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South African Journal of Botany 83 (2012) 151–158

SOUTH AFRICAN
JOURNAL OF BOTANYwww.elsevier.com/locate/sajb

Response of salt stressed okra (*Abelmoschus esculentus* Moench) plants to foliar-applied glycine betaine and glycine betaine containing sugarbeet extract

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Received 12 April 2012; received in revised form 14 August 2012; accepted 17 August 2012

Available online 23 September 2012

Abstract

The present experiment was aimed at examining the ameliorative effect of foliar-applied glycine betaine (50 mM GB) and glycine betaine containing sugarbeet extract (50 mM GB) on various physiological and biochemical attributes of okra plants under salt stress. The experiment comprised of two okra cultivars (Arka-anamika and Sabaz-pari), two salt levels (0 and 150 mM NaCl), and two GB sources (synthetic pure GB and sugarbeet extract) arranged in four replicates. Salt stress significantly suppressed the biomass production, yield, and different gas exchange attributes (A , E , C_i , and g_s). Glycine betaine and proline contents in leaves, and Na^+ and Cl^- contents in both leaves and roots increased, while K^+ and Ca^{2+} contents and K^+/Na^+ ratios decreased significantly. Foliar application of both pure GB and sugarbeet extract significantly reduced the adverse effects of salt stress on plant biomass production, plant yield, various gas exchange characteristics and leaf K^+ , Ca^{2+} , Cl^- and Na^+ contents. However, GB and sugarbeet extract showed differential effects on A , g_s , E , C_i , C_i/C_a ratio, leaf K^+ , Ca^{2+} , and Cl^- contents, and K^+/Na^+ ratio. Pure GB proved better than the sugarbeet extract in improving growth, while the reverse was true for plant yield under salt stress. However, with respect to different gas exchange attributes both GB and sugarbeet extract were found to be equally effective in reducing the adverse effects of salt stress on these photosynthetic attributes. Foliar-applied sugarbeet extract was found to be more effective as compared to pure GB in reducing the adverse effects of salt stress on K^+ and Ca^{2+} uptake and K^+/Na^+ ratio in shoot and root of both okra cultivars. Thus, sugarbeet extract could be used to induce salt tolerance in economically important crop plants.

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Keywords: Glycine betaine; Okra; Photosynthesis; Salinity; Sugarbeet extract

1. Introduction

Methods presently being used to induce salt tolerance in crop plants include conventional breeding, genetic engineering and exogenous use of organic and inorganic chemicals (Ashraf and Foolad, 2007). Exogenous use of organic osmolytes such as glycine betaine (GB), proline and trehalose is found to be very effective in inducing salt tolerance in a number of crops (Rhodes and Hanson, 1993; Ali et al., 2007). GB acts as a catabolic source of methyl groups to facilitate various biochemical processes (Craig, 2004) as well as an osmolyte to protect cells under stressful environmental conditions. It increases the water retention of cells and can protect the plant cells against osmotic stress (Ashraf and

Foolad, 2007; Mäkelä, 2004; Sakamoto and Murata, 2002). Glycine betaine has improved stress tolerance in a number of species including cotton (Naidu, 1998), tomato (Mäkelä et al., 1998), rice (Rahman et al., 2002), wheat (Mahmood et al., 2009; Raza et al., 2007), sunflower (Iqbal et al., 2008), and maize (Ali and Ashraf, 2011; Ali et al. 2008; Nawaz and Ashraf, 2009). These studies used synthetic GB purchased from chemical manufacturing companies.

Under stress conditions, GB synthesis in most plant species occurs in cell chloroplasts (Park et al. 2007; Rathinasabapathi et al., 1997). Although GB naturally occurs in many crop plants, its concentration is genus- or species-specific. It occurs abundantly in wheat (McDonnell and Wyn Jones, 1988), spinach (Weigel et al., 1986), bean (Gadallah, 1999), and alfalfa (Wood et al. 1991). In contrast, many economically important crop plants are unable to accumulate GB (Zwart et al., 2003) or its production is too low for the plant requirements.

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Sugarbeet contains high levels of GB e.g. 100 mmol GB/kg fresh plant tissue (Mack et al. 2007) or 0.2–0.3% (Korteniemi, 2007). Sugarbeet root extract is composed of a variety of other inorganic nutrients and organic compounds such as 2.8% fiber, 9.56% carbohydrates, 0.003% vitamin E (tocopherols), 0.005% vitamin C, 0.016% calcium, 0.325% potassium, 0.023% magnesium and 0.040% phosphorus (<http://www.botanicalonline.com/commonbeet.htm>). However, very little information is available about its exogenous use in alleviating the adverse effects of stresses on plants. Keeping in mind the useful nutritional composition of sugar beet extract, we hypothesized that foliar application of sugarbeet extract could minimize salt-induced adverse effects on the growth of okra. Thus, the principal objectives of the present study were to examine whether exogenous application of sugarbeet extract could protect okra plants against salt stress, and to study the role of sugar beet extract in regulating growth and affecting key physiological processes involved in salt tolerance of okra as compared to synthetic GB.

