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GLYCINEBETAINE-INDUCED MODULATION IN SOME BIOCHEMICAL AND PHYSIOLOGICAL ATTRIBUTES OF OKRA UNDER SALT STRESS

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Abstract

Role of glycinebetaine (GB) in okra (*Abelmoschus esculentus* L. Moench) cv. Subz-pari plants grown under salinity stress was investigated under field conditions. The crop was planted under varying levels (0, 200 and 400 mg NaCl per kg of soil) of salinity stress. Foliar application of 75 mM GB was employed at two phases i.e. after 30 and 60 days of sowing. Imposition of salinity stress significantly increased leaf GB and proline contents but significantly reduced leaf chlorophyll content and physiological characteristics such as rate of photosynthesis (Pn), rate of transpiration (E), stomatal conductance (gs) and leaf relative water content (LRWC). Exogenous application of GB significantly increased GB content but decreased proline content of leaves and improved various gas exchange characteristics/physiological parameters. The present results thus indicated that foliar application of GB (75 mM) can modulate various biochemical and gas exchange parameters of okra, grown under salt stress.

Key words: *Abelmoschus esculentus*, Gas exchange, Proline, Soil salinity.

Introduction

Okra (*Abelmoschus esculentus* L. Moench), a member of Malvaceae family, is mainly grown in most areas of the world with tropical or subtropical environments (Doymaz, 2005). It is planted in summer season i.e. from March to June in Pakistan and is considered as a popular summer vegetable. Okra crop is a good source of mineral elements like calcium, potassium and unsaturated fatty acids (Martin, 1982).

Salinity stress is a main threat for the production of crops because excessive loss of yield is due to salinity (Abbasi *et al.,* 2012). In some parts of world, especially in arid environments, salt stress is a major abiotic stress as it injuriously upsets the growth and development of plants (Anitha & Usha, 2012). Salt stress disturbs different photosynthetic activities, rate of transpiration and water status in plants (Abbasi *et al.,* 2014). Under salt stress conditions, reduced plant growth may be due to the presence of toxic ions such as Na^+ and Cl^- around the root zone or due to low water potential (Silveira *et al.,* 2009).

Plants synthesize compatible solutes such as proline, glycinebetaine (GB) and sugars when exposed to different abiotic stresses (Park *et al.,* 1995). GB is an important compatible solute that is accumulated in different plants grown under abiotic stress conditions. In salt-stressed plants, GB is commonly recognized to enhance the activity of antioxidants (Banu *et al*., 2009), sustain stomatal conductance and rate of transpiration (Raza *et al.,* 2006), protect photosynthetic apparatus (Manaf, 2016) and maintain ions (Cuin & Shabala, 2005). However, a number of plants synthesize low GB content as compared to required level. The required endogenous GB level in such type of plants may be attained through exogenous GB application. Several reports demonstrated that exogenous GB application to lower accumulating or non-accumulating plants may be beneficial for lowering the injurious effects of environmental stresses (Makela *et al.*, 1998; Chen & Murata, 2008). A number of researchers reported that foliar GB application could alleviate the damaging effects of salinity stress in several crops such as tomato (Makela *et al.*, 1998, 1999), maize (Kausar *et al.*, 2014), pea (Nusrat *et al.*, 2014) and lettuce (Yildirim *et al.*, 2015). GB when applied exogenously to salt stressed plants, enhances photosynthetic capacity, stomatal conductance and water use efficiency (Athar *et al.*, 2015) and regulates some chemical attributes and antioxidant activities (Shams *et al.*, 2016), indicating a relationship between physiological and biochemical characteristics. Most of such types of research have been taken under controlled laboratory or greenhouse trials in pots or hydroponic systems. Present study was, therefore, an attempt to assess the role of GB application in mitigating the injurious effects of salinity stress in okra crop under field conditions and study correlation among the physiological and biochemical attributes studied.

Materials and Methods

Experimental site and conditions: Two field trials were conducted during the summer seasons of two consecutive years using the same treatment combinations. Prior to sowing, field was systematically ploughed and prepared. Soil samples were collected for physico-chemical analysis. The soil analysis showed that it was clayey in texture (sand 22%, silt 30% and clay 48%) and had EC 2.4 dSm^{-1}, pH 8.2, organic matter 0.73% and total N 0.045%. Okra cv. Subz-pari seeds were obtained from the local market. The ridges were prepared 50 cm apart and seeds were sown (on 18^{th} March 2009 and 25^{th} March 2010) on both sides of the ridges. After 15 days of sowing, thinning was carried out to retain vigorously growing plants at a distance of nearly 20 cm apart. The crop was irrigated at 7 to 10 days of intervals subject to the required situations. Fertilizers such as N, P_2O_5 and K_2O (120: 75: 60 kg ha^{-1}) were used as urea (46% N), single super phosphate (18% P_2O_5) and muriate of potash (60% K_2O), respectively.

