The effect of humic acid application on stevia (*Stevia rebaudiana*) growth and metabolites under drought stress

Ameneh Zamani [1], Mojtaba Karimi[1]*, Ali Abbasi-Surki[1] and Fatemeh Direkvand-moghadam[2]

1. Department of Agronomy, Faculty of Agriculture, Shahrekord University, Shahrekord, Iran
2. Central Lab, Ramin Agricultural and Natural Resources University, Ahvaz, Iran

Abstract

The soil water limitation is a relevant abiotic stress that affects the plants life. In this respect, adoption of an efficient strategy may improve the plant growth during this stress. The present study was conducted in order to find the likely useful effect of the humic acid (HA) on stevia (*Stevia rebaudiana*) in a pot trial, especially under drought stress condition. Therefore, five levels of humic acid (0, 1, 1.5, 2, and 2.5 mg.kg^{-1} soil) were applied to the soil planted with Stevia seedling under normal (well-watered) and drought stress condition (resulted from 10-day irrigation intervals), based on a factorial experiment with three replications. Drought stress significantly reduced the stevia leaf production and steviol glycosides (SVglys) content and compositions. Stevioside (Stev) and Rebaudioside A (Reb A) contents significantly decreased under drought stress and all concentrations of HA. On the other hand, HA (in concentrations more than 1.5 mg.kg^{-1} soil) strongly increased leaf production in stevia and resulted in a significant increase in SVglys yield (44% in comparison with no HA). Results clearly demonstrated that although HA showed an inhibitory effect for SVglys content in stevia leaf, it can increase the SVglys yield through a remarkable increase in leaf growth in stevia, especially under drought stress condition.

Keywords: Stevia, Stevioside, Rebaudioside A, Steviol glycosides, leaf yield

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Introduction

Plants usually experience many biotic and abiotic stresses during their life. One of the most important abiotic stresses are caused by soil water shortage which annually loses a huge part of the crops products in the world. Coping with drought stress has always been a main concern for the farmers and plant researchers who endeavour to inhibit the drought stress damages in plants and consequently improve the food security in the world. Adding the water absorbing compounds in the soil is one of the strategies to increase the water availability of the soil and also improve the crop productivity.

Humic acid (HA) is a relatively stable product of organic matter decomposition (Mackowiak et al., 2001) with some beneficial effects on plant growth and production. These include chelating soil nutrients, improving nutrient uptake, removing toxins from soils, stimulating soil biological activities, solubilizing the minerals, improving soil structure, and improving water-holding capacity for better drought resistance and

*Corresponding author
E-mail address: m.karimi@sku.ac.ir
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reduction of water usage consumption (Mayhew, 2004; Liu and Cooper, 2000). Accordingly, application of HA in crop production has been intensively increasing during the last decades. There are many documents showing that HA is able to improve water availability for the crop root (increase water use efficiency) and consequently increase the crop yield, e.g. in *Echinacea purpurea* (Khorasaninejad et al., 2018), chickpea (Rasaei et al., 2013), rice (García et al., 2012), jerusalem artichoke (Ezzat et al., 2015), tomato (Osman and Ewees, 2008), bentgrass (Cooper et al., 1998), potato (Alenazi et al., 2016), cotton (Rady et al., 2016), and wheat (Malik and Azam, 1985). In addition, some beneficial effects of HA on biochemical and physiological processes in plants have been also reviewed (Vaughan and Malcolm, 1985; Aso and Sakai, 1963; Nardi et al., 2002).

Stevia (*Stevia rebaudiana*) is a perennial plant belong to the Asteraceae family, which natively grows in Paraguay (Kinghora et al., 1986). Stevia leaves contain some sweet compounds named steviol glycosides (SVglys) which pharmaceutically are so valuable regarding to diabetic patients (Chatsudthipong and Muanprasat, 2009). There are several types of the SVglys in Stevia leaf, but stevioside (Stev) and Rebaudioside A (Reb A) are most important regarding the quantity and quality (Ceunen and Geuns, 2013). Stevia cropping is intensified in the last decades due to its natural sweeteners and farmers are interested in producing higher yields of this plant, especially under varying environmental condition. There is some evidence showing that drought stress is able to reduce the stevia yield (leaf production) and metabolites (Pandey and Chikara, 2015; Yang et al., 2015; Gupta et al., 2015). Accordingly, finding strategies to prevent the yield losses of stevia under drought stress condition is an important aim in stevia cultivation. HA application could be expected to improve the stevia growth and metabolites, especially under water shortage situation. Therefore, the present study was carried out in order to find the effect of HA application on yield and quality of stevia.

