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IMPACT OF GLYCINE BETAINE ON SALINITY TOLERANCE OF STEVIA (*STEVIA REBAUDIANA* BERTONI) UNDER *IN VITRO* CONDITION

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ABSTRACT. Stevia (*Stevia rebaudiana* Bertoni), with great potential as a natural sweeteners source, has a high content of sweeteners, which are up to 150 times sweeter than sugar, but virtually with no calories. Stevia also suitable to be cultivated in semiarid climates and coastal areas, which are characterized by the low quality of the irrigation water. Soil salinity occupies a prominent place among the soil problems that threaten the sustainability of agriculture over a vast area in the world. Glycine betaine is an osmoprotectant, that plays an important role and accumulates rapidly in many plants during salinity or drought stress. In order to evaluation of glycine betaine amending effects on salinity stress in stevia under *in vitro* condition, a factorial experiment was conducted in 2015. Four NaCl levels, including 0, 50, 75 and 100 mM, along with 0, 1, 12.5, 25 and 50 mM of glycine betaine concentrations were used in Murashige and Skoog (MS) medium. The results showed that salinity levels had significant reduction effects on plant height, root length, shoot fresh weight, number of leaf, total chlorophyll, rebaudioside A and stevioside of the stevia genotype. Due to increasing of glycine betaine, levels all the traits were increased. Owing to amending effect of glycine betaine, its high concentrations made less hazarding effects of salinity on the researched traits. The highest mean value of rebaudioside A (10.62rt) and stevioside (23.38rt) determined at 50 mM of glycine betaine with 0 mM of NaCl concentration.

Keywords: factorial experiment; osmoprotectant; stevioside; stress; sweeter.

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INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni), the nature's sweetest gift, belongs to the family Asteraceae really stands out in that it has numerous health benefits (Rashid *et al.*, 2013). Stevia is also known as by the name of sweet leaf, honey leaf, sweet herb, honey yerba etc (Hossain *et al.*, 2008). There are nearly 300 species in the genus of *Stevia* dispersed all over the world. Of these, only *S. rebaudiana* contains the secrete of stevioside, which makes it the sweetest herb in the world (Soejarto *et al.* 1983; Kolb *et al.,* 2001). The leaves naturally enclose a complex mixture of eight sweet diterpene glycosides, including stevioside, steviolbioside, rebaudiosides (A, B, C, D, E) and dulcoside A (Goyal & Samsher, 2010). Rebaudioside A is approximately 250 to 300 times sweeter than sucrose. Research has shown that rebaudioside A does not contribute calories or carbohydrates to the diet and does not influence blood glucose or insulin response, which permits people with diabetes to consume a greater variety of foods and obey with a healthful meal plan. Stevioside is also 300 times sweeter than sucrose at 0.4% sucrose concentration (Soejarto *et al.,* 1983; Liu & Li 1995). Stevioside is chemically stable and happens in the dried leaves of *S. rebaudiana* at about 42% (w/w). *S. rebaudiana* essential oil and extracts possess high antioxidant, anti-inflammation and antimicrobial properties (Muanda *et al.,* 2011). The propagation through seeds is not sufficient leading to a very low seed germination percentage (Taware *et al.,* 2010). Salinity is a main abiotic stress and is likely to boost in severity as a consequence of universal warming (Ashraf & Harris, 2004; Sairam & Tyagi, 2004). Three primary components verify salinity tolerance: osmotic tolerance, Na+ exclusion and tissue tolerance. All three components are important, but supply differently to overall salinity tolerance (Sherif *et al.,* 2007; Khosravinejad *et al.* 2009; Kapoor & Srivastava 2010; Radi *et al.,* 2013).