Soil salinity levels and GB sprays: Salinity treatments i.e. 0, 200 and 400 mg per kg of soil were applied by adding calculated amounts of NaCl into the soil prior to sowing the seeds. GB (75 mM) was employed as foliar application to the plants after 30 and 60 days of sowing.

GB and proline estimation: Leaf samples collected 90 days after sowing were used for GB and proline estimation. GB estimation (µmol g^{-1} dry wt.) was done by the procedure of Grieve & Grattan (1983), whereas proline (µmol g^{-1} fresh wt.) was assessed as described by Bates *et al.* (1973).

Leaf chlorophyll content (SPAD values): Chlorophyll meter (SPAD-502, Minolta, Japan) was used to measure leaf chlorophyll content after 65 days of salinity stress.

Gaseous exchange parameters: Transpiration rate (E), net photosynthetic rate (Pn) and stomatal conductance (gs) were estimated by means of C-340 Hand-Held Portable Photosynthesis System (CID, USA) after 65 days of salinity stress. Data were collected during the period 9.30 AM to 11.50 AM under the following environments: atmospheric pressure 99.24 kPa, area of leaf surface 6.3 cm^2 and leaf chamber temperature from 33.1 to 41.3°C.

Leaf relative water content (LRWC): LRWC was measured after 65 days of salinity stress. For this purpose, the top most matured leaves of two plants from every treatment were taken. The leaves were cut just beneath the lamina and put in plastic bags, and then readily taken to the laboratory within the next hour. The leaves' fresh weight (FW) was recorded with an electric balance and then these were saturated in purified water. After 18 hours, leaves were cautiously dried with the help of tissue paper, then turgid weight (TW) was measured. After it, leaves were placed in an oven at 70 °C for drying and then leaves dry weight (DW) was measured. LRWC was determined by the formula:

$$\text{LRWC (\%)} = (\text{FW}-\text{DW})/(\text{TW}-\text{DW}) \times 100$$

Experimental design and statistical analysis: The experiments were set up in a two factor factorial randomized complete block design (RCBD) having three replications. Each plot was measured as 4 m × 3 m (12 m^2) with 1 m distance between plots and 1.5 m gap between replications. Collected data were analysed for ANOVA by using Statistix ver. 8.1. The treatment means were compared by applying the least significance difference (LSD) test at 0.05 level of probability. The effect of years and their interaction with other two factors i.e. salinity levels and GB sprays was non-significant, therefore not mentioned in the next parts. Pearson's correlation coefficients were estimated and matrix was constructed through software XLSTAT.

Experimental Results

Increased salt stress significantly increased the leaf GB titre; therefore okra plants grown under higher salinity level had higher GB concentration. Foliar GB (75 mM) application also enhanced the internal leaf GB content and greater leaf GB content was recorded in two sprayed plants than those of single or unsprayed plants. As far as cumulative effect of NaCl treatments and GB sprays is concerned, higher levels of GB was observed in plants cultivated under higher salinity level and sprayed twice, while the lower leaf GB content recorded in plants developed under non-saline soil and without GB application (Table 1).

Leaf proline level increased with the increase of soil salinity level. Plants grown under higher salinity level resulted in greater leaf proline content, while the plants grown in normal soil synthesized low level of leaf proline content. Foliar GB application significantly reduced leaf proline concentration. The minimum leaf proline concentration was recorded in two sprayed plants than those of single or unsprayed plants. The synergistic effect of NaCl levels and GB sprays has no influence on leaf proline content in plants developed under non-saline soil. However, the effect of GB sprays was significant on leaf proline content in NaCl stressed plants only (Table 1).

Leaf chlorophyll content was decreased with the increase of salinity level in the soil. The reduction in leaf chlorophyll content was greater in plants developed under higher salinity stress conditions. The maximum leaf chlorophyll content was recorded in plants developed under normal soil conditions. Foliar GB application increased leaf chlorophyll content. Higher leaf chlorophyll content was measured in two sprayed plants as compared to single or unsprayed plants. However, the cumulative effect of soil salinity levels and GB applications was non-significant on this parameter (Table 1).