Materials and Methods

A pot trial was conducted under controlled conditions in a greenhouse (humidity and temperature were 65/45% and 28/20 °C, in day/night, respectively) using a factorial experiment based on a completely randomized design with three replications. Identical stevia (*Stevia rebaudiana*) seedlings were transplanted into the pot (12 kg capacity) filled with a loam soil where ultimate density of the plant was 4 per pot. A commercial type of humic acid (HA) was prepared and five concentrations (0, 1, 1.5, 2, and 2.5 $mg.kg^{-1}$ soil) were applied to the pot soil before seedling transplanting. A half of the pots were irrigated normally (every 4 days) and the others were exposed to drought stress using irrigation period. The irrigation for drought stress was applied when the wilting symptoms were appeared on the plants, which generally occurred with a 10-day irrigation interval (on average). In order to quantify the drought stress treatment, the soil moisture and leaf relative water content (RWC) of plants were recorded before every irrigation and the means of soil moisture content (weight) and leaf RWC for drought stress treatment were 12% and 68%, respectively.

Just before flowering, the plants were harvested (after 138 days from transplanting) and some important agronomic traits were recorded, namely plant height, leaf and stem dry weights, and harvest index (HI, as the ratio of the leaf dry weight to total stevia above the ground weight). The leaves were dried and thereafter powdered in for steviol glycosides (SVglys) extraction and analysis. The SVglys extraction and assay was carried out based on our previous work (Karimi et al., 2016) with some modifications. Ethanol (70%) was added to 100 mg powdered leaves and mixed properly. The mixture was fully homogenized and filtered by a syringe-attached filter (0.45 µm) before it was submitted to SVglys analysis using high performance liquid chromatography (HPLC). The SVglys assay was carried out using a Knauer HPLC system (NH_2 column (Kromasyl, 5 µm, 100 Å, 25 cm × 4.6 mm); diode-array detector (DAD)). The mobile phases included distilled water and acetonitrile under isocratic conditions which started from 20:80 of water:acetonitrile (pH:3) in flow rate 1 mm/min. Two standards of Stev and

Reb A (Sigma Aldrich) were used and injected into the HPLC system before plant sample assay. Thereafter, 20 µL of the plant sample solution was injected and the SVglys absorbance was recorded at 210 nm. The chromatograms area was calculated and converted to SVglys content based on a pure standard and expressed as leaf dry matter percentage. Finally, Stev, Reb A and C were quantified.

The data were analyzed by SAS 9.4 software and the means were compared using least significant differences (LSD, $p \leq 0.05$). The histograms and graphs were prepared by Excel. In addition, the interaction effects were presented by means error bars.

Results

Among the stevia traits recorded in this study, only leaf dry weight, harvest index (HI), and Reb A were significantly affected by the interaction of drought stress and HA concentrations (Table 1, $p \leq 0.05$). The other traits showed a significantly independent response regarding to drought stress treatment and HA concentrations. Also, none of the experimental treatments had a significant effect on Reb C.

Stevia height and stem growth significantly decreased due to the drought stress, by 10% and 19%, respectively (Figs. I.A and I.D) while the HA (in all concentrations) increased the plant height (Fig. I.B) and stem growth (Fig. I.E).

