# The effect of soil moisture depletion on Stevia (*Stevia rebaudiana* Bertoni) grown in greenhouse conditions: Growth, steviol glycosides content, soluble sugars and total antioxidant capacity

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## Abstract

The purpose of the present study was to determine threshold values of soil moisture content for Stevia (*Stevia rebaudiana* Bertoni) and to evaluate the effects of drought stress on the main metabolites of this species. For these purposes, a greenhouse experiment was carried out with four soil moisture levels and plant growth, steviol glycoside (SVglys) contents, soluble sugars and antioxidant capacity were investigated at variable soil moisture content. Irrigation was scheduled at 3, 6, 9 and 12-day irrigation intervals, based on soil moisture content at 90, 75, 60 and 45% of field capacity (FC) respectively. The results showed that soil water depletion up to 60% FC (9-day irrigation interval) had no negative effect on plant growth and leaf dry weight, whereas a significant growth reduction occurred at 45% FC (12-dayirrigation interval). Similarly, the total SVglys content increased when soil moisture was depleted to 60%FC (9-day irrigation interval), but these metabolites contents decreased by 45% FC treatment. Although Stevia growth and SVglys content significantly decreased under severe drought stress (45% FC), the total antioxidant capacity and soluble sugars increased in the identical condition. The obtained results suggest that Stevia plants can grow well with a soil water content near to 60% FC, showing a good SVglys content. The Stevia tolerance to mild water stress is noteworthy, especially in water limited regions. In addition, it was found that soil water depleted to 45% FC was detrimental to Stevia in greenhouse conditions. The improvement of antioxidant capacity and soluble sugar content by soil water stress conditions could be considered as physiological and biochemical responses to a progressive drought stress in Stevia and maybe an acclimation response to drought stress.



*Key words*: Drought stress; Secondary metabolites; Stevioside.


*Abbreviations*: SVglys, Steviol glycosides; Stev, Stevioside; Reb A, Rebaudioside A; Reb F, Rebaudioside F; Reb C, Rebaudioside C; Dulc A, Dulcoside A; HI, Harvest index; FC, Field capacity; WP, Wilting point; TSS, Total soluble sugars.

## 1. Introduction

Soil water reduction is one of the major limiting factor to plant growth in many parts of the world. The inevitability, the stress caused by soil moisture reduction would be experienced by plants in their life cycle. In this respect, several strategies have been identified in plants in response to the soil water deficiency (Chaves et al., 2003). Most research to date has focused on studying the responses to soil moisture variation for well-known crops, but these aspects have not been fully investigated in new or specialty crops. Understanding how the plants respond to soil water limitation can play an important role in improving crop management and performance, especially since the climate-change scenarios suggest an increase in aridity in many areas of the globe (Chaves et al., 2003).

Stevia (*Stevia rebaudiana* Bertoni) is a perennial plant belonging to the *Asteraceae* family, native to the Rio Monday valley in Paraguay. The leaves of *S. rebaudiana* have been used by indigenous people of Paraguay and Brazil to sweeten beverages for centuries (Lewis, 1992; Soejarto, 2002). In fact, the plant is the source of a great number of sweet ent-kaurene diterpenoid glycosides (Kinghorn, 2004) called steviol glycosides (SVglys), a group of no-calorie and intensely sweet compounds (Crammer and Ikan, 1987; Gregersen et al., 2004). The SVglys represent an excellent alternative to artificial sweeteners (Fujita and Edahiro, 1979; Yadav et al., 2011) and they have been approved for use as sweeteners in many countries, including United States, Canada, China, Japan, South Korea, Australia, New Zealand and Europe (Singh and Rao, 2005; EFSA 2010; Woelwer-Rieck, 2012). Generally, the total SVglys content ranges from 4-20% of leaf dry weight, depending on many factors (Brandle et al., 1998; Starratt et al., 2002). The major constituents of SVglys in Stevia leaf are Rebaudioside A and Stevioside (Reb A and Stev, 2-4% and 5-10% of leaf dry weight, respectively), the former is more potent and more pleasant-tasting than Stevioside (Jenner and Grenby, 1989).

Due to the short time of Stevia domestication and introduction as a new crop, its agronomic and physiological traits have not been extensively studied. Knowledge about Stevia response to abiotic stress, such as drought stress, could improve the Stevia spread in the world. Most of the studies have been focused on Stevia metabolite production under optimal conditions, while the effect of drought stress on Stevia metabolites was poorly investigated. It has been reported that, under in vitro culture condition and using polyethylene glycol to stimulate drought stress, fresh and dry weight, water content, chlorophylls, carotenoids and anthocyanins were negatively affected by drought stress (Hajihashemi and Ehsanpour, 2013). On the contrary, antioxidant activity and enzymatic defense systems (catalase, ascorbate peroxidase, polyphenol oxidase and peroxidase) increased (Hajihashemi and Ehsanpour, 2014). The increase of secondary metabolites under