2. Material and methods

The experiment was conducted under natural conditions in the net-house of the Botanical Garden, University of Agriculture, Faisalabad, Pakistan, during 2008–2009, where the average photosynthetically available radiation (*PAR*) measured at noon ranged from 1028 to 1365 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The average day/night relative humidity (RH) and temperature were 50/71% and 24/8 °C, respectively.

Two cultivars of okra [Sabaz-pari and Arka-anamika (an Indian variety)], two salt stress treatments (control and 100 mM NaCl) and two sources of GB (50 mM pure GB and sugarbeet extract containing 50 mM GB) in 0.1% solution of Tween-20 were used. Seeds of both cultivars of okra were obtained from the Ayub Agricultural Research Institute, Faisalabad.

Seed samples were surface sterilized with 10% sodium hypochlorite solution for 5 min and washed three times with sterilized distilled water. The seeds were sown in earthen pots (25 cm in diameter and 28 cm in height) lined with a polyethylene sheet, each containing 8 kg of well washed sand. The total number of pots was 48, comprising four replications of each treatment. Fifteen seeds were sown in each pot. One week following seed germination, plants were thinned to 5 plants per pot. Sodium chloride (analytical grade, Merck, Germany) was used for salt stress treatment. Salt stress treatments (control, 100 mM NaCl) were started 20 days after seed germination and regularly watered with salt solution every 5 days. Different concentrations of GB (0 and 50 mM pure GB, prepared in 0.1% (v/v) Tween-20 and sugarbeet extract containing 50 mM GB) were used for exogenous application. Glycine betaine of Sigma-Aldrich, Japan was used. For the extraction of sugarbeet juice, fresh sugarbeet roots were procured from the local market. After washing well, the roots were extracted using an electric extractor (Model Ju-209, Guangdong, China). The extract was filtered using a fine sieve (0.3 mm) and stored at –20 °C for further use. Glycine betaine in the sugarbeet extract was measured by high performance liquid chromatography (HPLC) following Gibon et al. (1997).

The GB concentration determined was 50 mmol GB/kg. The juice was extracted 1 day before its foliar application. Glycine betaine and sugarbeet extract (60 ml per pot) were applied as foliar spray only once 10 days after the salt treatment was initiated. The data for different growth and gas exchange attributes was recorded at the booting stage, 3 weeks after foliar application of GB and sugarbeet extract.

After recording the data for shoot and root lengths and fresh weights of roots and shoots, the plants were placed in an oven at 70 °C for 5 days and dry weights (g) of shoots and roots were determined.

2.1. Gas exchange characteristics

Measurements of CO_2 assimilation rate (*A*), sub-stomatal CO_2 concentration (C_i), stomatal conductance (g_s) and transpiration rate (*E*) were made on the 2nd leaf from the top of stalk of the plants using an LCA-4 ADC portable infra-red gas analyzer (Analytical Development, Hoddesdon, UK). Two plants per replicate were used for these gas exchange measurements. These measurements were made from 10.30 a.m. to 1.30 p.m. with the following specifications/adjustments: leaf surface area, 11.25 cm^2 ; ambient temperature, 35 ± 2.5 °C; ambient CO_2 concentration, 348 $\mu\text{mol mol}^{-1}$; leaf chamber temperature varied from 35.15 to 48.25 °C; chamber gas flow rate (*U*), 248 $\mu\text{mol s}^{-1}$; molar flow of air per unit leaf area (*Us*), 221.06 $\text{mol m}^{-2} \text{s}^{-1}$; relative humidity (RH) of the chamber ranged from 35.5 to 43.2%; *PAR* (Q leaf) at leaf surface during noon was maximum up to 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$; and ambient pressure, 98.8 kPa.