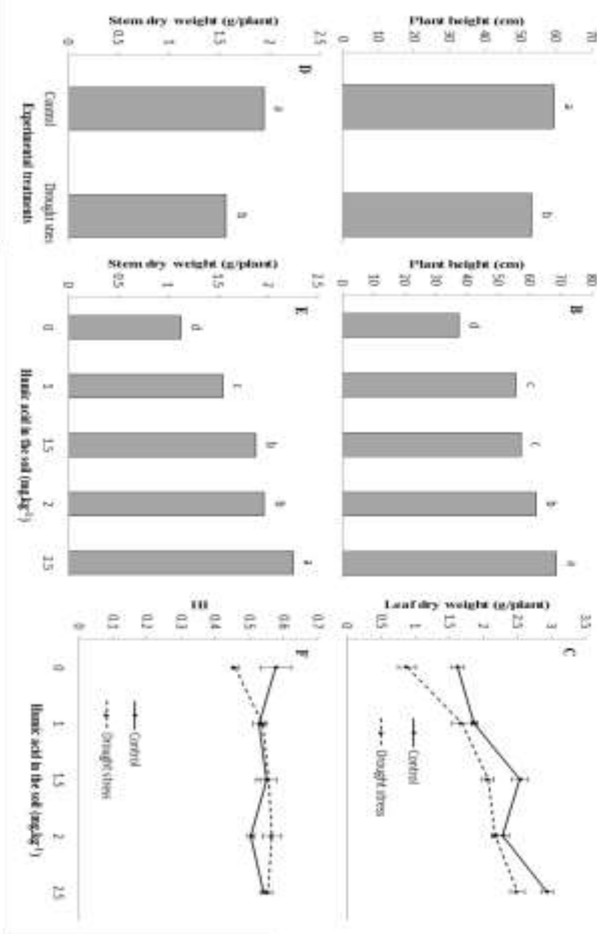

Fig. I. Effect of drought stress and humic acid concentrations on stevia height (A, B), leaf dry weight (C), stem dry weight (D, E), and harvest index (F); difference letters show significance differences between treatments ($p \leq 0.05$). The standard errors of each mean (from three replications) are also presented for interaction effects of the drought stress and humic

Table 1
Analysis of variance for stevia traits affected by drought stress and different concentration of humic acid (HA)

Source of Variation	df	Mean of Squares (MS)									
		Plant height	Leaf dry weight	Stem dry weight	HI	Totals SVglys content	Stev	Reb A	Reb C	Reb A/Stev	SVglys yield
Drought stress	1	294**	1.12**	1.05**	0.0002	2.88*	4.34**	0.13	0.0002	0.06**	0.014**
Humic Acid	4	819**	1.87**	1.12**	0.001	6.9**	4.36**	0.42**	0.00007	0.008	0.004**
Drought stress × HA	4	17.5	0.09*	0.04	0.006*	0.56	0.15	0.17*	0.00007	0.005	0.001
Error	20	9.9	0.03	0.07	0.001	0.51	0.44	0.06	0.06	0.004	0.0004
Coefficient of Variation (CV%)	-	5.6	8.4	15.5	7.3	8.9	12	11	2	15	13
Coefficient of determination (R^2)	-	0.94	0.93	0.79	0.51	0.76	0.71	0.67	0.24	0.57	0.80