Significantly decreased rate of photosynthesis was recorded in salinity stressed plants. The reduced rate of photosynthesis was observed in NaCl stressed plants and higher value of the parameter was found in non-stressed plants. Foliar spray of GB increased the rate of photosynthesis. The findings revealed that two sprayed plants resulted in higher rate of photosynthesis. The aggregate effect of NaCl levels and GB sprays indicated that two sprayed plants under non-stressed conditions resulted in higher rate of photosynthesis, while lower rate of photosynthesis was observed in plants developed under higher salinity stress conditions without GB application (Table 2).

As the level of salinity increased in the soil, transpiration rate decreased. The lower rate of transpiration was observed in plants developed under higher soil salinity stress conditions and the maximum rate of transpiration was found in non-stressed plants. Exogenous GB application improved rate of transpiration. Greater rate of transpiration was recorded in two sprayed plants as compared to single or unsprayed plants. The interaction between NaCl treatments and GB applications revealed that higher rate of transpiration was noted in two sprayed plants developed under non-saline soil, while lower value was found in unsprayed plants grown under higher salinity level (Table 2).

Table 1. Effect of soil salinity levels and number of GB (75 mM) sprays on leaf GB and proline contents and chlorophyll content.

NaCl added to soil (mg kg^{-1} of soil)	No. of GB sprays 0	1	2	Mean
	Leaf GB content (µmol g^{-1} dry wt.)			
0	1.174 i	1.861 h	2.180 g	1.738 c
200	2.810 f	3.061 e	3.224 d	3.032 b
400	3.481 c	3.803 b	4.021 a	3.768 a
Mean	2.488 c	2.908 b	3.142 a	-
	Leaf proline content (µmol g^{-1} fresh wt.)			
0	11.177 f	11.170 f	11.174 f	11.173 c
200	17.953 c	14.360 e	11.193 f	14.502 b
400	22.548 a	18.322 b	15.720 d	18.863 a
Mean	17.226 a	14.617 b	12.696 c	-
	Leaf chlorophyll content (SPAD value)			
0	53.20	56.73	58.32	56.08 a
200	47.98	51.67	53.17	50.94 b
400	44.82	48.40	50.12	47.78 c
Mean	48.67 c	52.27 b	53.87 a	-

Means with different letters in each group are statistically significant at $p \leq 0.05$ (LSD test)

Table 2. Effect of soil salinity levels and number of GB (75 mM) sprays on photosynthesis and transpiration rates, stomatal conductance and leaf relative water content.

NaCl added to soil (mg kg^{-1} of soil)	No. of GB sprays 0	1	2	Mean
	Rate of photosynthesis (µmol CO_2 m^{-2}s^{-1})			
0	12.267 c	16.000 b	17.867 a	15.378 a
200	8.200 g	10.633 d	12.117 c	10.317 b
400	6.817 h	9.050 f	10.317 e	8.728 c
Mean	9.094 c	11.894 b	13.433 a	-
	Rate of transpiration (mmol H_2O m^{-2}s^{-1})			
0	3.910 c	5.170 b	5.780 a	4.953 a
200	2.700 g	3.468 d	3.813 c	3.327 b
400	2.215 h	2.920 f	3.298 e	2.811 c
Mean	2.942 c	3.853 b	4.297 a	-
	Stomatal conductance (mmol m^{-2}s^{-1})			
0	20.843 c	26.815 b	30.237 a	25.965 a
200	14.138 g	18.142 d	20.612 c	17.631 b
400	11.942 h	15.267 f	17.472 e	14.893 c
Mean	15.641 c	20.074 b	22.773 a	-
	Leaf relative water content (%)			
0	76.33	80.39	83.17	79.97 a
200	68.97	73.74	76.45	73.06 b
400	64.08	69.93	72.09	68.70 c
Mean	69.80 c	74.69 b	77.24 a	-

Means with different letters in each group are statistically significant at $p \leq 0.05$ (LSD test)

Stomatal conductance reduced as soil salinity level was increased. The decreased stomatal conductance was measured in salinity stressed plants and its higher value was found in plants grown under normal soil. Foliar GB application improved stomatal conductance. The higher value of stomatal conductance was recorded in two sprayed and the lower value was found in unsprayed plants. The collective effect of NaCl stress and application of GB indicated that the plants developed under normal soil with two GB sprays resulted in greater stomatal conductance and the minimum was recorded in unsprayed plants grown under higher level of NaCl in the soil (Table 2).

