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Productivity of new sweet plant in Morocco (*Stevia rebaudiana* Bertoni) under water stress

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Abstract
Because of the prospect of global climate change most crop will be exposed to negative impacts caused by water stress. A pot experiment was conducted to evaluate the effect of different irrigation regimes on growth, yield and quality parameters of stevia (*Stevia rebaudiana* Bertoni) plants in the Regional Centre of Agronomic Research of Rabat in Morocco (INRA). We applied three irrigation treatments calculated to be 100, 80 and 50% of the mean maximum evapotranspiration (ETm) calculated daily. The results showed that water stress significantly reduced plant height, stem diameter, number of leaves plant $^{-1}$ and leaf area plant $^{-1}$ by 22.44%, 36.54%, 45.10% and 58.15 % at I3 (50% ETm), respectively as compared to I1 (100% ETm). All of the above responses led to reduced fresh biomas and dry leaf yields. Similarly, the total steviol glycosides yield decreased by 37.66% at I3 as compared to I1. By contrast, the total steviol glycosides content significantly increased in I3 stevia leaves than I1 (24.71%). These findings demonstrate that stevia is sensitive to water deficit but adopts adaptive strategies that maintains its yield and increases the content of steviol glycosides.

Keywords: Stevia; Water stress; Leaf area; Steviol glycosides; Dry leaf yield; Morocco.

1. Introduction
Water stress occurs when the demand for water exceeds the available amount during a certain period or when poor quality restricts its use. It frequently occurs in areas with low rainfall and high population density or in areas where agricultural or industrial activities are intense. Water stress leads to changes in morphological, physiological, and biochemical responses of plants. Consequently, plant growth and crop production are negatively affected (Yousfi *et al*., 2016; Stagnari *et al*., 2014; Chrysargyris *et al*., 2016) [45, 39, 9]. It is also characterized by the reduction of leaf water status, which affects photosynthesis through the limitation of the efficiency of the photosystem II (PSII) activity (Fini *et al*., 2013) [12]. In this respect, several strategies have been identified in plants in response to water stress (Chaves *et al*., 2003) [8]. Most research to date has focused on studying the responses to water stress for well-known crops, but these aspects have not been fully investigated in new crops. Understanding how plants respond to water stress 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) [8].
Stevia (*Stevia rebaudiana* Bertoni) is a perennial irrigated summer plant belonging to the *Asteraceae* family, native to the Rio Monday valley, an area in north-eastern Paraguay (Soejarto, 2002; Reis *et al*., 2015) [37, 30]. Its leaves are the economic part of the plant (Ramesh *et al*., 2006) [29], with a high concentration of steviol glycosides (SG), possible substitutes of synthetic sweeteners (Yadav *et al*., 2011 ; Ahmed *et al*., 2007; Ramesh *et al*., 2006) [44, 1, 29] which gives stevia a great importance as a natural food sweetener supplier crop. The major SG in stevia leaf are stevioside (STV) (5–10% of dry leaf weight), which is about 300 times sweeter than sucrose (Crammer and Ikan, 2003) [10] and rebaudioside A (Reb A) (2% - 4%), which is more suited than STV for use in foods and beverages due to its pleasant taste (Tanaka, 1997) [41], but virtually with no calories (Cardello *et al*., 1999) [6]. Therefore, stevia has its own significance in the diet of obese and diabetic people. Other products from stevia are used for pharmaceutical and cosmetic industries (Ahmed *et al*., 2007) [1]. The SG have been approved for use as sweeteners in Japan, China, Brazil, Paraguay, Mexico, Russia, Indonesia, Korea, United States, India, Tanzania, Canada and Argentina (Pal *et al*., 2015) [26]. Though China is the largest stevia producer in the world market, Japan and Korea are the main

consumers (Pal *et al*., 2015) [26]. Stevia is relatively unknown in Morocco, where it can be a new sweet crop. Generally, the produvtivity of stevia depends on many factors (Starratt *et al*., 2002) [40] such as water availability (Vasilakoglou *et al*., 2016; Crammer and Ikan, 2003) [43, 10].