2.2. Free proline determination in fresh leaves

Free proline was determined following the method of Bates et al. (1973). Fresh leaf material (0.5 g) of each treatment (four replicates of each treatment) was homogenized for 20 min in 10 ml 3% sulfo-salicylic acid using a pestle and mortar. After filtration with filter paper (Whatman No. 1), 2.0 ml of the filtrate was mixed with 2.0 ml acid ninhydrin solution and 2 ml glacial acetic acid in a test tube. The reaction mixture was incubated at 100 °C for 1 h and then cooled using an ice bath. Four milliliters of toluene was added to the solution which was then mixed vigorously for 1–2 min. The toluene layer was removed and its absorbance measured at 520 nm using a spectrophotometer (IRMECO U2020). The proline concentration was determined from a standard curve and calculated on a fresh weight basis using the following formula:

$$\mu\text{mol proline g}^{-1} \text{ fresh weight} = (\mu\text{g proline ml}^{-1} \times \text{ml of toluene}/115.5)/(\text{g of sample}).$$

2.3. Glycine betaine determination in leaves

Glycine betaine estimation was carried out following the method of Grieve and Grattan (1983). Oven-dried (40 °C) leaf material (1.0 g) was ground in 10 ml distilled water. After filtration, 1 ml extract was mixed with 1 ml 2 N HCl. A 0.5 ml

portion of this mixture was mixed with 0.2 ml potassium tri-iodide solution which was then shaken and cooled in an ice bath for 90 min with occasional shaking. Two milliliters of ice cooled distilled water and 20 ml 1,2 dichloromethane (cooled at -10°C) were added to the mixture. The two layers formed in the mixture were then mixed thoroughly for 1–2 min at 4°C . The upper aqueous layer was discarded and the optical density of the organic layer was measured at 365 nm. The concentration of GB was calculated using a standard curve.

2.4. Determination of Na^+ , K^+ , Ca^{2+} and Cl^- in leaves and roots

The oven-dried leaves and roots (0.1 g portions) were digested with 2 ml of H_2SO_4 and H_2O_2 mixture according to the method of Wolf (1982). Potassium, sodium and calcium in the digests were determined with a flame photometer (PFP-7, Jenway Ltd. Felsted, Dunmow, Essex, UK). The Cl^- in the leaves and roots was determined using a chloride analyzer (Sherwood, Model 926).

2.5. Yield and yield components

Different yield attributes such as number of pods per plant and weight of total pods per plant were measured at maturity.

2.6. Statistical analysis

Analysis of variance technique was employed for carrying out statistical analysis of the data collected using a Costat 6.33 computer package (Cohort Software, California, USA). The mean values were compared with the least significant difference test (LSD) following Snedecor and Cochran (1980).

3. Results

Shoot and root lengths of both okra cultivars decreased significantly due to root zone salinity (Fig. 1a and b). Foliar application of GB or sugarbeet extract significantly increased the shoot and root lengths of both okra cultivars, but their effect was not the same in both okra cultivars as well as under stress and non-stress conditions. In cv. Arka-anamika shoot length increased with foliar application of GB under saline conditions, but not with foliar application of sugarbeet extract. In cv. Sabaz-pari, this increase in shoot length under saline conditions was recorded with foliar application of both the GB and sugarbeet extract. A significant increase in root length of both okra cultivars was also recorded due to foliar-applied GB or sugarbeet extract both under saline and non-saline conditions, but this increase was cultivar-specific. A relatively larger increase in root length was observed in cv. Arka-anamika as compared to that in cv. Sabaz-pari due to foliar application of pure GB or sugarbeet extract.

Shoot and root fresh and dry weights of both okra cultivars decreased significantly due to root zone salinity (Fig. 1c, d, e and f). When GB or sugarbeet extract was applied as a foliar spray to both okra cultivars, an increase in shoot and root fresh

and dry weights was observed. Generally, pure GB was found to be more effective than sugarbeet extract in increasing shoot and root biomass of salt stressed plants of both cultivars. However, the reverse was true under non-stress conditions wherein sugarbeet extract was relatively more effective except in increasing shoot dry weight. Comparison of the responses of the two cultivars showed that cv. Arka-anamika was more responsive than cv. Sabaz-pari to exogenously applied GB and sugarbeet extract in terms of shoot and root biomass.

The number of fruits and fruit weight of both okra cultivars decreased markedly due to root zone salinity (Fig. 1g and h). Foliar-applied GB and sugarbeet extract significantly increased most of these yield attributes in both cultivars under saline or non-saline conditions. The response of the cultivars to exogenous GB or sugarbeet extract was variable under different stress conditions. For example pure GB was more effective in increasing number of pods and pod weight as compared to sugarbeet extract under non-stress conditions, whereas the reverse was true under stress conditions. Again cv. Arka-anamika was more responsive to exogenously-applied GB or sugarbeet extract as compared to cv. Sabaz-pari in these two yield attributes.