Many plants build up compatible solutes or osmoprotectants, which serve as nontoxic solutes for cytoplasmic osmoregulation, and can also partly reverse the harmful effects of salts on proteins and membranes (Bohnert & Shen 1999; Yancey, 1994). The metabolic engineering of accretion of osmoprotectant has therefore attracted wide attentions, as a way to improve crop stress resistance (Russell *et al.,* 1998). One of the most comprehensively studied compatible solutes is glycine betaine, which not only acts as an osmoregulator, but also stabilizes the structures and activities of enzymes and protein complexes, and retains the integrity of membranes against the damaging effects of extreme salt in many plant species (Sakamoto & Murata 2002). Glycine betaine is a common well-matched solute that accumulates in many species of *Poaceae*, *Amaranthaceae*, *Asteraceae* and *Chenopodiaceae*, but is missing in such plant species as carrot, soya

bean, castor bean and mustard (Flower & Yeo, 1986; Rhodes & Hanson, 1993). Levels of glycine betaine in the *Poaceae* are associated with salt tolerance. Highly tolerant *Spartina* and *Distichlis* mount up the highest levels, moderately tolerant species accumulate intermediate levels, and susceptible species accumulate low levels or no glycine betaine (Rhodes *et al.,* 1989). Genetic indication that glycine betaine improves salinity tolerance has been acquired in barley and maize (Rhodes *et al.* 1989; Grumet & Hanson 1986). Many genes related with glycine betaine synthesis were isolated and introduced into plants, and the metabolic engineering of glycine betaine biosynthesis noticeably improved the tolerance of transgenic plants to salt, drought and tremendous temperature stresses (Quan *et al.,* 2004; Sakamoto & Murata, 2001; Sulpice *et al.,* 2003; Shen *et al.,* 2002).

Nevertheless, most of these studies focused on model plants, and there have been very few information on cotton. In order to efficiently maximizing of plant propagation *via* direct organogenesis, it is important to study the effect of stress factors on the growth and development of *S. rebaudiana* grown *in vitro*. The effect of salt stress on biochemical parameters on *in vitro* regenerated plants of stevia has less been investigated. The objective of the present study was to salinity tolerance of stevia under *in vitro* culture at different glycine betaine concentrations.

MATERIALS AND METHODS

This study was carried out in Plant Tissue Culture Laboratory, Faculty of Sari Agricultural and Natural Resources Sciences, in 2015. The present work was conducted to assess glycine betaine on salinity tolerance of stevia (*S. rebaudiana*) under in *vitro* condition. The terminal shoots were gathered from growing plants, which were 2-3 months age and were cut into 1-1.5 cm pieces.

Explants sterilization

Shoot tip explants ranging in size from 0.5 to 1 cm of stevia were rinsed under running tap water with soap for 5 min to remove all the remaining detergent and then washed with sterilized distilled water. The explants were soaked for 10 min in 20% Clorox concentration for explants surface sterilization, afterward 1.5 g l^{-1} ($HgCl_2$) mercuric chloride for 1 min, then wash with sterilized distilled water for 3-4 times to eliminate all traces. All steps of the sterilization had been done under aseptic situations, inside the culture cabinet (Laminar air flow hood), when 10 explants were cultured in each jar to enclosing 200 ml medium.

Medium for all cultures contained 1.1 g l^{-1} MS (Murashige & Skoog, 1962) inorganic salts, complemented with 0.2 g l^{-1} myo-inositol, 30.0 g l^{-1} sucrose and 6.0 g l^{-1} agar. The pH of the medium was adjusted to 5.7 and autoclaved at 1.2 kg cm^{-2} and 121ºC, for 20 min.

For salinity experiment MS medium was supplemented with different concentrations of NaCl at 0, 50, 75 and 100 mM, along with 0, 1, 12.5, 25 and 50 mM of glycine betaine concentrations. The jars for experiment (salinity stress) were incubated at 25ºC, under 16/8

light/dark photoperiod regime with intensity about 2000 lux. After 30 days of incubation, direct regeneration plants were assessed in the basis of quantity and quality traits, including plant height, root length, shoot fresh weight, number of leaf, total chlorophyll, rebaudioside A and stevioside.