Stevia does not require higher levels and frequency of irrigation for higher biomass production but is much sensitive and susceptible to water stress during the crop growth period (Shock, 1982) [36]. Donalisio *et al*. (1982) [11] also reported that stevia can survive in areas of continuous moisture but not withstand the prolonged water logging conditions. It has been stated that stevia growth was optimal at soil water content of 43-47.6% (Goenadi, 1983) [14]. Midmore *et al*. (2012) [25] reported that plant height, leaf biomass and stem yield increased up to field capacity (FC), but all decreased at 120 % of FC. Under *in vitro* culture condition and using polyethylene glycol to stimulate drought stress on stevia, it has been reported that fresh and dry leaf weights, water content, and chlorophylls were negatively affected by drought stress (Hajihashemi and Ehsanpour, 2013) [16]. In addition, pot data indicated that the soil moisture level near 45% FC (12-day irrigation interval) is a threshold level of soil moisture for stevia, since it caused a significant reduction in stevia plant height and dry leaf yield (Karimi *et al*., 2015) [18]. Shi and Ren (2012) [33] also indicated that there was no appreciable impact on the dry leaf yield in the mild drought (without water for 5_days), but with the drought stress continuing, the dry leaf yield per plant became less and less.

The increase of secondary metabolites under water stress has been frequently reported for many plants (Stagnari *et al*., 2014; Ghane *et al*., 2012) [39, 13], but this response has not been sufficiently investigated in stevia. Guzman (2010) [15] have reported that SG concentration is not responsive to plant water stress. Aladakatti *et al*. (2012) [3] found that the different irrigation schedules did not influence significantly the stevioside and rebaudioside A content of the leaves at harvest, but marginally lower irrigation frequency recorded higher stevioside content and rebaudioside content compared to higher irrigation frequency. Karimi *et al*. (2015) [18] reported that soil moisture reduction up to 60% FC was not harmful to stevia metabolites, while a soil moisture level around the 45% FC represented a stressful condition for stevia, leading to quality reduction. Hajihashemi and Geuns (2016) [17] have reported that polyethylene glycol-induced drought stress has a negative effect on the content of SG. Midmore *et al*. (2012) [25] also observed an increase in SG content with 120% of FC.

Because of the prospect of global climate change, most crop will be exposed to negative impacts caused by water stress. Therfore, the present work was conducted in order to evaluate the effect of different irrigation regimes on growth, yield and quality parameters during the growing cycle of stevia plants in sub humid region and to determine the best level for irrigation achieved a higher dry leaf and SG yields.

2. Materials and methods

2.1. Growing conditions and treatments

A pot experiment was conducted during stevia growing period from 10 June to 28 August 2015, in open field at the Regional Centre of Agronomic Research of Rabat in Morocco (INRA) (34.21 N, 6.40 E, 10.5 m above mean sea-level). Weather data were measured by an automatic weather station (iMETOS, Pessl Instruments, Austria), located near the experimental plot. The mean temperature ranged from 21.9 to 23.8 °C and relative humidity (RH) ranged from 74.3 to 87.4%. The INRA stevia variety was sown into plug trays filled with land and commercial substrate on April 1st, 2015 and watered to field capacity (FC) by tap water. After 70 days (on June 10, 2015), the uniform seedlings were transplanted into pots, with one plant per pot. The 20 L pots were filled with 2 kg of gravel at bottom for drainage and 15 kg of same soil as for study area. Before filling, the soil was collected from 0 to 30 cm soil depth and was analysed. The soil contained 5. 1% clay, 11.4% silt, and 83.5% sand. The organic matter content was 2.2%, the pH was 7.8 and the N, P and K contents were 120, 106.6 and 154 ppm, respectively. Soil moisture at field capacity was 13.41 % and soil moisture at permanent wilting point was 4.32%. Soil moisture was maintained near the field capacity for the first four weeks and then the irrigation treatments were applied as 100%, 80% and 50% of the maximum evapotranspiration (ETm) calculated daily by water balance method and transformed into three developmental stages in earlier research (Benhmimou *et al*., 2016) [5]. Water loss was determined by weighing the pots at the interval of two days using an electronic weighing device, and then water was added to re-establish initial weight. Weighing of pots was done between 7:00 and 8:00 am. All pots were irrigated manually using graduated cylinder of 250 ml and beaker 1L based. There were three levels of water stress, 3 replications and 3 pots per replicate of stevia plants totalling 27 experimental pots arranged according to a randomized complete block design. The plants of the whole pots were harvested manually 10 cm above the base of the stem (Megeji *et al*., 2005) [24] at 80 days after transplanting, leaves and stems were separated and used for further data analysis.