Salinity stress caused a marked increase in proline content in the leaves of both okra cultivars (Fig. 1i). Exogenous application of GB and sugar beet extract significantly decreased the leaf proline contents in both okra cultivars particularly under saline conditions. A difference in cultivar response in this attribute was not observed. Pure GB and sugarbeet extract applications altered proline contents to the same extent under stress and non-stress conditions.

Like leaf proline, GB content also increased in both okra cultivars due to salt stress (Fig. 1j). Foliar applied GB and sugarbeet extract increased the leaf GB contents in both okra cultivars under stress and non-stress conditions. Under non-stress conditions a larger increase in GB contents was observed due to foliar applied GB as compared with that due to sugarbeet extract.

Of different gas exchange attributes, net CO_2 assimilation rate (A) and transpiration rate (E) of both okra cultivars were reduced significantly due to imposition of salt stress to the growth medium (Fig. 2a and b). For the most part exogenous application of GB and sugarbeet extract as foliar spray significantly increased the photosynthetic rate (A) and transpiration rate (E) of both okra cultivars except cv. Arka-anamika which showed a decreasing response in photosynthetic rate (A) due to foliar application of sugar beet extract under non-saline conditions. The maximum increase in leaf A and E under saline and non-saline conditions in both okra cultivars was due to foliar application of GB except in salt stressed plants of cv. Arka-anamika in which foliar-applied sugarbeet extract caused a maximum increase in leaf A .

Salt stress caused a marked reduction in stomatal conductance (g_s) and sub-stomatal CO_2 concentration (C_i) in both okra cultivars (Fig. 2c and d). Exogenous application of GB and sugarbeet extract significantly enhanced the intrinsic C_i in the stressed and non-stressed plants of both cultivars. In contrast, pure GB or sugarbeet extract containing GB had no significant effect on g_s of both okra cultivars.

