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In Vitro Evaluation of Some Traits in *Stevia rebaudiana* (Bertoni) under Drought Stress and Their Relationship on Stevioside Content

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Abstract

The experiment was conducted to evaluate the differences of plant growth as well as sweetener material of two types of *Stevia rebaudiana* under conditions of absence and presence of drought stress. *In vitro*, the experiment was conducted as drought traits using four levels of polyethylene glycol (0.0, 10,000, 20,000, 30,000 ppm). It was observed that survival % and No. of shoots should be considered as the important factors associated with stevioside content in stevia plant. According to path coefficient analysis and positive direct effect of studied traits, survival % recorded the highest value (0.5386), whereas No. of shoots recorded the highest value (−0.8827) as negative direct effect in related to stevioside content. The results showed that all traits were affected, especially under 30,000 ppm level of polyethylene glycol. Generally, the most of studies traits exhibited a recorded clearly difference between the two types and used drought levels on stevia plants. Therefore, selection based on survival % would be more effective to improving stevioside content of stevia plants in drought stress conditions.

Keywords

Stevia, Drought Stress, Polyethylene Glycol, Stevioside, Path Coefficient

1. Introduction

In recent years, an increasing demand has been noted for new natural substitute sweeteners such as sucrose or synthetic sweeteners. Leaves of stevia plant were used historically in northern of Paraguay as sweeteners and

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herbal remedy, and sweeteners contain compounds about 200 to 350 times higher than sucrose. It is a sweet gaining significance in different parts of the world because of the non-caloric sweeteners extracted from its leaves.

Stevia plant is still one of the plants recently domesticated. It is believed that yield improvements can be achieved by selection plant breeding for increasing stevioside content, leaf-to-stem ratio, and the degree of plant response to environmental stress conditions as detected by Clinton [1]. The viability of stevia seed shows a poor percentage [2] and a very low germination percentage was recorded by Goettemoeller and Ching [3]. Hence, the propagation through seeds is not adequate, due to a very low seed germination percentage [4]. In the same context, [5] reported that, germination rates of stevia seeds vary greatly.

For such, plant cell and tissue culture has been a useful tool for propagation to study stress tolerance mechanisms under *in-vitro* conditions which are characterized by a limited space, a short period of time and homogeneity of stress application [6]. Polyethylene glycol (PEG) of high molecular weights has been long used to simulate drought stress in plants as non-penetrating osmotic agents lowering the water potential in a way similar to soil drying [7] [8]. Osmotic solution, such as PEG which has been used to impose water stress by exposing the root system of plants, can resolve the problem. Addition of PEG to nutrient solution produces osmotic stress over a period of 3 - 4 weeks. PEG is used successfully to decrease the water potential of plants as it does not enter into the root [9].

In the plant life cycle, seed germination and seedling stages are sensitive to environmental stress [10]. In the same manner, plant species differ in their sensitivity or tolerance to salt stress [11].

Correlation measured the mutual association between two variables which aids in determining the most effective procedures for selection of superior genotypes. However, correlation analysis is not enough to give an exact picture of relative importance of direct and indirect influence of each component character on seed yield. Path coefficient analysis is an important tool for plant breeders, which helps in partitioning the correlation coefficient into components of direct and indirect influences and provides better insight on character and their relationship with yield. The objective of this study was to estimate the association of yield contributing characters, their direct contribution to yield and indirect effects through other characters on pigeon pea yields under rainfed situations [12] [13].

This paper presents to study the effect of water deficit on two types of *Stevia rebaudiana* (Bertoni) grown under *in vitro* using different polyethylene glycol levels as a degree for drought stress.

2. Materials and Methods

2.1. *In Vitro* Conditions

Actively growing shoots of *S. rebaudiana* (Bertoni) were collected as source of explants from plants grown in the greenhouse at the Desert Research Center, Cairo, Egypt. Stem nodal segments of about 1.5 cm in length were isolated from the shoots. The explants were washed in running tap water and then washed again thoroughly by adding a few drops of Tween-20 to remove the superficial dust particles as well as fungal and bacterial spores. They were then surface sterilized with 1.5% sodium hypochlorite solution for 20 min and finally rinsed six times with sterile double distilled water inside the laminar air flow chamber. The explants were then inoculated aseptically into MS [14] medium with different concentrations and combinations of growth regulators. Stem node segments were inoculated on MS medium supplemented with 0.5 mg/L 6-benzylaminopurine and 0.1 mg/L kinetin as mentioned by Abd Alhady [15]. The most vigorous shoots were taken from the establishment's stage. Stem node segments (2 - 3 cm long) containing two axillaries buds were cut and cultured in various media. MS medium containing 2 mg/L BAP and 0.5 mg/L kin supplemented with different concentration (0.0, 10, 20, 30 g/L PEG).

