.51b	0.410 ± 0.04c	0.337 ± 0.01ab	323.42 ± 7.33ab
SA + Zn	4.36 ± 0.49c	0.349 ± 0.04d	0.356 ± 0.02a	322.59± 8.42ab
Fe + Zn	5.71 ± 0.74ab	0.458 ± 0.06b	0.330 ± 0.01ab	310.58 ± 5.68bc
SA + Fe + Zn	6.14 ± 0.77a	0.568 ± 0.07a	0.338 ± 0.01ab	335.86 ± 10.23a
LSD (p= 0.05)	0.569	0.046	NS	22.32
Interaction effect				
LSD of SWP × FA	1.13	0.093	0.068	NS

The data are means ± standard error (n = 8 for soil water potential; n =4 for the foliar application). Means in each column with the same alphabetical letter (s) are not significantly different at 0.05 probability level according to LSD test. Abbreviations: LSD: Least significant difference; NS: non-significant; SVglys: Steviol glycosides; Stev: Stevioside; Reb A: Rebaudioside-A; SWP: Soil Water Potential; SA: Salicylic acid; Fe: iron; Zn: zinc.

are extremely important because the higher ratio of Reb A / Stev is so desirable to use in the sweetener industry. Due to the commercialization of Reb A, the different agronomic practices have yielded altered SVglys in the stevia leaves [52]. Although some studies have been carried out to evaluate the function of different parameters on this ratio, no evidence is reported about the effect of Fe, Zn, and SA on the Reb A/ Stev ratio. *Pal et al.* [18] reported that the Reb A/ Stev ratio was not affected by the application of three major macronutrients (nitrogen, phosphorus, and potassium), while *Tavarini et al.* [52] reported the positive effect of nitrogen on the improvement of Reb A/ Stev ratio. Since the physiological and molecular mechanisms of SVglys biosynthesis in response to the application of macro and microelements have not been yet clarified, it is difficult to explain the reason for the changes in the concentration of SVglys and the Reb A/ Stev ratio.

Effect of soil water potential and chemical treatments on total sugar

Analysis of variance showed (Table 3) that the content of total sugar in the stevia leaves was significantly affected by Soil Water Potential (SWP) and the foliar application (FA) of SA, Fe, and Zn, but the interaction effect of SWP × FA on this trait was not statistically significant ($p \leq 0.05$, F test). As can be seen in Table 3, the sugar content was significantly increased in the moderate stress (-3.5 atm) ($p \leq 0.05$, LSD), and thereafter decreased, when the drought stress became more severe. No significant change was observed between the soil water potentials of -0.5 and -10 atm.