2.2. Data collection

2.2.1. Growth parameters

The plant height was measured with a meter ruler from ground to the base of the fully opened leaf and the stem diameter was measured with slide calipers up to 0.01 mm accuracy. Fully opened leaves were counted in each plant and the mean was computed to get the average number of leaves $plant^{-1}$. 20 leaves were randomly selected from each plant and leaf area was obtained using leaf area meter. Leaf area per leaf was calculated and multiplied with respective number of leaves $plant^{-1}$ to get leaf area $plant^{-1}$ and expressed in cm^2.

2.2.2. Yield parameters

Stevia total fresh biomass, fresh leaf yield, fresh stem yield, dry leaf yield and leaf to stem ratio (fresh leaf yield / fresh stem yield) were determined in each plant. We estimated the fresh biomass, fresh and dry leaf yield per plant using one digital scale with precision of 0.01 g. Leaves were dried at 50°C temperature in hot air dryer for 6 hours and stored in clean gunny bags. At this temperature, the quality of dried leaves produced, in terms of colour, sweetness and nutrient content, was better compared with drying at 70°C (Samsudin and Aziz, 2013) [32]. Dry leaf had an important role in stevia extract in term of quality (Yadav *et al*., 2011) [44].

2.2.3. Quality parameters

Dry leaves of stevia obtained during this trial were ground in a laboratory grinding mill to produce powder particles of 0.10 mm in size, and were kept at ambient temperature until they were used for the analysis to assess the contents of stevioside (STV), rebaudioside A (Reb A) and total steviol glycosides (STV; Reb, A, B, C, D and F; steviolbioside; rubudioside and dulcoside A) as influenced by water stress. STV (%), Reb A (%) and total SG (%) were determined in the powdered stevia leaves sent to the STEVIA NATURA Company of France.

The SG yield was calculated by multiplying dry leaves yield by the concentration of SG in leaves.

2.3. Statistical analysis

Data obtained were analyzed by the analysis of variance (ANOVA) using Statistical Analysis System ver. 9.1 (SAS Institute Inc., Cary, NC., USA), and means were compared using Duncan's multiple range test (DMRT) at the 0.05 significance level.

3. Results

3.1. Growth parameters

The ANOVA performed for the stevia plant height, stem diameter, number of leaves plant^{-1} and leaf area plant^{-1} at harvest indicated that these growth parameters were significantly affected by the irrigation levels (Table 1). Irrigation regime I1 (100% ETm) recorded significantly higher plant height (64.89 cm) which was on par with the irrigation regime I2 (80% ETm) (61.40 cm), whereas irrigation regime I3 (50% ETm) resulted in a reduction of plant height of 18.02% and 22.44% compared to I2 and I1, respectively. Stem diameter, number of leaves plant^{-1} and leaf area plant $^{-1}$ data showed the same trend as plant height (Table 1). Irrigation regime I1 recorded significantly higher stem diameter, number of leaves plant^{-1} and leaf area plant^{-1} (10.92 mm, 466.11, and 10443.204 cm^2, respectively) followed by irrigation regime I2 (8.911 mm, 404.56, and 8128.804 cm^2). Lower stem diameter, number of leaves plant^{-1} and leaf area plant^{-1} were recorded with water regime I3 (6.93 mm, 255.89, and 4370.904 cm^2).

Table 1: Effect of water stress on growth parameters of stevia at harvest.