Determination of stevioside and rebaudioside by HPLC

Diterpene glycosides (stevioside and rebaudioside A) contents were determined using High Performance Liquid Chromatography (HPLC), according to Nishiyama *et al.* (1992) method. The HPLC system was a Unicam-Crystal-200 chromatograph. The analytical column was Cosmosil 5 NH_2 -MS column (15 cm × 4.6 mm I.D., 5 μm, Germany). Pure stevioside and rebaudioside A extraction from leaves were carried out by soaking 1g of dry leaves in 1liter water, at 85°C, for 30 min. Then use Buchner filtration for separation the resulting liquid fraction and wash the residue with an additional volume of hot water (50 ml). Lyophyilization was used concentrated the aqueous solution to 50 ml and defatted by ethyl acetate that extracted with isobutyl alcohol (150 ml). The aqueous phase was discarded and the organic solution was evaporated by rotary evaporator at 70°C till drying. The resulting dried extracted was dissolved in hot methanol (100 ml) and kept overnight to crystallize. These crystals were separated by filtration and re-dissolved again in boiling methanol (60 ml). The active charcoal become steady for clarifying the solution and left to recrystallize and, finally, all previous steps of procedure were repeated till observation of colorless crystals. An isocratic mobile phase with 30% H_2O/methanol (50:50) and 70% acetone was utilized. Separation was performed with a waters and methanol-water (63: 35 v/v), as the elusion solvent, at flow rate of 2 ml min^{-1} and the detection wavelength was 219 nm.

Chlorophyll content assessment

Leaf chlorophyll content is an indicator of photosynthetic activity, stress condition and nutritional status of a plant. The efficacy of a hand-held chlorophyll meter (CCM-200) for nondestructive estimation of total chlorophyll and nitrogen content in the stevia leaves has been evaluated.

Statistical analysis

Data were statistically analyzed by using factorial experiment based on completely randomized design (CRD), according to Steel & Torrie (1990). Mean separations were done by using SAS computer program V.9 (SAS INSTITUTE INC, 2004) and to compare between means least significant differences was used.

RESULTS AND DISCUSSION

Analysis of variance

Significant mean squares of the salinity levels were determined for plat height, root length, shoot fresh weight, number of leaf, total chlorophyll, rebaudioside A and stevioside of the stevia genotype (*Table 1*), indicating all the traits significantly affected by salinity levels. Glycine betaine levels had also significant effect on all the traits.

Table 1 - Summary of analysis of variance for quantity and quality traits of stevia

S.O.V	df	M.S						
		Plant height	Root length	Shoot fresh weight	Number of leaf	Total chlorophy ll	Rebaudio-side A	Stevioside
Salinity (S)	4	10.51**	10.76**	1.70**	33.89*	4.89*	40.61**	183.67**
Glycine betaine (B)	3	84.18**	1.80**	3.67**	28.44**	15.84**	21.84**	78.85**
S×B	12	1.02*	0.52**	0.48**	1.54**	0.26*	1.07**	1.07**
Error	40	0.16	0.03	0.006	0.12	0.13	0.14	0.23

*, ** Significant at p< 0.05 and 0.01 levels of probability, respectively.

Salinity effect on the traits

It is evident from the data that all the measured growth criteria demonstrate gradual decline with the increase of salinity level from 0 to 100 mM, in directed regenerated stevia genotype. Plant height of the regenerated genotypes varied from 8.85 to 3.45 cm, at 0 to 100 mM of salinity levels, respectively (*Table 2*). Root length ranged from 2.36 to 1.74 cm, at 0 to 100 mM of salinity levels, respectively, and it was classified in two statistical groups for four salinity levels. Shoot fresh weight of regenerated genotypes of stevia decreased at high salinity levels in medium culture. The first and second salinity levels had the same effect on root length of regenerated genotypes of stevia. Shoot fresh weight mean value, in fourth salinity level, was about half its mean value in the first salinity level of medium culture (*Table 2*). Number of leaf values of regenerated genotypes of stevia decreased at high salinity level of medium culture and therefore photosynthesis was decreased at high salinity levels. Mean value of total chlorophyll varied from 4.04 to 1.63 mg g^{-1}, at 0 and 100 mM of salinity levels and it was separated in four statistical groups. Similar findings obtained by Ali *et al.* (2015) that increasing salinity during stevia development would delay vegetative growth and chlorophyll content reduction and formation of thinner roots. The addition of NaCl to the culture medium resulted in marked alteration of biochemical constituents and, accordingly their levels, varied with NaCl concentrations. Change in chlorophyll contents due to salinity is the most obvious biochemical response (Sairam & Tyagi, 2004). Rebaudioside A increased with increasing of salinity levels in medium culture and it ranged from 5.42 to 9.63rt at 0 and 100 mM of salinity levels, respectively. Stevioside, as another diterpene glycodide, was increased at high salinity levels. In each salinity level, the mean value of stevioside was two times of rebaudioside A.