drought stress has been frequently reported for many plants (Jaleel et al., 2007a; Jaleel et al., 2007b; Larson, 1988; Mewis et al., 2012), but this phenomenon has not been sufficiently investigated in Stevia. Aladakatti et al. (2012) reported that an irrigation based on the full replenishment of crop evapotranspiration resulted in a maximum leaf yield and, these authors suggested that an irrigation at 5-days interval was suitable for summer cultivation of Stevia in the semi-arid regions of India. It has been also reported that an irrigation at 5-days interval did not significantly influence the net photosynthesis, the transpiration rate and the leaf dry matter, while the highest decrease of these traits was observed with an irrigation at 10-day-intervals (Shi and Ren, 2012). By studying the effects of different irrigation levels (irrigation with 33, 66 and 100% restitution of water consumption), it was found that the harvest index and water use efficiency in Stevia decreased with the increase in irrigation regime, while the Stevioside and the Rebaudioside A contents were unaffected by irrigation regimes (Lavini et al., 2010).

The development of water deficit in higher plants generates a series of plant responses that enable them to tolerate or resist the water deficiency. Osmotic adjustment is usually considered as a physiological process that helps to preserve water in plant tissues under soil water depletion (Sharp and Davies, 1979) and it is believed to be a primary acclimation response through the increase of soluble cellular solutes such as soluble sugars, in the cytosol (Chaves et al., 2003; Feng et al., 1994; Kerepesi and Galiba, 2000). Moreover, the soluble sugar production is an important process in Stevia due to the glucose units enrichment in SVglys chemical structure (Shibata et al., 1995; Shibata et al., 1991). Under in vitro condition, it has been reported that water soluble carbohydrates and reducing sugars, were reduced by drought stress (originating by polyethylene glycol) (Hajihashemi and Ehsanpour, 2013). However, the soluble sugars variation in Stevia leaves encountering water deficit stress has not been clearly studied in fully developed Stevia plant.

The stimulation of antioxidant systems due to water deficiency has been reported in many plants and it is believed that the tolerance to water-deficit stress is dependent on antioxidant system induction (Fu and Huang, 2001; Jagtap and Bhargava, 1995; Reddy et al., 2004). It has been reported that aqueous and alcoholic extracts of Stevia leaves have a potent antiradical activity (Gopalakrishnan et al., 2006; Kim et al., 2011; Tavarini and Angelini, 2013a), and could prevent oxidative DNA damage (Ghanta et al., 2007). It was found that antioxidant capacity of Stevia was stimulated by drought stress induced by polyethylene glycol (Hajihashemi and Ehsanpour, 2014). However, little is available in the literature regarding to soil moisture variation effects on Stevia growth and on its antioxidant systems.

Since water resources become increasingly scarce in many regions of the world, the managing of water consumption in plants needs an efficient knowledge about the plant response to water-deficit stress. Moreover, studying the physiological processes of stressed-plants can help the plant breeder to select more efficient biotechnological methods to produce drought tolerant cultivars. Accordingly, in order to clarify the Stevia response to water deficit, we investigated the effect of soil water depletion on Stevia yield, SVglys, soluble sugars and antioxidant capacity, trying to define a threshold soil moisture level below which Stevia growth and SVglys accumulation can be inhibited.

## 2. Materials and methods

### *2.1. Growing conditions and treatments*

A greenhouse experiment was carried out at the Agricultural Biotechnology Research Institute of Iran (ABRII- Central of Iran, Esfahan) using completely randomized designs with three replications. Greenhouse temperature, humidity and air $CO_2$ concentration were 25/22 °C, 60/40% and 400/500 ppm, during day/night, respectively. Stevia propagation was carried out by tissue culture. The seedlings obtained by tissue culture were initially cultivated in peat moss medium to select the well-established plantlets. After three weeks, the uniform seedlings were transplanted into pots containing a loam soil (50% sand, 15% clay; field capacity 20.2%; wilting point 10.5%; bulk density 1.38 $g.cm^{-3}$). The 20L pots were filled with soil up to 2 cm below its surface and then, three seedlings were transplanted into each pot. Soil moisture was maintained near the field capacity for the first two weeks and then the irrigation treatments were applied as 90, 75, 60 and 45% of field capacity (FC). Soil moisture content was measured using the gravimetric method. A preliminary experiment showed that the above-mentioned treatments could be obtained through 3, 6, 9, 12-day irrigation intervals (correspond to 90, 75, 60 and 45% FC, respectively). So, in our experiment, the 90, 75, 60 and 45% FC were equal to 3, 6, 9 and 12-day irrigation intervals, respectively. The irrigation water volume was also increased during the growth period of the Stevia, due to its increasing water consumption. Irrigations for each treatment were done in order to replenish 100% of soil field capacity. The plants were harvested at 62 days after transplanting into the pot, leaves and stems were separated and weighted, and used for further assays.

### *2.2. Determination of SVglys*

The SVglys were determined according to the Food and Drug Administration method (FDA, 2009) and the procedures used by previous researchers (Ceunen and Geuns, 2013; Karimi *et al.*, 2014a, 2014b).