Water regime	Parameters			
	Plant height (cm)	**Stem diameter (mm)**	**Number of leaves plant $^{-1}$**	**Leaf area plant $^{-1}$ (cm^2)**
I1 (100% ETm)	64.89^A	10.92^A	466.11^A	10443.20^A
I2 (80% ETm)	61.40^B	8.91^B	404.56^B	8128.80^B
I3 (50% ETm)	50.33^C	6.93^C	255.89^C	4370.90^C

*Means followed by different letters in each column are significantly different (Duncan multiple range test at the 5 % significance level).

3.2. Yield parameters

The fresh biomass yield, fresh leaf yield, fresh stem yield and dry leaf yield at harvest were significantly affected by water stress (Table 2). Increasing water regime increased fresh biomass, fresh leaf yield, fresh stem yield and dry leaf yield up to ETm but they decreased at the lower water regime I3. Water regime I1 resulted in higher fresh biomass (155.37 g plant $^{-1}$), fresh leaf yield (85.68 g plant^{-1}), fresh stem yield (69.69 g plant^{-1}) and dry leaf yield (24.79 g plant^{-1}), which was followed by water regime I2 (126.79, 70.04, 56.74, and 20.36 g plant^{-1}, respectively). The lower water regime I3 significantly decreased fresh biomass yield, fresh leaf yield, fresh stem yield and dry leaf yield until 50.65%, 49.99%, 51.47% , and 53.85%, respectively, compared to I1. On the contrary, fresh leaf to stem ratio was not affected by water regime (Table 2). The highest ratio was obtained at I1 (1.31) closely followed by I3 (1.27) and I2 (1.24). The result of leaf to stem ratio indicated that the contribution of leaf to the overall fresh biomass yield of stevia is higher in all treatments.

Table 2: Effect of water stress on yield parameters of stevia.

Water regime	Parameters				
	Fresh biomass yield (g plant^{-1})	**Fresh leaf yield (g plant^{-1})**	**Fresh stem yield (g plant^{-1})**	**Dry leaf yield (g plant^{-1})**	**Ratio Leaf/Stem**
I1 (100% ETm)	155.37^A	85.68^A	69.69^A	24.79^A	1.31^A
I2 (80% ETm)	126.79^B	70.04^B	56.74^B	20.36^B	1.24^A
I3 (50% ETm)	76.67^C	42.85^C	33.82^C	11.44^C	1.27^A

* Means followed by different letters in each column are significantly different (Duncan multiple range test at the 5 % significance level).

3.3. Quality parameters

Water stress caused a significant effect on total steviol glycosides (SG) content in stevia dry leaves (Table 3). The highest value of total SG content was obtained in plant irrigated at 50% ETm (12.87% of the leaf dry weight) followed by I2 (11.17%) and I1 (9.69%). The analysis of SG compositions showed that STV and Reb A were significantly affected by water regime (Table 3). STV content values showed the same trend as total SG content. The highest content of STV was obtained in 50 % ETm treatment (6.26%) followed by I2 (4.59%) and I1 (4.30%). On the contrary, Reb A increased with 80% ETm treatment and decreased with I1.The water stress caused a significant effect on SG yield (SG production per plant leaves) and the highest values of STV and total SG yields were observed in plants grown under 100% ETm (1.07 and 2.39 g plant^{-1}, respectively), while the highest Reb A yield was recorded with I2 (1.02 g plant^{-1}) followed by I1 (0.93 g plant^{-1}) and I3 (0.53 g plant^{-1}). However, there was no significant difference between 100% and 80% ETm treatments, regarding Reb A and total SG yields of stevia (Table 3).

Table 3: Effect of water stress on quality parameters of stevia.

Water regime	Parameters					
	Stevioside (%)	**Rebaudioside A (%)**	**Total SG (%)**	**Stevioside (g plant^{-1})**	**Rebaudioside A (g plant^{-1})**	**Total SG (g plant^{-1})**
I1 (100% ETm)	4.30^C	3.74^C	9.69^C	1.07^A	0.93^A	2.39^A
I2 (80% ETm)	4.59^B	5.00^A	11.17^B	0.94^B	1.02^A	2.27^A
I3 (50% ETm)	6.26^A	4.66^B	12.87^A	0.72^C	0.53^B	1.49^B

* Means followed by different letters in each column are significantly different (Duncan multiple range test at the 5 % significance level).