2.2. Characters Studied

The data were recorded in vitro on the following parameters after 25 days as follow:

1—Survival (%). 2—Length of shoots (cm). 3—No. of shoots/explant. 4—Fresh weight/20 shoots (g). 5—Dry weight (g)/20 shoots at 60˚C for 48 hour. 6—Stevoside content (ppm): fresh leaves harvested by cutting from explants of *Stevia rebaudiana* after brown and yellow leaves were removed from the explants then, methanol was added to ground leaves at ratio (4:1 v/w) and remained for 7 h, then filtered through Whatman No. 4 filter paper, then stored at 4˚C ± 1˚C in a refrigerator until used. Stevioside standard determination was carried out according to Nishiyama *et al.* [16] using HPLC. Pure stevioside powder obtained from N. U. Natural Inc., USA as standard.

2.3. Experimental Design and Statistical Analysis

The experimental design was arranged in factorial based a randomized complete block design (RCBD) with five replications where the first factor was four polyethylene glycol 6000 potential levels (0.0, 10,000, 20,000, 30,000 ppm) and the second factor was the types of *Stevia rebaudiana* (Bertoni). Statistical analysis of variance, Means separation was conducted by f-test for both drought levels (L) and the interaction between drought levels and types (L*T) while, the two group means of types (T) conducted using t-test according to MSTATC software program [17]. For means comparison, LSD values were calculated following the method of Gomez and Gomez [18]. Statistical analyses for all studied traits were done following Singh and Chaudhary [19] for correlation coefficient and Dewy and Lu [20] for path analysis. Regarding, survival (as a percentage) data were transformed by arcsine before analysis of variance.

3. Results and Discussions

The significance levels for measured traits, including survival (%), length of shoots (cm), No. of shoots, fresh weight (g), dry weight (g) and stevioside content (ppm) are shown in **Table 1**. The analysis of variance showed that two types of stevia (T) and the treatments as drought stress levels (L) differed significantly for all studied characters. At the same time, the results also exhibited that, the interaction between drought levels treatments and types of stevia (L*T) were significant for all traits except No. of shoot. The differential responses of different concentrations levels of PEG on two types of stevia and the interaction (L*T) can be seen more clearly through **Table 2** and **Figures 1(a)-(f)** for all studied traits. Generally, treatments of stevia seeds with 6000 ppm polyethylene glycol showed clearly reduction in all traits except stevioside content (**Figure 1(f)**) which recorded high value (124.753 ppm) with increasing to highest drought level (30,000 ppm) and this result agreement with different investigation [4] [10] [21] which they reported that both seed and seedling stages are key developmental stages conditioning the final yield of crops because of its sensitive to environmental stress. Also, [22] reported that, water deficit affects the germination of seed and the growth of seedling negatively. These results provided the evidence for the significant responses variability present for these traits under drought stress conditions. These results are in agreement with those of Shokoofeh and Ali [23] they studied the effect of polyethylene glycol (PEG; 0% - 6% w/v) on *Stevia rebaudiana* to stimulate drought stress and they reported that drought stress reduced fresh and dry weight, water content, chlorophylls, carotenoids. In the same trend, [24] decided that, the effect of drought stress without irrigation for 10 days showed maximum decrease in net photosynthetic, transpiration rate and dry leaf production of stevia germplasms, they decreased for 28.85%, 47.04%, 43.03%, 43.61% and 42.25% than in drought stress without irrigation 5 days respectively. Also, [25] reported that, callus and suspension biomass cultured of stevia plant on salts showed less growth when compared with control. As well as, they found that abiotic stress induced by the salts increased the concentration of steviol glycoside significantly. In callus, the quantity of steviol glycoside got increased from 0.27 (control) to 1.43 and 1.57% with 0.10% NaCl, and 0.025% Na_2CO_3, respectively.

Drought stress is physiologically related, because induced osmotic stress and most of the metabolic responses of the affected plants are similar to some extent [26].

Simple correlation coefficients among all traits were estimated in **Table 3** and the results clearly indicate that survival % (−0.805), length of shoots (−0.807), No. of shoots (−0.928), fresh weight (−0.941) and dry weight (−0.942) has significant and negative relation with stevioside content under overall studied treatments conditions and types. It could be concluded that, dry weight followed by fresh weight should be considered as the important traits with stevioside content under drought stress.