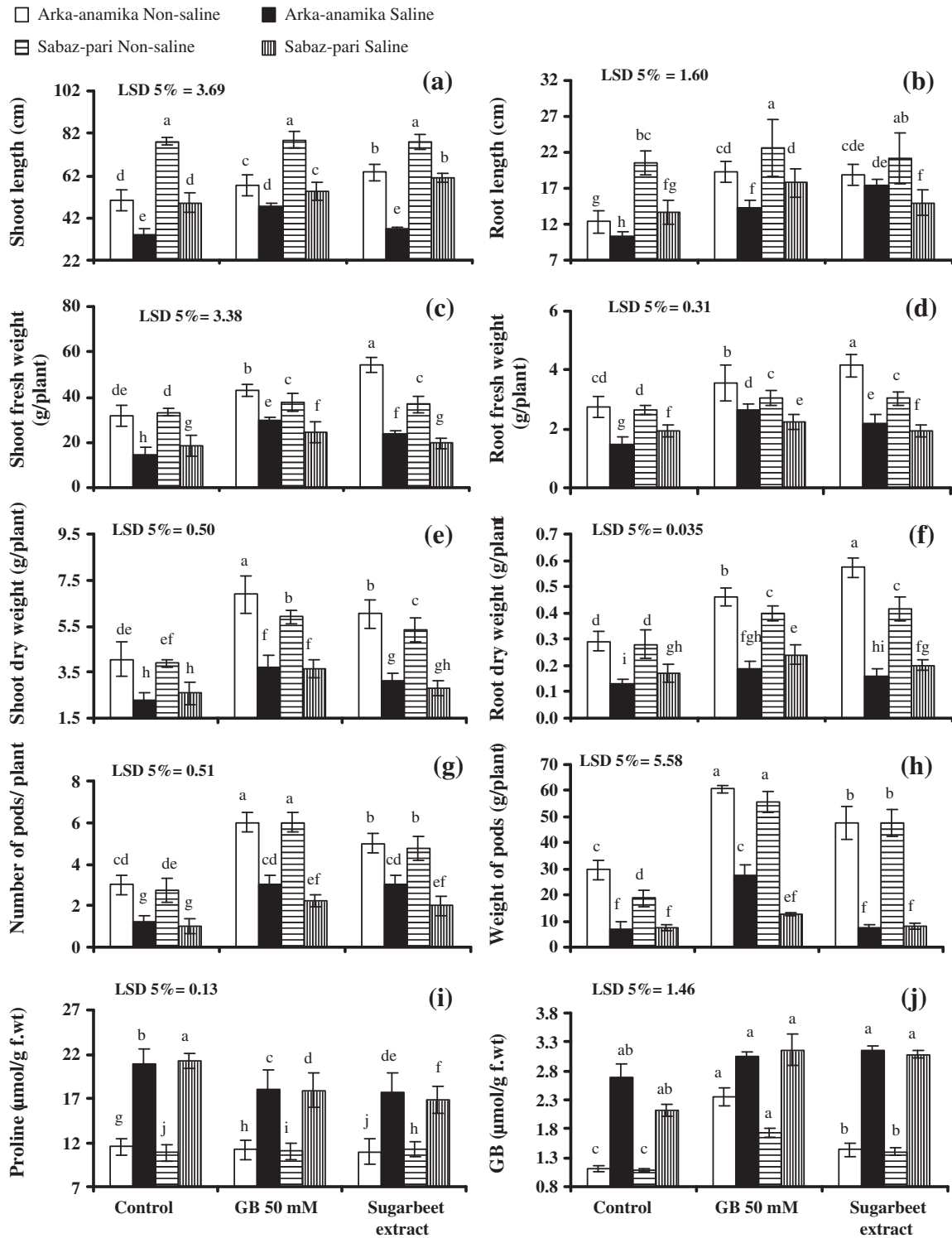


Fig. 1. Changes in growth, yield and biochemical attributes of two cultivars of okra due to foliar application of GB and sugarbeet extract under salt stressed and non-stressed conditions (mean±S.E. where $n=4$).

Imposition of salt stress significantly increased water use efficiency (A/E) of both okra cultivars. Exogenously applied GB and sugarbeet extract significantly affected the A/E ratio of both cultivars under stress and non-stress conditions (Fig. 2e).

Under non-saline conditions, leaf A/E ratio decreased significantly due to foliar application of sugarbeet extract, but not with pure GB. In contrast, under saline conditions, leaf A/E ratio of cv. Sabaz-pari increased due to exogenous application