Table 2 - Salinity levels effect on quantity and quality traits of stevia

Salinity levels (mM)	Plant height (cm)	Root length (cm)	Shoot fresh weight (g)	Number of leaf	Total chlorophyll mg g^{-1}	Rebaudioside A rt (min)	Stevioside rt (min)
0	8.85a	2.36a	1.66a	10.62a	4.04a	8.71a	18.78a
50	5.53b	2.41a	1.73a	8.65b	3.02b	7.88b	17.64b
75	4.30c	1.84b	0.82b	7.96c	2.32c	6.99c	15.64c
100	3.45d	1.74b	0.85b	7.50d	1.63d	5.90d	13.58d

Means, in each column, followed by at least one letter in common are not significantly different at the 1% level of probability- using Duncan's Multiple Range Test.

Glycine betaine effect on the traits

One of the most extensively studied compatible solutes is glycine betaine, which not only acts as an osmoregulator, but also stabilizes the structures and activities of enzymes and protein complexes, and maintains the integrity of membranes against the damaging effects of excessive salt in many plant species (Sakamoto & Murata, 2002). The average plant height elevated with increasing of glycine betaine levels. Plant height varied from 4.23 to 6.66 cm at 0 and 50 mM of glycine betaine levels, respectively (*Table 3*). Root length ranged from 1.33 to 3.53 cm at first and fifth glycine betaine levels. As a result of increasing of plant height, shoot fresh weight of stevia was increased at high level of glycine betaine. Number of leaf also increased at high concentrations of glycine betaine and its means value were separated in five statistical groups. Due to increasing of glycine betaine levels in medium culture of stevia, total chlorophyll content increased and it ranged from 1.97 to 3.68 mg g^{-1}.

Rebaudioside A increased at high levels of glycine betaine and its mean value, at 50 mM, was about two times of its amount at 0 mM (*Table 3*). Stevioside was also increased with increasing of glycine betaine levels at medium culture and its highest mean value detected at 50 mM of glycine betaine level.

Table 3 - Glycine betaine levels effect on quantity and quality traits of Stevia

Salinity levels (mM)	Plant height (cm)	Root length (cm)	Shoot fresh weight (g)	Number of leaf	Total chlorophyll mg g^{-1}	Rebaudioside A rt (min)	Stevioside rt (min)
0	4.23e	1.33d	0.97d	6.61e	1.97d	5.42d	12.52e
1	5.07d	1.33d	0.96d	7.67d	2.55c	5.82d	13.45d
12.5	5.61c	1.69c	1.13c	8.53c	2.54c	7.13c	15.04c
25	6.09b	2.55b	1.41b	9.70b	3.02b	8.85b	19.55b
50	6.66a	3.53a	1.86a	10.90a	3.68a	9.63a	21.47a

Means, in each column, followed by at least one letter in common are not significantly different at the 1% level of probability- using Duncan's Multiple Range Test.

Salinity and glycine betaine interaction effects on the traits

The results in *Table 4* showed that the high mean values of plant height were observed at 0 mM of NaCl concentrations (control), under all of glycine betaine concentrations. The rate of decline of plant height decreased with increasing of NaCl concentrations (*Fig. 1*).