Table 1. Observed mean square from ordinary analysis of variance for studied traits.

S.O.V	d.f	M.S					
		Survival (%)	Length of shoots	Number of shoots	Fresh weight	Dry weight	Stevioside
Replicates	4	0.186^{ns}	0.002^{ns}	0.212^{ns}	0.168^{ns}	0.0001^{ns}	0.026^{ns}
Treatments (L)	3	3618.59^{**}	62.597^{**}	1251.37^{**}	264.77^{**}	0.591^{**}	1687.852^{**}
Types (V)	1	42.44^{**}	0.210^{**}	26.24^{**}	7.225^{**}	0.014^{**}	127.215^{**}
L*V	3	4.99^{**}	0.015^{**}	3.175^{**}	0.343^{ns}	0.005^{**}	4.546^{**}
Error	28	0.199	0.003	0.192	0.130	0.0001	0.0 39

ns, **: non-significant and significant at 0.05 and 0.01 level of probability, respectively.

Table 2. Performance of two types stevia under different drought levels and interaction between them.

	Survival (%)	Length of shoots (cm)	Number of shoots	Fresh weight (g)	Dry weight (g)	Stevioside (ppm)
Drought levels (L)						
L1	89.960	7.880	31.780	16.040	1.305	104.474
L2	83.300	5.990	34.400	15.900	1.295	98.465
L3	71.900	4.331	19.700	8.110	0.913	122.238
L4	46.720	1.989	10.280	6.210	0.850	124.753
LSD (0.05)	0.409	0.050	0.401	0.330	0.009	0.181
Types (T)						
T1	74.000	5.120	24.850	11.990	1.109	114.266
T2	71.940	4.975	23.230	11.140	1.072	110.699
t-test	**	**	**	**	**	**
Interaction (L*T)						
L1*T1	90.000	7.940	33.400	16.280	1.310	106.293
L2*T2	89.920	7.820	30.160	15.800	1.300	102.656
L3*T1	85.000	6.040	35.000	16.200	1.300	99..329
L4*T2	81.600	5.940	33.800	15.600	1.290	97.600
L1*T1	73.000	4.462	20.400	8.740	0.928	124.716
L2*T2	70.800	4.200	19.000	7.480	0.898	119.760
L3*T1	48.000	2.038	10.600	6.740	0.900	126.725
L4*T2	45.440	1.940	9.960	5.680	0.800	122.780
LSD (0.05)	0.578	0.070	0.568	0.467	0.013	0.256