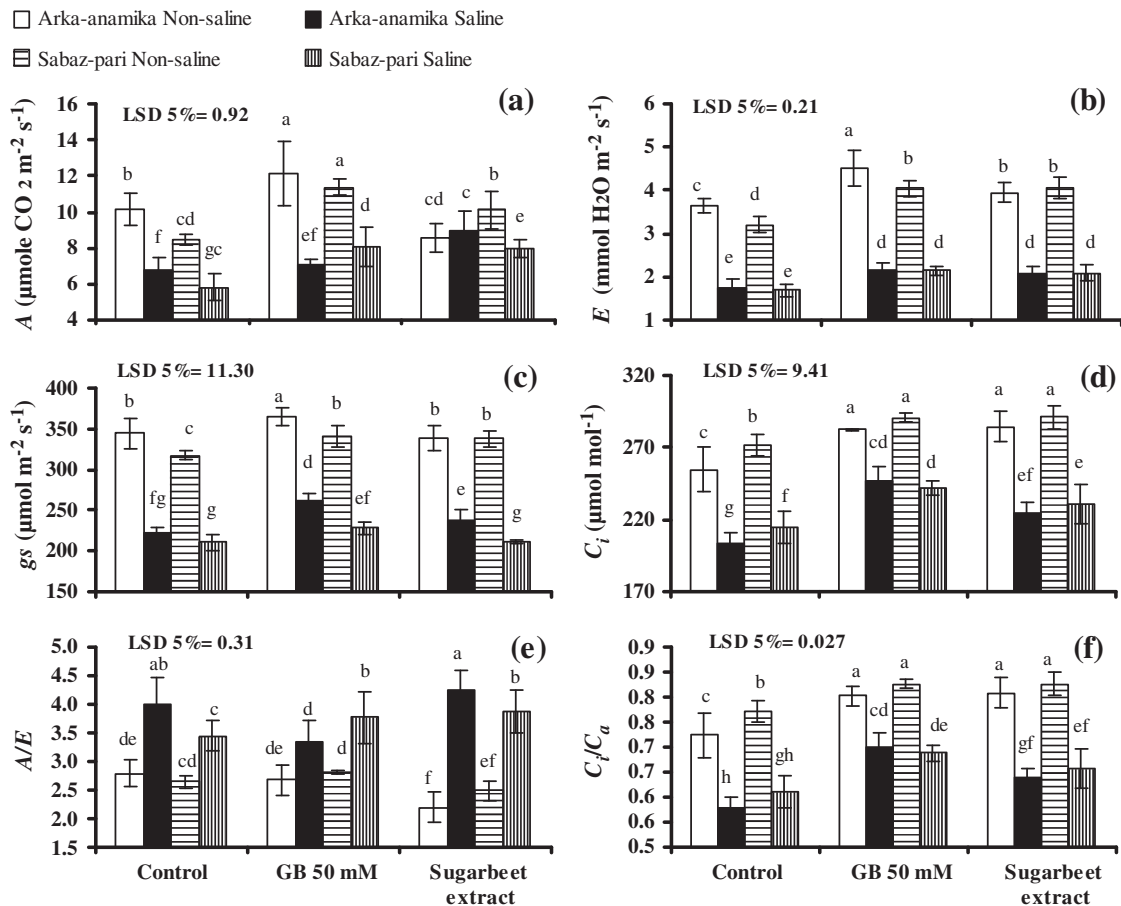


Fig. 2. Changes in gas exchange attributes of two cultivars of okra due to foliar application of GB and sugarbeet extract under salt stressed and non-stressed conditions (mean \pm S.E. where $n=4$).

of GB as well as sugarbeet extract, however, in cv. Arka-anamika leaf A/E ratio decreased significantly due to foliar application of GB.

Leaf C_i/C_a ratio (Fig. 2f) of both okra cultivars reduced markedly due to imposition of salt stress to the rooting medium. Exogenous application of GB as well as sugarbeet extract increased the leaf C_i/C_a ratio of okra cultivars both under stress and non-stress conditions, but maximum increase in C_i/C_a ratio of both okra cultivars was observed due to foliar-applied GB as compared with sugarbeet extract under stress conditions.

Potassium (K^+) content in the roots and shoots of both okra cultivars was markedly reduced due to salt stress (Fig. 3a and b). However, exogenous application of GB and sugarbeet extract affected the root and shoot K^+ content in both okra cultivars under saline and non-saline conditions, but not in a uniform way in both roots and shoots. For example shoot K^+ content increased due to foliar application of both GB and sugarbeet extract except in cv. Arka-anamika in which a decrease in K^+ uptake was observed. However, in contrast in the roots an increase in K^+ uptake was observed due to foliar application of only GB in both cultivars under saline and non-saline conditions.

Accumulation of Na^+ and Cl^- in the shoots and roots of both okra cultivars increased significantly due to root zone salinity

(Fig. 3c and d). Exogenous application of both GB and sugarbeet extract markedly decreased the accumulation of Na^+ and Cl^- in shoots and roots of both okra cultivars, but this effect was observed only under salt stress. Foliar spray of both GB and sugarbeet extract showed similar decreasing trend in Na^+ uptake in roots and shoots in both cultivars. The decreasing effect in Cl^- uptake in shoots and roots of both cultivars was more pronounced for foliar application of sugar beet extract as compared with GB.

Ca^{2+} content in shoots and roots of both okra cultivars decreased due to root zone salinity (Fig. 3e and f). Foliar applied GB and sugarbeet extract significantly affected the Ca^{2+} uptake in roots and shoots of both okra cultivars. Both cultivars showed differential response in Ca^{2+} uptake in shoots due to foliar application of GB and sugarbeet extract under saline and non-saline conditions. In cv. Arka-anamika under non-saline conditions foliar application of both GB and sugarbeet extract increased the Ca^{2+} uptake. Whereas, in cv. Sabaz-pari an increase in Ca^{2+} was observed with foliar application of sugarbeet extract. The reverse was observed for shoot Ca^{2+} contents under saline conditions. Similarly, root Ca^{2+} contents in both okra cultivars under saline and non-saline conditions were also increased due to foliar application of GB and sugarbeet extract under saline and non-saline conditions except in cv. Sabaz-pari which showed no