Significant decrease in LRWC was found in plants grown under the soil salinity stress conditions. The highest LRWC was noted in plants grown under control soil conditions. GB sprays significantly increased LRWC. The two sprayed plants resulted in greater LRWC as compared to 1 sprayed or unsprayed GB plants. The mutual effect of soil NaCl regimes and GB sprays was non-significant on the parameter (Table 2).

Pearson's correlation matrix indicated that rate of photosynthesis had significant positive correlation with leaf chlorophyll content, rate of transpiration, stomatal conductance and leaf relative water content (r = 0.992, 0.999, 0.999, and 0.993, respectively). Leaf proline content had significant negative correlation with, rate of photosynthesis, rate of transpiration, stomatal conductance, leaf relative water content and leaf chlorophyll content (r = -0.959, -0.958, -0.962, -0.984 and -0.982, respectively). Leaf GB content had non-significant correlation with all the other parameters (Table 3).

Table 3. Pearson's correlation coefficients of physiological and biochemical attributes of okra.

Variables	Rate of photosynthesis	Rate of transpiration	Stomatal conductance	Leaf relative water content	Leaf GB content	Leaf proline content	Leaf chlorophyll content
Rate of photosynthesis	1						
Rate of transpiration	0.999*	1					
Stomatal conductance	0.999*	0.999*	1				
Leaf relative water content	0.993*	0.993*	0.993*	1			
Leaf GB content	-0.253	-0.267	-0.261	-0.261	1		
Leaf proline content	-0.959*	-0.958*	-0.962*	-0.984*	0.324	1	
Leaf chlorophyll content	0.992*	0.993*	0.992*	0.999*	-0.283	-0.982*	1

*Correlation was significant at $p<0.05$

Discussion

Under salinity stress conditions, plants accumulate organic osmolytes such as proline, glycinebetaine (GB) and sucrose. GB plays an important role in maintaining osmotic adjustment, protecting membranes, structures of proteins and pH of the cells (Murata *et al.,* 1992). Salt stressed plants significantly increased the leaf GB content. Bano *et al.* (2012), Shaheen *et al.* (2013) and Batool *et al.* (2013) observed increased synthesis of GB under salt stress conditions in different plants such as carrot, eggplant and cauliflower, respectively. In the present study, GB spray of 75 mM significantly enhanced internal GB level in salt stressed plants as well as in non-stressed plants. The plants sprayed twice with GB indicated greater leaf GB concentration as compared to plants with single GB spray. Raza *et al.* (2006) found a positive link between degree of salt tolerance and internal level of GB in plants. The improved salinity tolerance in GB sprayed okra plants was apparently due to increased endogenous GB levels.

Plants, grown under salinity stress, synthesize organic substances in the cytoplasm for osmotic adjustment (Yildiztugay *et al.,* 2014). The accumulation of proline content is the significant modification in plants grown under salt-stressed conditions (Misra & Saxena, 2009). In salinity stressed plants, the higher accumulation of leaf proline content may be due to stimulation of its synthesis and prevention of its breakdown. Lutts *et al.* (1996) demonstrated that under abiotic stress conditions, the synthesis of proline content greatly varies within or between the species of plants. Ozdemir *et al.* (2004) also found that the proline accumulation under salt stress has been related to osmotic adjustment, membrane stability, ammonia detoxification, and enhanced protection of some enzymes present in mitochondria and cytoplasm. GB application decreased the leaf proline concentration in salt-stressed plants. Leaf proline content decreased more in two sprayed plants as compared to single one. However, GB sprays had no impact on leaf proline level in plants developed under non-saline soil. Under foliar application of GB, the decline in leaf proline concentrations may be due to GB interference in regulation of osmotic stress leading to reduced proline concentration. Transgenic tomato plants have been reported to accumulate greater GB content but lower proline content under salinity stress conditions, whereas normal plants of tomato synthesize lower GB content but greater proline content (Zhou *et al*., 2007). These findings proposed that the accumulation of proline content in tomato plants grown under salinity stress conditions was due to salt-induced damage, while higher synthesis of GB content in transgenic tomato plants was responsible for the protection of plants from salt injury by lowering the proline level.