Enhancing glycine betaine content in plant tissues through exogenous glycine betaine application or genetic selection was considered a possible way to improve tolerance to abiotic stresses in cotton (Naidu *et al.,* 1998), but further research demonstrated much limited improvement by either exogenous application. Due to salinity concentration enhancing, root length decreased, but its intensity of reduction was low at high concentrations of glycine betaine.

Table 4 – Interaction effect of glycine betaine and salinity on quantity and quality traits of Stevia

Treatments		Plant height (cm)	Root length (cm)	Shoot fresh weight (g)	Number of leaf	Total chlorophyll mg g^{-1}	Rebaudioside A rt (min)	Stevioside rt (min)
Glycine betaine (mM)	Salinity levels (mM)							
0	0	8.58a	2.24e	2.10b	9.86de	2.98c-f	7.61efg	15.72f
	50	3.30fg	1.48ghi	0.98jk	6.13ij	2.34fgh	5.43j	14.5g
	75	2.77gh	0.95jk	0.29n	5.47jk	1.68hij	4.35k	10.75i
	100	2.26h	0.66k	0.51m	4.98k	0.86k	4.28k	9.12j
1	0	8.62a	1.53gh	1.12hij	10.20cd	3.75bc	7.30gh	15.65f
	50	5.33cd	1.66gh	1.36fg	7.25h	2.77ef	6.59hi	14.33g
	75	3.45fg	1.28hij	0.69l	6.67hi	2.30fgh	5.78ij	12.89h
	100	2.87gh	0.84jk	0.67lm	6.56hi	1.37jk	3.61k	10.95i
12.5	0	8.88a	1.65gh	1.53ef	10.61cd	3.45cde	8.16def	17.43e
	50	5.67cd	2.35e	1.41f	8.38g	3.00c-f	8.40de	16.33f
	75	4.84de	1.73fg	0.76l	8.08g	2.20f-i	6.48hi	14.18g
	100	3.03gh	1.05ijk	0.84kl	7.05h	1.49ijk	5.49j	12.23h
25	0	8.96a	2.95cd	1.68de	10.80bc	4.40b	9.86ab	21.73bc
	50	6.15c	2.31e	1.76cd	10.06cde	3.38cde	9.52b	20.91c
	75	5.11d	2.12ef	1.15hi	9.30ef	2.53fg	8.55d	19.16d
	100	4.15ef	2.81d	1.03ij	8.64fg	1.78g-j	7.46fg	16.39ef
50	0	9.18a	3.42b	1.87c	11.63a	5.60a	10.62a	23.38a
	50	7.18b	4.26a	3.13a	11.45ab	3.58cd	9.47bc	22.12b
	75	5.33cd	3.11bcd	1.24gh	10.25cd	2.87def	9.77b	21.2bc
	100	4.93de	3.33bc	1.19ghi	10.26cd	2.65ef	8.65cd	19.19d

Means, in each column, followed by at least one letter in common are not significantly different at the 1% level of probability- using Duncan's Multiple Range Test.