### *2.2.1. Extraction and quantification of SVglys*

Stevia leaves were dried using a hot air oven at 65 °C for 48 h and then the dry samples were ground in a laboratory grinding mill to produce powder particles of 0.10 mm in size. Thereafter, 0.1 g of powdered leaves were transferred to 15 mL tubes, 3 mL distilled water were added and kept in a water bath for 30 min at 80°C. Resultant solution was firstly centrifuged at 12,000 *g* for 5 min and the supernatant recovered. Then, 3 mL distilled water was added to the pellet and was centrifuged as above. This process was repeated three times and the supernatant from each process was pooled. The pooled supernatant was centrifuged again (12,000 *g* for 5 min) and the new resultant supernatant was transferred to new tubes. Thereafter, 1 mL of distilled water was added to the remainder, centrifuged as the previous time and the supernatant obtained was added to the new pool. The volume of the final supernatant was exactly diluted to 10 mL using distilled water and filtered using a 0.45 µm nylon filter attached to a syringe. A $C_{18}$ cartridge was used for SVglys purification. The $C_{18}$ cartridge was firstly washed with 3 mL methanol and then conditioned with 3 mL of distilled water. Thereafter, 0.5 mL of the filtered supernatant was loaded into the $C_{18}$ cartridge and then the $C_{18}$ cartridge was washed with acetonitrile/water mixture (20:80, v/v). Finally, SVglys were eluted from $C_{18}$ cartridge with 1 mL of acetonitrile/water (80:20, v/v) and kept in 1.5 mL tubes at -20 °C until further analysis.

### *2.2.2. High-performance liquid chromatography (HPLC)*

For the chromatographic analysis of SVglys, two reverse-phase $C_{18}$ columns were connected in series and a UV-Vis detector set at 202 nm was used. A solvent gradient of acetonitrile and water, as mobile phases, was created with a flow rate of 0.5 mL $min^{-1}$. The acetonitrile ratio was increased into the solvent gradient in 50, 65, 80, 80 and 50% during 0-10, 10-18, 18-22, 22-24 and 24-30 minutes, respectively. 40 µL of the purified extract was injected into the HPLC pump. Rebaudioside A (Reb A), Stevioside (Stev), Rebaudioside F (Reb F), Rebaudioside C (Reb C) and Dulcoside A (Dulc A) were detected. For quantification purposes, pure Stevioside and

Rebaudioside A (purity>99%) were used as external standards. Then, Reb F, Reb C and Dulc A were quantified by their molecular weight ratio to Reb A, because it has been shown that all SVglys have similar molar extinction coefficients (Geuns and Struyf, 2009; Geuns, 2010). The HPLC peak area was calculated by Chromstar 7.0 software and the SVglys were expressed as percentage of leaf dry weight (W/W), using the calibration curves obtained from the relationship between external standards (ppm) and their relative HPLC peak area.

### *2.3. Soluble sugars quantification*

Soluble sugars were quantified only in control and 45% FC treatments. The dried leaves (0.04 g) were grounded and extracted as described by Tobias et al. (1992) and Karimi et al. (2014c). Soluble sugars were assayed by coupled enzymatic assay methods (Guglielminetti et al., 1995) and measuring the increase in $A_{340}$. The absorption of known amounts of glucose was used as standard. Incubations of samples and standards were carried out at 37°C for 30 min. The reaction solution (1 mL) for glucose assay was as follows: 100 mM Tris-HCl, pH 7.6; 3 mM $MgCl_2$; 2 mM ATP; 0.6 mM NADP; 1 unit Glc-6-P dehydrogenase and 1 unit hexokinase. Fructose was assayed as described for glucose plus the addition of 2 units of phosphoglucose isomerase. Finally, the increase in $A_{340}$ was recorded. Sucrose was first broken down using 85 units of invertase (in 15 mM sodium acetate, pH 4.6) and the resulting glucose was assayed as described above. Recovery experiments evaluated losses taking place during the extraction procedures. Two tests were done for each metabolite by adding a known amount of authentic standards to the samples prior to the extraction. The concentrations of the standards added were similar to those estimated to be present in the tissues in preliminary experiments. Data were expressed as micromoles hexoses equivalent per gram dry weight (DW).