4. Discussion

The reason for higher fresh biomass yield, fresh leaf yield, fresh stem yield and dry leaf yield at harvest with the water regime I1 (100% ETm) was attributed to the higher growth parameters viz., plant height, stem diameter, number of leaves plant^{-1} and leaf area plant^{-1} produced at this level, as compared with those for I3 (50 % ETm). This might be due to more availability of water which facilitate nutrient accumulation, maintained cell turgidity and increased number of leaves which converted more solar energy and fixed more CO2 to produce more photosynthates, and thus greater growth (Souch and Stephens, 1998) [38]. On the basis of our findings, the irrigation level at 50% ETm could be considered as a drought stress level for stevia cultivation under pot condition, effectively inhibiting the fresh biomass and dry leaf yields in comparison to full evapotranspiration. The reduction of yield as a result of water stress can be caused by reduced leaf area and reduced photosynthesis per unit of leaf area (Rouphael *et al*., 2012) [31].

Our findings were in agreement with Karimi *et al*. (2015) [18], who found that the soil moisture level near 45% FC (12-day irrigation interval) is a threshold level of soil moisture for stevia, since it caused a significant reduction in stevia plant height and dry leaf yield in pot experiment. Shi and Ren (2012) [33] also indicated that there was no appreciable impact on the dry leaf yield in the mild drought (without water for 5 days), but with the drought stress continuing, the dry leaf yield per plant became less and less. Donalisio *et al*. (1982) [11] reported also, in Brazil, higher dry leaf yield of stevia under irrigation due to better plant growth with higher number of leaves plant^{-1}. On the contrary, Shock (1982) [36] reported that stevia does not require higher levels and frequency of irrigation for higher biomass production but opined that the crop is much sensitive and susceptible to water stress during the crop growth period. The higher dry leaf yield in this study was higher than the yield reported by Midmore *et al*. (2012) [25] who recorded a dry leaf weight of 3.0 g plant^{-1} for the December harvest.

The results of reduced leaf number in water deficit conditions is similar to the findings by Shilpi and Malvika (2014a) [34] who observed that the stress conditions caused a decrease in the number of leaves of stevia compared to non-stress conditions and maximum decrease was found in severely stressed plants having the lowest values. An experiment carried out in south Italy showed that more leaf senescence was observed through decreasing irrigation volumes (Lavini *et al*., 2008) [21]. Ramesh *et al*. (2006) [29] also reported more number of leaves plant^{-1} of stevia with adequate irrigation. However, Pordel *et al*. (2015) [28] found that the number of leaves of stevia in non-stress conditions was 239.08.

The value of leaf area plant $^{-1}$ obtained in this study is higher than the value reported by Aladakatti (2011) [2] who recorded an average leaf area of 4360 cm^2 plant^{-1} in an experiment conducted in India. Pordel *et al*. (2015) [28] found that the leaf area and plant height of stevia in non-stress conditions were 239 cm^2 and 81.7 cm, respectively. Shilpi and Malvika (2014b) [35] also reported that the stevia leaf area and plant height recorded during the experimental period were highest in well watered plants and decreased with decreasing water levels.

Evaluation of leaf to stem ratio revealed that all water regimes could record higher leaf to stem ratio. This might be due to higher temperature (21.9 to 23.8 °C) and longer photo periods that prevailed during the crop growth period, resulting in advanced growth with high number of leaves plant^{-1}. This had positive impact on the biomass yield and dry leaf yield which has an important role in stevia extract in term of quality (Yadav *et al*., 2011) [44]. Several research revealed that temperature, length and intensity of photoperiod significantly affected stevia biomass production as is evident from the remarkable increase in yield during summer cuttings than that of winter cuttings (Allam *et al*. 2001) [4]. The sensitivity of stevia crop to day length, photoperiod and temperature was also reported by Lester (1999) [22] in Australia and, Valio and Rocha (1977) [42] in Japan. In this experiment stevia leaf/stem ratio ranged from 1.24 to 1.31 and was slightly greater than those reported by Kumar *et al*. (2014) [20] and Megeji *et al*. (2005) [24], who found that this ratio ranged from 0.9 to 2.0 and 0.79 to 1.14, respectively.