**Significant at 0.01 level of probability.

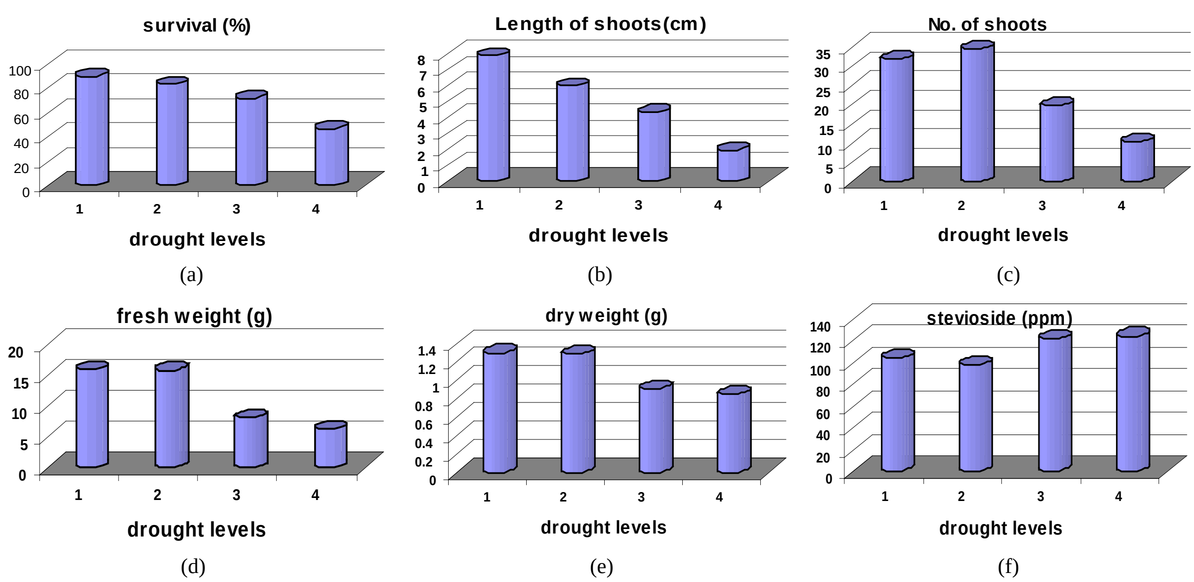

Figure 1. Effect of drought levels using polyethylene glycol concentration on studied traits. Where, 1 = control (L1); 2 = 10,000 ppm polyethylene glycol (L2); 3 = 20,000 ppm polyethylene glycol (L3); 4 = 30,000 ppm polyethylene glycol (L4); No. of shoots = number of shoots.

Analysis of simple correlation explained, that relationship seed yield to a single variable may not provide a complete understanding about the importance of each component in determining seed yield [20]. They added that path coefficient analysis is a statistical technique of partitioning the correlation coefficient into its direct and indirect effects, so that the contribution of each character to stevioside content could be estimated. Total correlation coefficient between both fresh weight and dry weight traits with stevioside content (−0.941 and −0.942 re-

Table 3. Correlation coefficient among studied traits under overall treatments and types.

Traits	Survival	Length of shoots	Number of shoots	Fresh weight	Dry weight
Length of shoots	0. 97**				
Number of shoots	0.942**	0.913**			
Fresh weight	0.900**	0.918**	0.968**		
Dry weight	0.881**	0.906**	0.955**	0.993**	
Stevioside	−0.805**	−0.807**	−0.928**	−0.941**	−0.942**

**Significant at 0.01 level of probability.

Table 4. Partition of correlation coefficients into direct and indirect effect for mean stevioside content under overall treatments and types of stevia plant.

Traits	Direct effect	Indirect effect via					Correlation with stevioside content
		Survival %	Length of shoots	Number of shoots	Fresh weight	Dry weight	
Survival (%)	0.5386	-	−0.0082	−0.8315	−0.2360	−0.2692	−0.805
Length of shoots	−0.0084	0.5229	-	−0.8059	−0.2407	−0.2768	−0.807
Number of shoots	−0.8827	0.5073	−0.0077	-	−0.2539	−0.2918	−0.928
Fresh weight	−0.2623	0.4847	−0.0077	−0.8544	-	−0.3034	−0.941
Dry weight	−0.3055	0.4745	−0.0076	−0.8429	−0.2604	-	−0.942

spectively) were mainly due to No. of shoots. It emphasizes that selection of both dry weight and fresh weight alone (direct selection) would not reliable criteria for improving stevioside content (**Table 4**).

Direct effect of all traits except survival % trait was negative and ranged from the lowest in the length of shoots (−0.0084) to the highest in No. of shoots (−0.8827) values. Direct effect of No. of shoots on stevioside content (−0.8827) is almost equal to correlation between them (−0.928) indicates that direct selection of No. of shoots may be reliable criteria for improving stevioside content. These results agreement with Singh and Chaudhary [19] they reported that, if the correlation between a causal factor and the effect is almost equal to its direct, then correlation explains the true relationship and a direct selection through this trait well be effective. On the other hand, direct effect of survival % was positive and recorded high value with stevioside content (0.5386) and recorded the highest value by negative indirect effect through No. of shoots (−0.8315). It indicates that survival % may be used as reliable criteria for screening high content of stevioside directly but we cannot using any traits as indirect effect for improve stevioside content (**Table 4**). Correlation coefficient may be negative but the direct effect is positive and high so, under these circumstances, a restricted simultaneous selection model is to be followed, *i.e.* restrictions are to be imposed to nullify the undesirable indirect effects in order to make use of the direct effect [27].

In the same manner, the direct effect of length of shoots was negative and negligible (−0.0084), while indirect effect through survival % was positive and high (0.5229), so it indicates that direct selection of length of shoots may be used as limited reliable criteria for improving stevioside content under drought stress conditions and used indirect effect selection of survival % for high content of stevioside. While, it must be considered that both dry ad fresh weight through survival % trait very important to improve stevioside content in stevia plants under drought treatments. This trend is consistent with the plant breeders who prefer yield components that indirectly increase yield [13].

4. Conclusion

The previous data suggest that a biotic stress induced by the drought increased the concentration of stevioside content significantly. Also, survival % as a positive direct effect factor followed by No. of shoots as a negative direct effect factor can be relied upon as reliable criteria for improving stevioside content of stevia plant under drought stress conditions.

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