### *2.4. Total antioxidant capacity*

The total antioxidant capacity was determined by the DPPH (2, 2 diphenyl-1-picrylhydrazyl) assay according to Thaipong et al. (2006) and Karimi et al. (2014b). Stevia fresh leaves were powdered in liquid nitrogen with a laboratory grinding mill and then 0.3 g of powdered leaves were dissolved in 3 mL of methanol. The solution was homogenized using a laboratory homogenizer and homogenates were kept at -4 °C for 12 h and centrifuged at 23000 *g* for 20 min. The supernatants were recovered and kept at -20 °C until further analysis. A stock solution of DPPH was prepared by

dissolving of 2.5 g of DPPH in 4 mL of methanol. Working solution of DPPH was prepared by dissolving different amount of stock solution in 5 mL methanol to obtain an absorbance of 1±0.02 units at 517 nm. The HPLC grade methanol was used as blank sample. The reaction solution contained 422.5 µL methanol, 200 µL DPPH solution and 2.5 µL of leaf extract and it was kept in the dark at room temperature for 24 h. Then, the absorbance was taken at 517 nm using UV-VIS spectrophotometer. The $IC_{50}$ value was calculated as the sample concentration necessary to decrease the initial absorbance of DPPH by 50% and $1/IC_{50}$ was used as an index of total antioxidant capacity (Hasperué et al., 2011).

### *2.5. Statistical analysis*

The data were subjected to the analysis of variance (ANOVA) using SAS 9.2 software (SAS Institute Inc., SAS Campus Drive, Cary, North Carolina 27513). Mean values of treatments were compared using the least significant difference (LSD) at the $p \leq 0.05$ level. For soluble sugars, the *t*-test was performed. Bar charts were plotted using Sigma Plot 12.3 software. The means are given with standard error (SE) in histograms (Table 1).

## 3. Result

### *3.1. Morphological and growth properties*

Plant height, leaf dry weight and harvest index (HI, i.e. leaf dry weight/total dry weight ratio) were significantly ($p \leq 0.05$, F-test) affected by soil moisture depletion (Table 2). A significant ($p \leq 0.05$, LSD) reduction in plant height was observed from 75% FC of soil moisture onwards (Table 4). Although the leaf dry weight showed a reduction trend to progressive soil water depletion, a significant decrease was recorded only in 45% FC treatment (Table 4). On the contrary, the stem dry weight was not affected by soil water depletion. The highest value of HI was found with 75% FC treatment which was significantly greater than HI observed in plants grown under 60 and 45% FC.

### *3.2. SVglys production*

Soil moisture reduction caused a significant variation ($p \leq 0.05$, F-test) in the total SVglys content of Stevia (Table 2). The highest value of total SVglys content (5.52% of the leaf dry weight) was obtained in plant irrigated at 60% FC. As soil moisture drop to 45% FC, a slight reduction was occurred in the total SVglys content, in comparison with 60% FC treatment (Fig. 1a).

The analysis of SVglys composition showed that Reb A, Stev and Dulc A were significantly ($p \leq 0.05$, F-test) affected by soil moisture depletion (Table 2), while Reb C and Reb F showed no significant variations ($p \leq 0.05$, F-test) depending on the extent of the treatment. Reb A increased in 60% FC treatment and thereafter decreased, when the stress became more severe (Fig. 1b). On the contrary, Stev slightly increased with decreasing soil moisture. The highest content of Dulc A was obtained in 75% FC treatment (Fig. 1b). In terms of sweetness quality, Reb A/Stev ratio was significantly affected by soil moisture depletion (Table 2) with the highest value in 60% FC treatment (Fig. 1c).

The soil water depletion caused a significant effect on SVglys yield (Table 2) and the maximum SVglys yield was observed in plants grown under 75% FC (6-day irrigation interval). However, there was no significant ($p \leq 0.05$, LSD) difference between 75 and 60% FC treatments, regarding to SVglys yield of Stevia (Fig. 1d).

### *3.3. Soluble sugars and Total antioxidant capacity*

Since Stevia growth and SVglys production were inhibited by soil moisture at 45% FC, the soluble sugar evaluation was carried out in the control and 45% FC treatments, by using t-test analysis. The obtained results indicated that the content of total soluble sugars (TSS) significantly ($p \leq 0.05$, t-test) increased under soil moisture depletion at 45% FC (Table 3, Fig. 2a). Analysing the TSS composition, it is possible to note that glucose was responsible for the TSS increment registered in the 45% FC treatment (Table 3, Fig. 2b). Fructose and sucrose did not show any significant variation with soil moisture reduction. The plants grown under water stress conditions (45% FC) showed TSS and glucose contents higher than 28 and 29%, respectively, compared to those recorded for well-watered plants (Figs. 2a and b).

Soil moisture reduction caused a significant enhancement in total antioxidant capacity of Stevia leaf extracts (Table 2, Fig. 2c). In plants grown under 75, 60 and 45% FC, the antioxidant capacity was significantly higher than that observed for well-irrigated plants (Fig. 2c). In particular, the highest antioxidant capacity was observed in 60% FC treatment, with a value increased by 37% in comparison with the control (well irrigated plants).