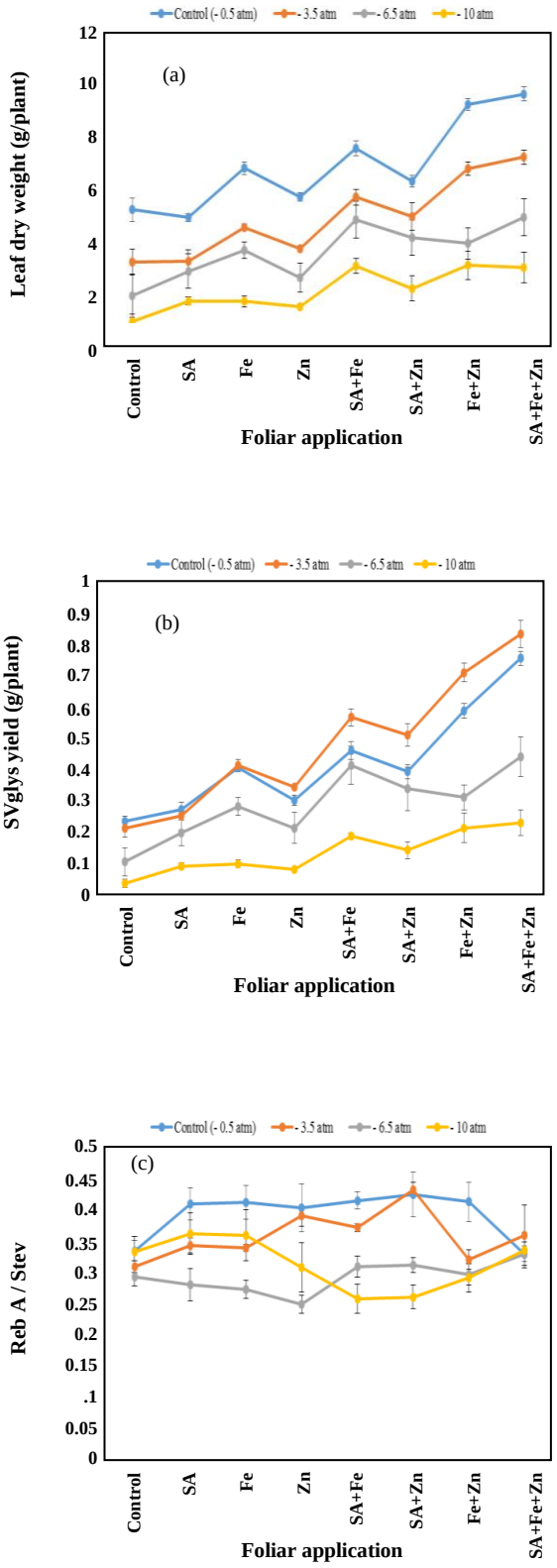

Fig. 3: Interaction effect of foliar application and different levels of soil moisture on leaf dry weight, SVglys yield and Reb A/ Stev ratio. Vertical bars indicate a mean standard error (±).

The plants subjected to water deficiency try to keep cell water potential through a net increase in solute concentrations, known as the osmotic adjustment [54]. Sugar accumulation prevents the oxidation of cell membrane and helps plants in maintaining the stability of the membrane under water deficiency. It also prevents the dehydration of proteins so as to remain functional [55]. Slower translocation of sugars out of the leaves and starch hydrolysis are some of the responsible factors for the increase of sugar content under stress conditions [54]. The lowest value for this trait (296.42 mg/g DW) was observed in plants grown under soil water potential of -6.5 atm. *Karimi et al.* [9] reported a significant increase in total soluble sugar of stevia leaves by the reduction of soil water content. On the contrary, *Hajihashemi* and *Ehsanpour* [32] observed the reduction of carbohydrates in the stevia leaves under drought stress stimulated by PEG. It has been reported that the rate of total sugar in plants is dependent on the environmental conditions, species even genotypes within same species [54].

Based on the results of the present study, all spraying treatments except the application of Fe and Zn separately were effective in the increase of total sugar in the stevia leaves compared to the control treatment (non-foliar application). Based on the mean comparison of foliar application effect, the content of total sugar varied between 282.93 mg g^{-1} DW (non-foliar application) and 335.86 mg g^{-1} DW (SA + Fe + Zn). Total sugar content in Fe + Zn, SA, SA + Zn, SA + Fe and SA + Fe + Zn treatments was increased by 8.9, 11.47, 12.29, 12.51 and 15.75 % relative to the control plants (non-foliar application) (Table 3). In agreement with our results, *Deswal and pandurangam et al.* [56] reported that the foliar application by microelements increased the total soluble sugar in *Zea mays* L. In another study, the highest value of total sugar in *Capsicum annuum* L. (38.70 mg^{-1} FW) was recorded in the application of SA + Zn. The favorable effects of Zn and Fe elements in the increase of total sugar content can be attributed to the role of these elements in the metabolism of starch and nucleic acid, as well as to the activities of different enzymes involved in these biochemical reactions [57, 58].

CONCLUSIONS

It is concluded from the findings of our research that the biosynthesis of SVglys in the stevia leaves is strongly

affected by growth conditions as well as nutritional variations. This research demonstrated novel findings depicting the positive effects of the exogenous application of microelements (Fe and Zn) and a signaling molecule (SA) on the accumulation of SVglys and total sugar content in stevia. Among the foliar spray treatments, the integrated application of Fe, Zn and SA (SA + Fe + Zn) was more effective in terms of the accumulation of SVglys and total sugar than using them individually that indicates an effective synergism between them. Also, it was observed that the reduction of soil moisture up to -3.5 atm caused the highest quantity of Stev, Reb A, Reb B, Reb C, Dulc A, total Svglys, SVglys yield and sugar content. In terms of sweetness quality (Reb A: Stev ratio), SA + Zn treatment under the soil water potential of -3.5 atm, as well as SA + Zn and SA + Fe treatments under soil water potential of -0.5 atm were the most favorable. The changes in the accumulation pattern of SVglys in response to the different levels of soil water potential, the application of micronutrients (Fe and Zn) and SA can provide the effective information for stevia producers especially those who grow this herb to use in the food industry and the production of low-calorie beverages. However, further studies are required to standardize the dose of Fe, Zn and SA to improve the quality and quantity of SVglys in the stevia plants.

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