Increased soil salinity level reduced the leaf chlorophyll content. Reddy & Vora (1986) reported that the decreased chlorophyll content may be due to active role of proteolytic enzymes like chlorophyllase. Foliar GB application clearly improved the leaf chlorophyll content. Under salinity stress conditions, exogenous GB application improved the availability of advantageous ions like Mg^{++} and K^+, and reduced the uptake of injurious ions such as Na^+ and Cl^-, resulting in an increased photosynthetic capacity (Shaddad, 1990).

Soil salinity stress significantly decreased the rate of photosynthesis (Pn). Salinity stress upsets the functioning of stomata by low uptake of K^+ ions. As K^+ ions perform a key function in stomatal opening and closing, therefore salinity affects the photosynthetic capacity by upsetting the gas exchange (Sudhir & Murthy, 2004). The decreased rate of photosynthesis may be the effect of decreased stomatal conductance (Etehadnia *et al.,* 2010).

Tezara *et al.* (2005) reported that the decreased rate of photosynthesis may be due to ion toxicity. Thus, greater toxicity of ions, higher osmotic stress, membrane injury, changed functioning of stomata and low accessibility of K^+ could have decreased the rate of photosynthesis in okra plants. However, GB application significantly increased the photosynthetic activity, which may be due to increased chlorophyll content. Makela *et al.* (1998, 1999) indicated that under salt stress conditions, enhanced rate of photosynthesis in plants after GB application may be due to reduced rate of photorespiration, greater gas exchange and higher carbon availability for photosynthesis. Improved salinity tolerance after GB application has been related with enhanced photosynthesis (Kausar *et al.,* 2014), which may be due to limitations of stomatal and non-stomatal activity (Dubey, 2005). The increased photosynthetic activity by GB application has been shown to be mainly due to the enhanced stomatal conductance (Taize & Zeiger, 2002).

As the soil salinity level increased, rate of transpiration significantly decreased. The greater reduction in rate of transpiration was recorded in plants under high level of salinity stress. Tezara *et al.* (2002) reported that the decreased transpiration rate under salinity stress conditions may be the effect of reduced turgidity of guard cells. Burman *et al.* (2003) revealed that the reduced turgidity of guard cells may be due to inequality of mineral nutrients such as low availability of K^+, as K^+ ions are needed to maintain the turgidity of guard cells. However, increased rate of transpiration after foliar GB application may be due to adjustment of osmotic stress resulting from enhanced uptake of useful mineral nutrients and accumulation of organic osmolytes.

Maintenance of water status is reflected as essential adaptation in plants grown under salinity stress conditions (Noreen *et al.,* 2010). Salt stress significantly reduced LRWC (Yildiztugay *et al.,* 2014). Decreased LRWC leads to drop turgidity, as availability of water for the enlargement of cell decreases (Katerji *et al.,* 1997). Duan *et al.* (2005) reported that salinity stress reduced the uptake of water and beneficial mineral nutrients which lead to cause toxicity of ions and osmotic effects. However, foliar GB application increased LRWC. The plants sprayed twice with GB had greater LRWC as compared to single sprayed plants. GB has been reported to lower cell damage from dehydration (Chen *et al.,* 2000) and prevent K^+ leakage from NaCl-stressed plants (Cuin & Shabala, 2005) .

A significant positive correlation was observed among all the parameters studied except for leaf proline and leaf GB contents. Leaf proline content was significantly negatively correlated with all the parameters except leaf GB content, which was not significantly correlated with any of the physiological or biochemical attribute. This is interesting to note that GB application had significant effect on all the physiological and biochemical characteristics of okra plants under salinity stress. However, leaf GB content was not correlated with any of the parameter indicating that the applied GB was either utilized by the plants or converted to some other products. Correlation between some of the growth, physiological and biochemical attributes and antioxidant and enzymatic activities has already been reported in mangrove (Takemura *et al.,* 2000) and rice plants (Chunthaburee *et al.,* 2016) under salt stress.

Conclusion

As the soil salinity level increased, the values of all the gaseous exchange parameters and leaf chlorophyll content were significantly reduced. However, leaf GB and proline contents have opposite trend and were significantly increased. Foliar spray of 75 mM GB on okra plants was helpful in ameliorating the injurious effects of soil NaCl stress on biochemical as well as on physiological parameters. Moreover, two foliar sprays of GB (75 mM) were more beneficial in their effectiveness as compared to single one.

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