## 4. Discussion

It is known that plant response to drought stress is associated with the time and intensity of stress, plant species, genotypes and environment conditions (Chaves et al., 2003; Chaves et al., 2002). In our experiment, Stevia growth and yield were found to be mutually related with intensification of soil moisture reduction, especially below the 60% FC. Our results indicated that soil moisture reduction up to 60% FC (9-day interval) was not limiting for Stevia because no significant reductions in Stevia growth, SVglys production and SVglys yield were recorded for this level of soil moisture content. Likewise, in a similar research carried out in greenhouse condition, it was also observed that moderate water-deficit stress (8-days irrigation period) did not significantly affect the SVglys content (Guzman, 2010). Accordingly, it can be outlined that, in Stevia cultivation, , especially in regions characterized by limited water resources, a soil moisture at 60% FC could be a good compromise between plant and SVglys yield and water consumption. Moreover, it has also been reported that Stevia has modest water needs, as growing in sandy soils in native habitat, Paraguay (Madan et al., 2010). This statement in Stevia response to water availability could be supported by our results. Furthermore, it can be argued that the soil moisture level near at 45% FC (12-dayirrigation interval) is a threshold level of soil moisture for Stevia, since it caused a significant reduction in Stevia growth, SVglys con-tent and yield. Our findings were in agreement with Shi and Ren (2012), which found that a 10-day interval irrigation caused the highest losses in Stevia leaf dry weight. On the basis of our findings, the soil moisture depletion near the at 45% FC could be considered as a drought stress level for Stevia cultivation under greenhouse conditions, due to effective inhibiting the leaf growth and SVglys yield in comparison to well-watered situation.

In addition, our results suggested that the soil moisture depletion had a different effect on Stevia plant organs. In fact, we found that the leaves were more sensitive to water stress than the stems. The same results have been observed in a field experiment carried out in south Italy (Lavini et al., 2010), where a more pronounced leaf senescence, in comparison to the whole plant biomass, with decreasing irrigation volumes, was found.

The leaf dry weight per plant and leaf SVglys concentration represent the two most important yield traits of Stevia. Thus, it is important to find an optimal balance between these two traits in order to optimize SVglys yield. Our study showed that an optimum SVglys yield could be

obtained with a soil moisture at 60% FC while a significant reduction in leaf dry yield and SVglys content occurred in presence of severe drought stress condition (45% FC). Since our results have confirmed the sensitivity of Stevia leaves to water deficiency, it is recommended that Stevia should not experience serious water stress during its vegetative growth, in parliamentary procedure to achieve the optimum yield.

The SVglys composition was also affected by soil moisture reduction. In this respect, the Reb A/Stev ratio increased in 60% FC treatment, but it was reduced with a soil moisture at 45% FC. Although the Reb A had an important role in Stevia extract in term of quality and taste (Sharma et al., 2009; Yadav et al., 2011), Stev is the most abundant steviol glycoside among 30 SVglys found in varying concentrations in Stevia leaf extract (Wölwer-Rieck, 2012). According to the Reb A and Stev variation depending on soil moisture reduction, a good quality can be obtained with a moderate drought stress (W3) which is also useful with respect to water saving. Among the SVglys, Dulc A appeared to be more sensitive to moderate and severe drought stress. It is difficult to explain the variation among the different compounds of Stevia because the physiological and molecular mechanisms of SVglys biosynthesis in response to drought stress have not been yet fully clarified.

Plants display a range of mechanisms to withstand drought stress and the osmolytes accumulation is one of the most important strategy for plant faced with water deficiency (Chaves and Oliveira, 2004). The soluble sugars are compatible osmolytes and their accumulation has been observed in many plants under drought stress conditions (Silva and Arrabaça, 2004; Souza et al., 2004; Zeid and Shedeed, 2006). It is also believed that soluble sugar production in plants is an acclimation mechanism in response to drought stress (Prado et al., 2000). It seems that osmolyte accumulation and the consequent osmotic adjustment, have been occurred in Stevia in response to drought stress, since a significant increase in the TSS content in stressed-plants was observed. Moreover, our findings showed that the TSS increment under drought stress mainly occurred through glucose production. The glucose deficiency could be the cause of SVglys reduction in Stevia under severe drought stress, and it can be assumed, that glucose units were used for osmotic adjustment in order to create an acclimation process in stressed tissues.

The stimulation of antioxidant systems and the increase in the biosynthesis of antioxidant metabolites are considered another important mechanism of drought resistance in plants (Cruz de Carvalho, 2008; DaCosta and Huang, 2007; Sairam and Saxena, 2000). It seems that this mechanism was also involved in Stevia response to water deficiency. In addition, SVglys may be linked up with antioxidant induction in Stevia, since a similar trend in SVglys and total antioxidant capacity during soil moisture reduction was detected. Previously, it has been reported that Stevia

leaf extract had the ability to scavenge the free radicals (Gopalakrishnan et al., 2006) and could improve the catalase and superoxide dismutase activity in rice leaves treated with steviol glycosides blending liquid at different concentrations (Congmin et al., 2009). Similar to our results, it has been also found that the antioxidant capacity of Stevia, under in vitro culture conditions and using polyethylene glycol, significantly increased as well as phenols and flavonoids, which were the main antioxidant compounds induced by drought stress (Hajihashemi *et al.*, 2012). Findings from our study are in agreement with the results reported by previous research and support that also in Stevia, as already observed in other species (Jaleel et al., 2007a; Jaleel et al., 2007b), the increase of antioxidant capacity could be considered as a drought stress acclimation. Nevertheless, the relationship between SVglys and total antioxidant activity should be further elucidated in order to clarify which of them are upstream.