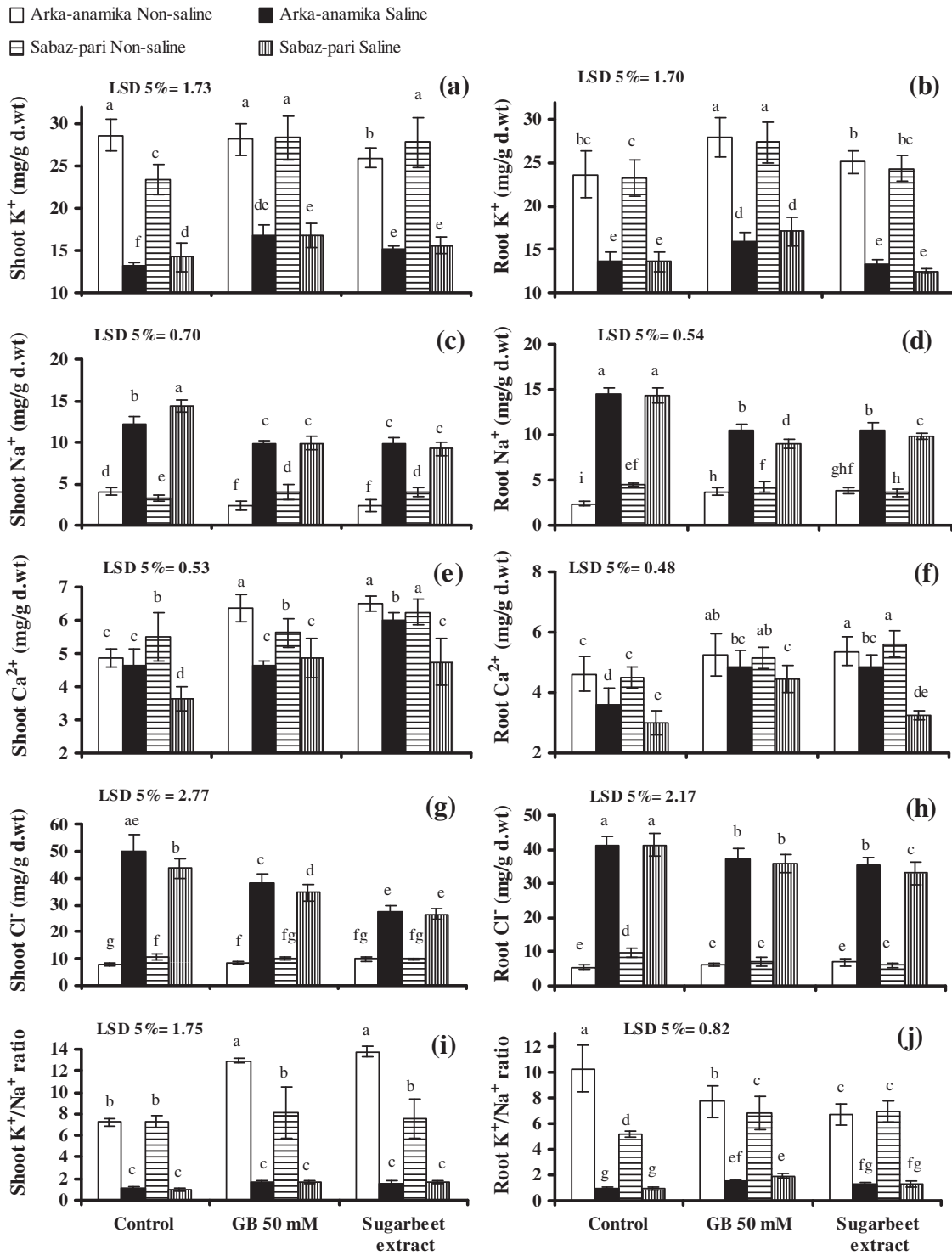


Fig. 3. Changes in nutrient uptake in roots and shoots in two cultivars of okra due to foliar application of GB and sugarbeet extract under salt stressed and non-stressed conditions (mean±S.E. where $n=4$).

response to exogenously applied sugarbeet extract under saline conditions.

The K^+/Na^+ ratio in roots and shoots of both okra cultivars decreased markedly due to root zone salinity (Fig. 3i and j). Exogenous application of GB or sugarbeet extract did not affect shoot or root K^+/Na^+ ratios under saline conditions in both

cultivars. Under non-saline conditions shoot K^+/Na^+ ratio remained unchanged in cv. Sabaz-pari, whereas with cv. Arka-anamika shoot K^+/Na^+ ratio increased significantly with the application of both pure GB and sugarbeet extract, more pronounced effect being with sugarbeet extract. In contrast root K^+/Na^+ ratio increased in cv. Sabaz-pari with the application of

GB and sugarbeet extract, where as the reverse was true in cv. Arka-anamika.

4. Discussion

During the last few decades considerable efforts have been made to reduce the adverse effects of salinity stress on crop production. The application of exogenous organic compounds to increase a plant's ability to tolerate salinity stress is a present focus in agriculture. Of different organic solutes being used to reduce the adverse effects of salt stress on plant growth and yield, exogenous use of GB is the most promising. In the present study, exogenously applied synthetic GB and that from sugarbeet extract played a vital role in improving okra growth under salt stress in terms of increased biomass production. Exogenously-applied pure GB and sugarbeet extract improved the fresh and dry weights as well as the shoot and root lengths of both okra cultivars under saline conditions. Pure GB was found to be more effective in enhancing plant growth under non-saline conditions, whereas sugarbeet extract was more effective under saline conditions. Okra plant biomass as well as fruit yield was considerably improved with foliar-applied sugarbeet extract under saline conditions. These findings can be supported by Mäkelä et al. (1998) and Abbas et al. (2010) who showed a substantial growth and yield improvements in tomato and eggplant, respectively when GB obtained from sugarbeet was applied as a foliar spray to salt stressed plants.