The STV and total SG contents of stevia leaves increased under severe drought stress (I3), while the Reb A content increased under moderate drought stress (I2). This increase in SG contents could be a mechanism to withstand drought stress, thus maintaining high cellular integrity in plant tissues.This would allow the plant to maintain water at the cell level as a result of an increase in the intracellular osmotic potential (Ceunen and Geuns, 2013) [7]. Similary, Karimi *et al*. (2015) [18] reported that the highest value of total SG content was obtained in plant irrigated at 60% FC, while Aladakatti *et al*. (2012) [3] found that the different irrigation schedules did not influence significantly the stevioside and rebaudioside A content of the leaves at harvest, but marginally lower irrigation frequency recorded higher stevioside content (11.69%) and rebaudioside content (5.79%) compared to higher irrigation frequency (11.33% and 5.3%). Megeji *et al*. (2005) [24] recorded a value of 9.94% for stevioside content in the leaves. Kovylyaeva *et al*. (2007) reported that levels of stevioside ranging from a minimum of 4.6% in Paraguay to a maximum of 15.5% in Vietnam, while rebaudioside A ranged from 0.3% in Canada to 3.8% in Vietnam and Paraguay. Pereira *et al*. (2016) also found that the highest concentration of stevioside was 12.16% while rebaudioside A was 7.01% in December. It is difficult to explain the variation among the different SG contents of stevia because the physiological and molecular mechanisms of SG biosynthesis responding to drought stress have not been yet fully elucidated.

Our study showed that the greatest SG yield could be obtained with a irrigation level at 100% ETm compared to those obtained in the presence of severe drought stress condition (50% ETm). This greater productivity could be attributed to greater dry leaf yield produced in I1, as compared with those in I3. Since our results confirmed the sensitivity of stevia leaves to water stress, it is recommended that stevia should not experience serious water stress during its vegetative growth, in parliamentary procedure to achieve the optimum yield. Similarly, Karimi *et al*. (2015) [18] found that the soil moisture level near at 45% FC (12-day irrigation interval) is a threshold level of soil moisture for stevia, since it caused a significant reduction in stevia SG yield. In contrast, Lavini *et al*. (2008) [21], and Aladakatti *et al*. (2012) [3] reported that SG yield is not responsive to water stress.

Our results also indicated that water regime at 80% ETm was not deterrent for stevia Reb A yield and total SG yield under pot conditions because no significant reductions in these traits were recorded at this regime of irrigation. As well, in a similar research carried out in pot condition, Karimi *et al*. (2015) [18] indicated that soil moisture reduction up to 60% FC (9-day interval) was not deterrent for stevia production because no significant reductions in stevia SG yield were recorded at this level of soil moisture content. It was also observed that moderate water-deficit stress (8-days irrigation period) did not significantly affect the SG yield (Guzman, 2010) [15]. Accordingly, it can be outlined that the stevia was able to tolerate moderate water sress (I80) at the end of the growing cycle. 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) [23].

Conclusions

In this research, it has been observed that the stevia biomass production and steviol glycosides (SG) yield were higher in the full irrigation treatment (100% ETm), while an evapotranspiration level around the 50% ETm represented a stressful condition for stevia, leading to growth and yield reduction. However, moderate stress (80% ETm) was not deterrent for stevia Reb A yield and total SG yield because no significant reductions in these traits were recorded at this regime of irrigation, while this regime diminished plant height, reduced leaf number, and biomass production of the plant. On the basis of SG content, reasonable values were obtained in a irrigation level at 50% ETm, regarding a more efficient use and saving of the water under pot conditions. The increase of SG in drought stressed-stevia was in favor of acclimation mechanisms and it seemed that did not efficiency used for SG production under the identical condition. These findings demonstrate that stevia is sensitive to water deficit but adopts adaptive strategies that maintains its yield and increases the concentration of SG.

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