## 5. Conclusion

In this research, it has been observed that soil moisture reduction up to 60% FC was not harmful to Stevia growth and its metabolites, while a soil moisture around 45% FC represented a stressful condition for. Stevia, leading to yield and quality reduction. On the basis of leaf yield and SVglys content, a soil moisture at 60% FC allowed to obtain good performances, with interesting implications regarding a more efficient use and saving of the water under greenhouse condition. The increase of soluble sugar production in drought stressed-Stevia plants could be an acclimation mechanism and it seemed likely used for SVglys production in drought stress situation. Accordingly, drought stressed-Stevia experienced a glucose limitation for SVglys production. Furthermore, in response to the oxidative damage caused by drought stress, the antioxidant capacity of Stevia leaves increases, with a consequent accumulation of secondary metabolites, such as phenols and flavonoids.

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## Table captions

**Table 1.** Irrigation interval and soil water content in the four treatments of soil moisture depletion.

**Table 2.** Analysis of variance for Stevia main traits under four treatments of soil moisture depletion (W1, W2, W3 and W4 are 90, 75, 60 and 45% FC which obtained from 3, 6, 9 and 12-days irrigation intervals, respectively).

**Table 3.** t-Test for soluble sugars in control (well-watered, W1) and 45% FC (W4) treatments (TSS, Total Soluble Sugars).

**Table 4.** Mean comparisons of plant height, leaf and stem dry weight and harvest index of Stevia under soil moisture variation (W1, W2, W3 and W4 are 90, 75, 60 and 45% FC which obtained from 3, 6, 9 and 12-days irrigation intervals, respectively).

## Figure captions

**Figure 1.** Total SVglys content (a), SVglys composition (b), Reb A/Stev ratio (c) and SVglys yield per plant of Stevia under soil moisture variation (W1, W2, W3 and W4 corresponding to 90, 75, 60 and 45% FC obtained by 3, 6, 9 and 12-days irrigation intervals, respectively). Standard error of means within treatment is reported as a vertical bar.

**Figure 2.** Total soluble sugars (a), Soluble sugars (b) and total antioxidant capacity (c) of Stevia under soil moisture variation (W1, W2, W3 and W4 corresponding to 90, 75, 60 and 45% FC obtained by 3, 6, 9 and 12-days irrigation intervals, respectively). Standard error of means within treatment is reported as a vertical bar.

538 **Table 1** Irrigation interval and soil water content in the four treatments of soil moisture depletion.

| Treatments | Irrigation interval (day) | Soil water content (%FC) | Soil water content of topsoil before irrigation (based on soil weight percentage) |
|---|---|---|---|
| W1 | 3 | 90 | 19.1 |
| W2 | 6 | 75 | 15.1 |
| W3 | 9 | 60 | 11.9 |
| W4 | 12 | 45 | 10.3 |

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540

**Table 2** Analysis of variance for Stevia main traits under four treatments of soil moisture depletion ((W1, W2, W3 and W4 are 90, 75, 60 and 45% FC which obtained by 3, 6, 9 and 12-days irrigation intervals, respectively).

| Source of Variation | df | Mean of Squares (MS) | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Plant height | Leaf dry weight | Stem dry weight | Harvest index | Reb A | Stev | Reb F | Reb C | Dulc A | Total SVglys | Reb A/Stev | SVglys yield | $1/IC_{50}$ |
| Treatment | 3 | 78.08$^{**}$ | 0.46$^{*}$ | 0.08 | 5.12$^{*}$ | 0.028$^{*}$ | 0.208$^{**}$ | 0.0001 | 0.05 | 0.01$^{*}$ | 0.83$^{**}$ | 0.001$^{*}$ | 0.001$^{*}$ | 0.142$^{*}$ |
| Error | 8 | 5.08 | 0.07 | 0.12 | 1.2 | 0.003 | 0.0209 | 0.0005 | 0.01 | 0.002 | 0.109 | 0.0002 | 0.0001 | 0.035 |
| CV | - | 2.82 | 4.95 | 6.49 | 2.2 | 8.16 | 5.49 | 54.5 | 11.08 | 10.5 | 6.54 | 5.46 | 4.87 | 14.95 |
| $R^2$ | - | 0.85 | 0.71 | 0.20 | 0.60 | 0.73 | 0.78 | 0.06 | 0.51 | 0.70 | 0.74 | 0.70 | 0.72 | 0.54 |

$^{**}$ Significant at 0.01 level; $^{*}$ Significant at 0.05 level; df, degree of freedom; CV, coefficient of variation; $R^2$, coefficient of determination.