More beneficial effects of sugarbeet extract than those of pure GB are expected as the sugarbeet extract not only contains GB, but also other inorganic and organic substances beneficial for plant growth (<http://www.botanical-online.com/commonbeet.htm>). A considerable reduction in photosynthetic rate in both okra cultivars was examined under salt stress in the present study. However, foliar applied GB or sugarbeet extract ameliorated the salt-induced inhibitory effect on photosynthetic rate of okra. In addition, increase in photosynthetic capacity due to foliar application of GB or sugarbeet extract was similar in both okra cultivars. Plant biomass production and fruit yield of okra were also positively linked with *A* and *E*, which indicated that exogenous GB-induced growth and yield improvement in okra was due to improvement in the rate of photosynthesis. Similar findings were also reported in different cultivars of eggplant where different GB sources significantly enhanced the photosynthetic rate (Abbas et al., 2010). Foliar-applied GB has been reported to be beneficial in improving *A* and *E* in crops such as tomato (Mäkelä et al., 1998) and wheat (Raza et al., 2006; Mahmood et al., 2009).

Glycine betaine has been reported to be taken up readily by leaf tissues when applied exogenously as foliar spray. For example foliar-applied GB to tomato plant leaves was translocated to the meristem containing tissues including floral buds and shoot apices (Park et al., 2006). Glycine betaine is translocated to actively growing and expanding portions of plants, and this long-distance translocation of GB is thought to occur by way of the phloem (Mäkelä et al., 1996). This is parallel to what has been observed in earlier studies (Ali and Ashraf, 2011; Mäkelä et al., 1996) in which a large amount of foliar-applied GB was transported to

meristem-containing tissues including floral buds and shoot apices. Similarly, in the present study, leaf GB contents of both okra cultivars also increased due to foliar application of pure GB or sugarbeet extract.

In the present study leaf proline concentration increased in the salt-treated plants of both okra cultivars, but significantly decreased due to foliar-applied GB obtained from both sources. Likewise, Heuer (2003) and Demiral and Türkan (2006) also reported that exogenously applied GB significantly reduced the proline content in salt-stressed tomato and rice plants, respectively, suggesting that this decrease in proline content due to foliar application of GB might be due to an interference of GB in the process of osmotic adjustment by proline accumulation.

In this investigation, accumulation of Na^+ and Cl^- in the leaves and roots of both okra cultivars increased significantly due to salt stress, while the levels of K^+ and Ca^{2+} decreased. However, application of GB lowered the accumulation of Na^+ and Cl^- and enhanced K^+ and Ca^{2+} in the leaves of both okra cultivars. Furthermore, the increasing or decreasing effects of exogenous GB on leaf K^+/Na^+ ratio was cultivar-specific. These results can be explained in the light of some previous reports in which it has been found that exogenous GB application reduces the accumulation of Na^+ and increases that of K^+ in the shoots of most plant species (Abbas et al., 2010; Mäkelä et al., 1999; Rahman et al., 2002; Raza et al., 2007; Zhou et al., 2007). Gadallah (1999) reported that GB-induced reduction in Na^+ accumulation and increase in that of K^+ in the leaves, takes place probably via reduction in g_s and *E*, which ultimately check ion transport. Overall, foliar applied GB (pure GB or that from sugarbeet extract) caused improvement in growth and yield of both okra cultivars, but it varied with the change in selection pressure. For example, under saline conditions GB containing sugarbeet extract was found to be more effective in enhancing plant growth but under non-saline conditions pure GB was more effective. The growth improvement due to GB or sugarbeet extract was found to be related to improved plant photosynthetic rate and stomatal conductance, high accumulation of K^+ , Ca^{2+} and tissue GB, maintenance of high K^+/Na^+ ratio, and low accumulation of Na^+ and Cl^- in the leaves.

5. Conclusions

It can be concluded that the natural source of GB (sugarbeet extract) and pure GB were found to be equally effective in reducing the adverse effects of salt stress on okra plants in terms of growth and some key physiological attributes. However, as compared to the synthetic GB, sugarbeet extract can be easily obtained by crushing and pressing fresh sugarbeet roots by simple means involving no sophisticated equipment. Furthermore, fresh sugarbeet roots are available in market throughout the year in most countries. Thus, sugarbeet extract can be used as an alternative cheaper source of natural GB as an ameliorative agent for protecting plants against the hazardous effects of salt stress.

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