* and **, significant at 0.05 and 0.01 , respectively

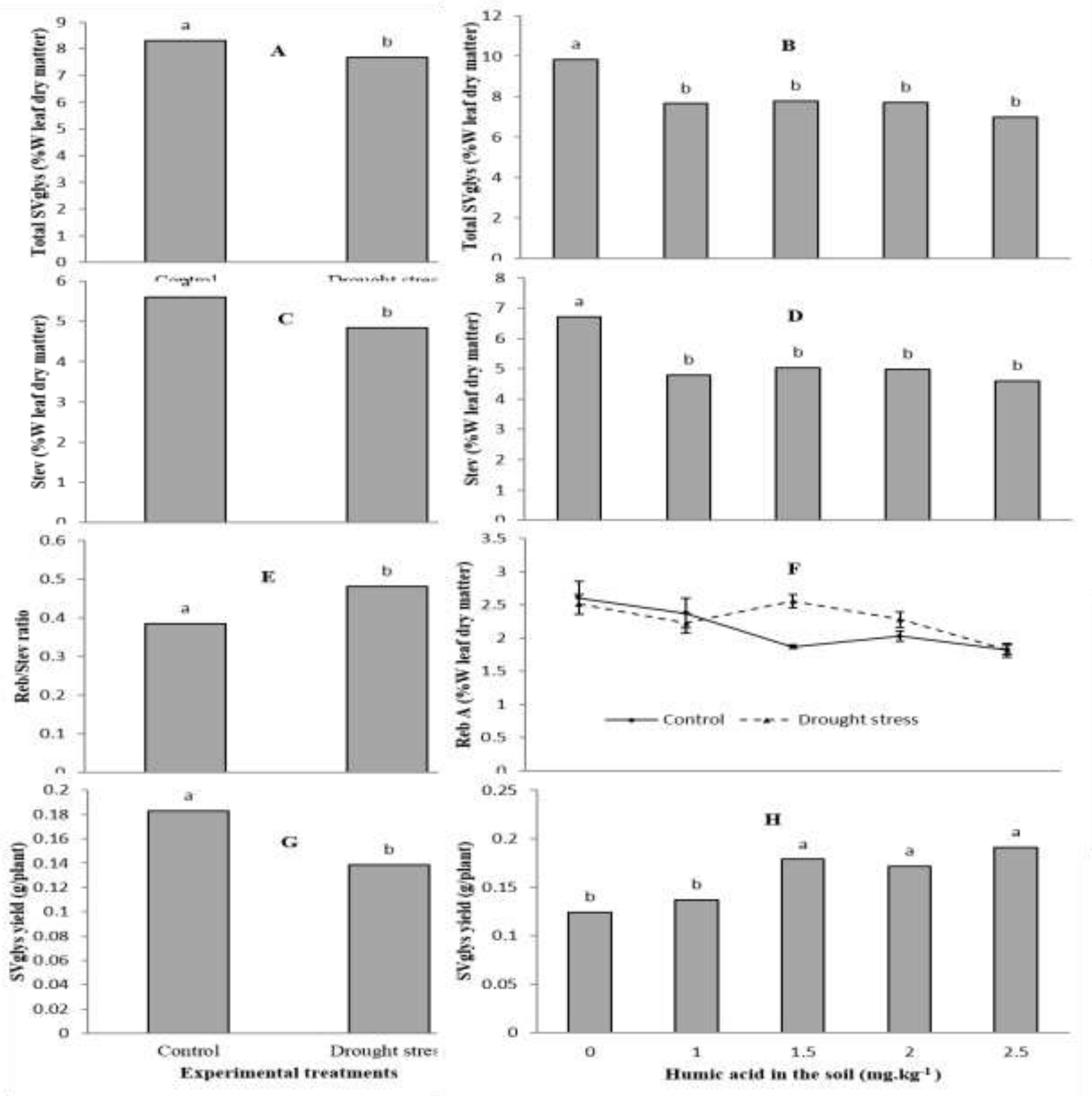

Fig. II. Effect of drought stress and humic acid concentrations on total detected steviol glycosides (SVglys, A, B), Stevioside (Stev, C, D), Rebaudioside A (Reb, F), Reb A/Stev ratio (E) and SVglys yield (G, H) of stevia; Different letters show significant differences between treatments (p≤0.05). The standard errors of each mean (from three replications) also are presented for interaction effects of the drought stress and humic acid.

The tallest stevia shoots were observed when 2.5 $mg.kg^{-1}$ HA was applied in the soil, which was approximately 80% more than the control treatment (no HA). In this line, the stem growth under HA treatment was twofold of the control treatment. There was a significant difference between leaf growth in drought-stressed plants and that of well-watered plants under no HA in the soil (Fig I.C), but HA improved this trait in the drought stressed plants, as drought-stressed plants were not significantly different from well-watered plants when 1 and 2 $mg.kg^{-1}$ HA were added to the soil (Fig I.C). It is notable that HA significantly increased the leaf dry weight under both irrigation conditions and the maximum leaf dry weight was obtained in the plants treated with 2.5 $mg.kg^{-1}$ HA. Under no HA treatment, the difference between well-watered plants and drought stressed plants was significant regarding to HI. Compared to the well-watered plants, drought-stressed plants showed a significant reduction in their HI while their variations were overlapped after adding HA in the soil (Fig. I.F).