546 **Table 3.** t-test for soluble sugars in control (well-watered, W1) and 45% FC (W4) treatments (TSS, Total Soluble
547 Sugars).

| | df | Glucose | | df | Fructose | | df | Sucrose | | df | TSS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Equality of Variances (Pr>F) | 2 | 0.4 | | 2 | 0.65 | | 2 | 0.39 | | 2 | 0.2 |
| t (Pooled) | 4 | -3.12* | | 4 | 1.2 | | 4 | -1.89 | | 4 | -4.33* |
| t (Satterthwaite) | 2.95 | -3.12 | | 3.56 | 1.2 | | 2.92 | -1.89 | | 2.44 | -4.33 |

548 ** Significant at 0.01 level; * Significant at 0.05 level.

549

550

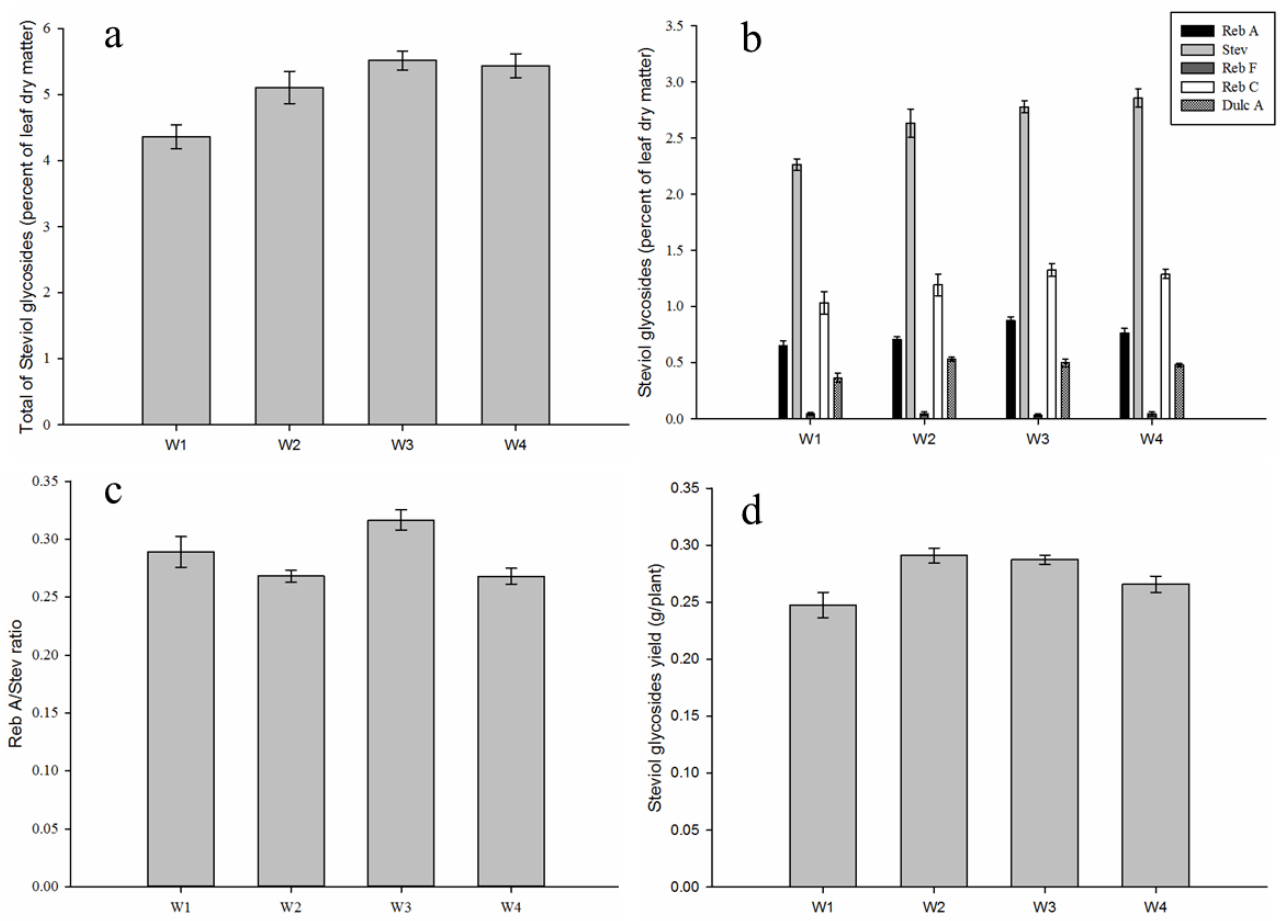
551 **Table 4** Mean comparison of plant height, leaf and stem dry weight and harvest index of Stevia under
552 soil moisture variation (W1, W2, W3 and W4 are 90, 75, 60 and 45% FC which obtained by 3, 6, 9 and
553 12-days irrigation intervals, respectively).