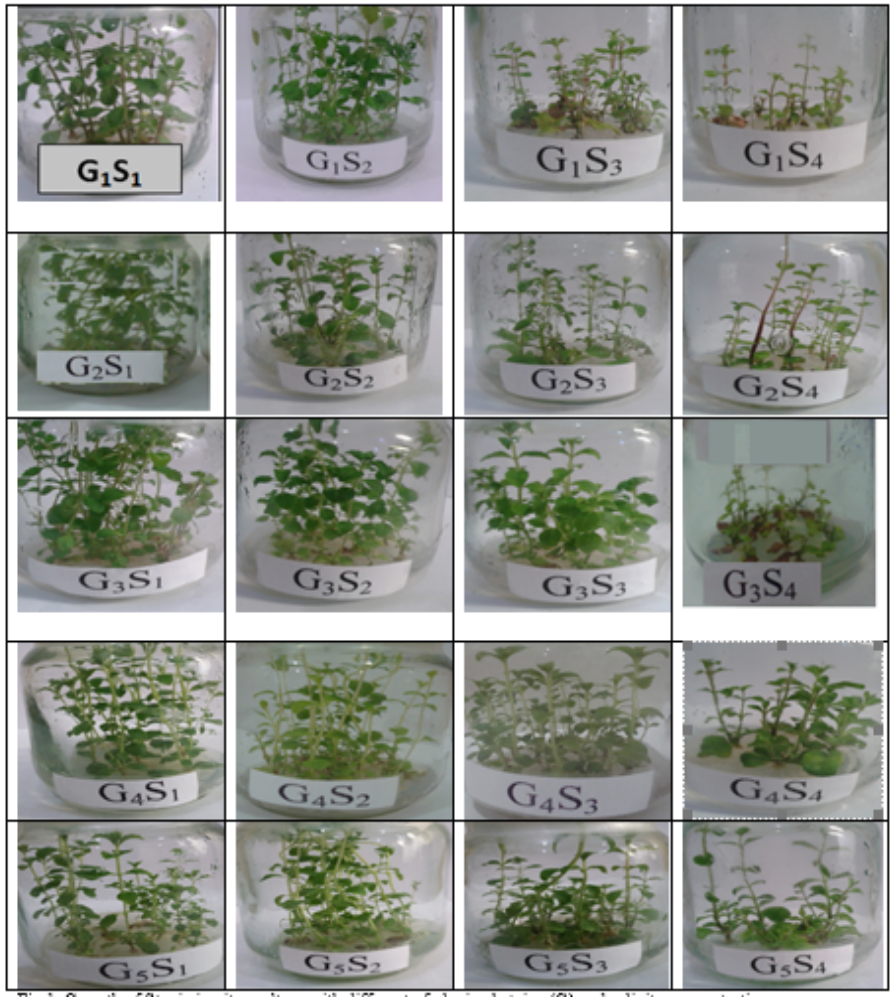

Figure 1 - Growth of stevia *in vitro* culture with different of glycine betaine (G) and salinity concentrations

G1,G2, G3, G4 and G5 indicating 0, 1, 12.5, 25 and 50 mM of glycine betaine concentrations, respectively.

S1, S2, S3 and S4 indication 0, 50, 75 and 100 mM of NaCl concentrations, respectively.

Concerning the shoot fresh weight, the treatment including 50 mM concentration of 50 mM of NaCl and glycine betaine gave the greatest values (3.13 g). For leaves number, the mean value decreased as NaCl levels increased, but increasing of glycine betaine made amending the hazardous effect of the salinity stress. High leaves number of stevia genotypes detected at 50 mM of glycine betaine concentration, along

with 0 and 50 mM of NaCl concentrations. Salinization can inhibit both cell division and cell expansion in growing tissues of roots, stems and leaves (Zidan *et al.,* 1990). The highest mean value of total chlorophyll content determined at 50 mM of glycine betaine and 0 mM of NaCl concentrations. Both diterpene glycosides (stevioside and rebaudioside A) contents were decreased due to increasing of salinity concentration. Most reduction effects of salinity on the both glycosides exhibited at 0 mM of glycine betaine concentration. These results are in agreement with those obtained by Rathore *et al.* (2014) in stevia, who found that chlorophyll content and both diterpene glycosides (stevioside and rebaudioside A) contents were decreased with increased salt concentrations. The highest mean value of rebaudioside A (10.62rt) and stevioside (23.38rt) determined at 50 mM of glycine betaine with 0 mM of NaCl concentration.

CONCLUSION

All the traits gradual decline with the increasing of the salinity level in directed regenerated stevia genotype. As result of glycine betaine, which not only acts as an osmoregulator, but also stabilizes the structures and activities of enzymes and protein complexes, therefore all the traits were increased at high concentration of glycine betaine. Due to amending effect of glycine betaine, its high concentrations made less hazarding effects of salinity on the researched traits.

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