Total SVglys observed in the drought stress treatment and HA (all tested concentrations) significantly reduced by 7% and 24% in comparison with control (no stress and no HA), respectively (Figs. II.A and B). The leaf Stev content showed a similar response to drought stress and HA, and reduced by 13% and 28%, respectively (Figs. II.C and D). Reb A content significantly decreased by HA concentrations, but this reduction was softened under drought stress condition (Fig. II.F). Under drought stress condition, the HA concentrations (up to 2 $mg.kg^{-1}$) played as a retentive factor for Reb A production in Stevia leaves. Reb A/Stev ratio increased by 25% in drought-stressed plants (Fig. II.E). Moreover, a decrease by 25% was observed in total SVglys content in plant leaves under drought stressed condition (Fig. II.G). It was also observed that 1.5, 2, and 2.5 $mg.kg^{-1}$ concentrations of HA significantly increased SVglys yield in stevia while 1 $mg.kg^{-1}$ HA did not show a significant change in SVglys yield.

Discussion

Soil water depletion has been shown to be an inhibiting factor for Stevia growth, especially in leaf production (Ren and Shi, 2012; Srivastava and Srivastava, 2014; Karimi et al., 2019). This has also been the case with the present study. On the other hand, HA was found to be a growth improvement factor for Stevia. The reason for the drought stress damages for Stevia is clear, but the mechanism of the HA on Stevia growth was not known till now. The effects of HA could be attributed to its bioactivities. In a study, HA was found to contain cytokinin hormone which resulted in more growth and greater drought resistance in bentgrass (Zhang and Ervin, 2004). In addition, indole-3-acetic acid, as a main plant growth regulator, was also found in HA (Zhang et al., 2005). Accordingly, the hormonal induction by HA in stevia is possible. Some useful effects of humic compounds on stevia growth and photosynthesis have been reported previously (Rashwan et al., 2017; Kakhki et al., 2019). The motivational effect of HA in many plants has been previously noticed. For example, HA application in soil stimulated the photosynthesis in maize and led to more yield (Haider et al., 2015). Similarly, the physiological improvement of chickpea due to HA has been demonstrated (Rasaei et al., 2013). In addition, an enhancement in leaf chlorophyll content of rice due to HA application has been reported (Tejada and Gonzalez, 2004). Results of the study showed that HA could play a supportive effect for stevia because it maintained the leaf growth at a desired level under drought stress condition. Similar to our finding, it has been formerly shown that HA had a limited promoting effect on durum wheat growth and yield (Delfine et al., 2005) and also increased the water use efficiency in potato (Alenazi et al., 2016). Altogether, the positive effect of HA on stevia growth and leaf production was confirmed in the study, especially in drought-stressed soil condition.

Approximately, the detected SVglys and their collection were decreased by drought stress and HA concentrations that was desired. It should be noted that increasing the Reb A/Stev ratio by drought stress was not through increasing the Reb A content, but it was due to the more reduction of Stev content due to drought stress, comparing to Reb A reduction. It means that Reb A was more stable under drought stress condition. There are some documents showing that HA enhanced the nutrition uptake thereby can increase the plant growth and development and in this way, nitrogen was the most important nutrition stimulated by HA (Nardi et al., 2000; Nikbakht et al., 2008; Jannin et al., 2012). Since leaf nitrogen content showed a negative correlation with leaf SVglys content (Barbet-Massin et al., 2015), it is possible to assume that HA decreased the SVglys content through the nitrogen improvement in the Stevia leaf. In addition, "dilution effect" can justify the SVglys reduction in the Stevia leaf caused by HA, because HA increased the leaf growth of Stevia. Although HA decreased the SVglys content in the Stevia leaf, finally, increased the SVglys yield in Stevia through a significant induction in leaf growth. In other words, HA compensated the SVglys yield through more leaf production in Stevia. The results are confirming that HA could be an amendment of the soil in the purpose of the SVglys yield enhancement in Stevia, especially under drought stress conditions.

Conclusion

Results of this study showed that although drought stress significantly suppressed stevia growth, HA was able to alleviate the adverse effects of drought stress, especially regarding the leaf production. In addition, it was found that HA had a negative effect on SVglys production in terms of stevia leaves while it finally increased the SVglys per plant (SVglys yield) through leaf growth improvement. The protective effect of HA for stevia production under drought stress was also outlined. Totally, it can be concluded that application of HA to the drought-treated soil can improve stevia yield.

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