| Treatments | Plant height (cm) | Leaf dry weight (g plant$^{-1}$) | Stem dry weight (g plant$^{-1}$) | Harvest index |
|---|---|---|---|---|
| W1 | 86.33 | 5.66 | 5.55 | 50.59 |
| W2 | 81.67 | 5.70 | 5.40 | 51.36 |
| W3 | 76.33 | 5.21 | 5.44 | 48.92 |
| W4 | 75.33 | 4.88 | 5.15 | 48.66 |
| LSD ($p \leq 0.05$) | 4.24 | 0.50 | N.S. | 2.09 |

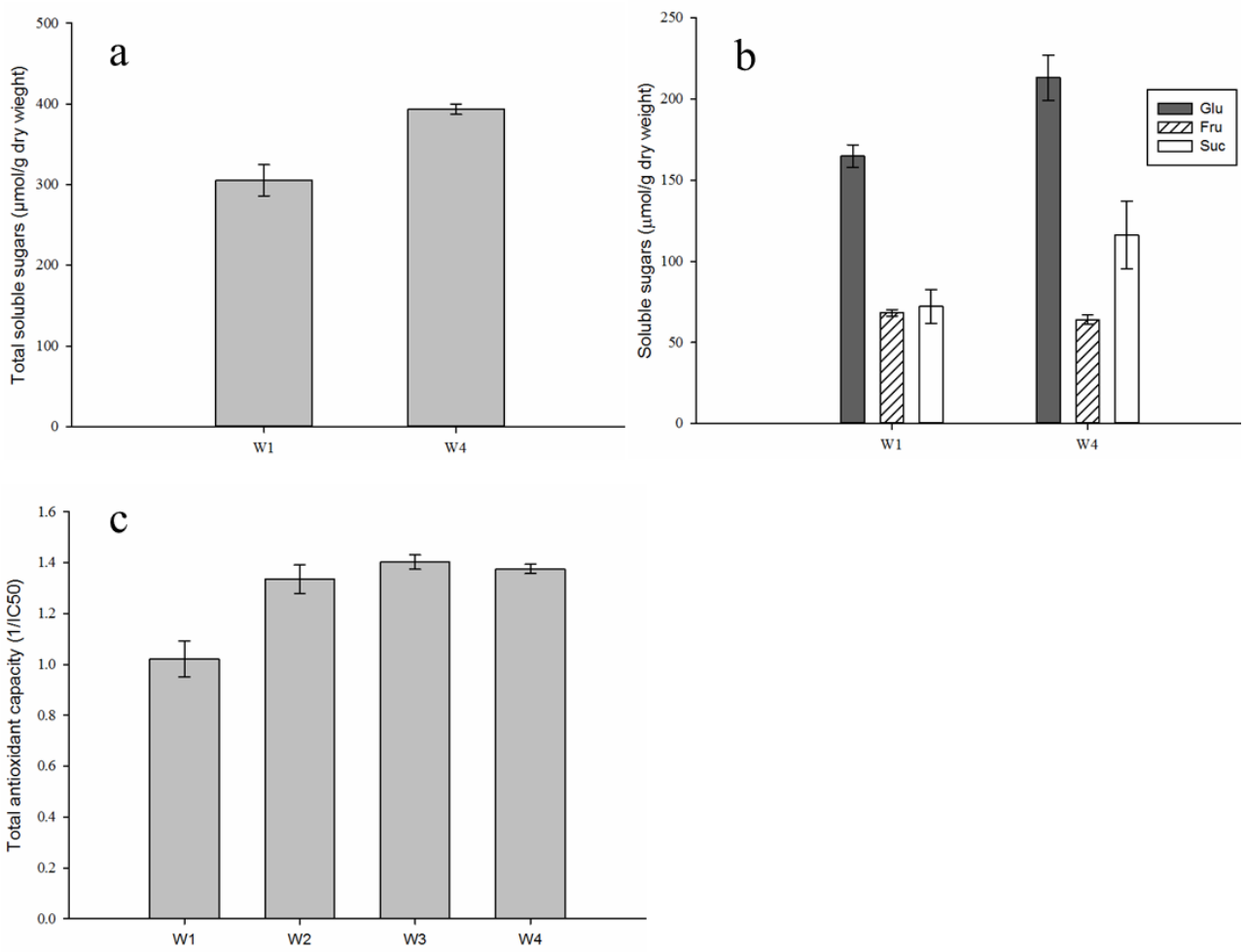
554 N.S.; Not Significant.

555

**Fig. 1**


a
Total of Steviol glycosides (percent of leaf dry matter)
W1
W2
W3
W4
b
Steviol glycosides (percent of leaf dry matter)
Reb A
Stev
Reb F
Reb C
Dulc A
c
Reb A/Stev ratio
d
Steviol glycosides yield (g/plant)

559 **Fig. 2**


a
Total soluble sugars (μmol/g dry wieght)
500
400
300
200
100
0
W1
W4
b
Soluble sugars (μmol/g dry weight)
250
200
150
100
50
0
Glu
Fru
Suc
W1
W4
c
Total antioxidant capacity (1/IC50)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
W1
W2
W3
W4

560