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# INFLUENCE OF EXOGENOUSLY APPLIED GLYCINEBETAINE ON THE PHOTOSYNTHETIC CAPACITY OF TWO DIFFERENTLY ADAPTED WHEAT CULTIVARS UNDER SALT STRESS

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## Abstract

Ameliorative effect of exogenously applied glycinebetaine (GB) on photosynthetic capacity of two wheat cultivars differing in salt tolerance grown under salt stress was assessed. Plants were grown under field conditions at 2.84, 15 dS/m in split-split plot design. Different levels of GB (no spray NS, 0.1% Tween 20 solution, 50 and 100 m*M* GB in 0.1% Tween 20 solution) were exogenously applied as a foliar spray to salt tolerant (S-24) and moderately salt sensitive (MH-97) wheat cultivars under saline and non-saline conditions. Salt stress reduced the photosynthetic capacity of both cultivars. Reduction in photosynthetic rate was primarily due to stomatal limitations. Cultivar S-24 excelled MH-97 under salt stress with respect to photosynthetic rate and leaf turgor potential. Salt-induced reduction in photosynthetic capacity was ameliorated by exogenous application of GB. High accumulation of GB mainly contributes to OA, which is one of the factors for improving photosynthetic capacity under salt stress. Better osmotic adjustment or plant water status due to GB application increased the stomatal conductance and thus favored higher $CO_2$ fixation rate. The protective effect of GB on photosynthetic pigments and GB induced reduction in transpiration rate are additional factors which might have contributed to better growth of wheat cultivars under salt stress. However, cv. S-24 was higher in photosynthetic capacity and plant water status or osmotic adjustment than MH-97, which could explain the ability of the salt tolerant S-24 to show higher salt tolerance than the moderately sensitive MH-97.

## Introduction

Salinity, one of the important growth limiting stress factors, reduces growth and grain yield in various crop species (Ashraf, 2004). Salt-induced reduction in plant growth and yield occurs due to various factors such as reduced photosynthetic metabolism, leaf chlorophyll content and photosynthetic capacity (Seeman & Critchley, 1985; Dubey, 2005), diversion of energy in the processes of osmotic adjustment and ion exclusion, and nutritional imbalance (Ashraf, 2004). There is an ample evidence that decline in net photosynthesis under salt stress is primarily due to stomatal closure (Makela *et al*., 1999; Dubey, 2005). However, non-stomatal limitations have also been reported to be responsible for reduced photosynthetic rate under saline conditions (Meyer & de Kouchkovsky, 1993).

The accumulation of glycinebetaine in salt stressed plants has been proposed to play an important role in salinity tolerance (Holmstrom *et al*., 2000; Ashraf & Harris, 2004; Ashraf, 2004). Glycinebetaine accumulates in the chloroplasts of many higher plants under environmental stress conditions, and it stabilizes quaternary structures of complex

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proteins such as those of PSII (Papageorgiou & Murata, 1995), and protects the membrane from high concentrations of $Na^+$ and $Cl^-$ (Rhodes & Hanson, 1993; Hanson *et al.*, 1995). Since the synthesis of organic solutes such as GB is energetically costly, exogenous foliar application of these compatible solutes is a possible shotgun approach to ameliorate salt-induced growth reduction (Makela *et al.*, 1996). Some crops, including wheat accumulates GB in moderate quantities (Wyn Jones & Storey, 1981). Makela *et al.*, (1998) reported that exogenous GB application caused a significant increase in growth and yield in green-house and field grown tomatoes. This improvement in growth and/or yield was linked to high endogenous GB level, improved water status of plants (Lopez *et al.*, 2002), increased photosynthetic capacity (Makela *et al.*, 1998; Yang & Lu, 2005). However, adverse effects of exogenous GB on growth of tomato have also been reported (Heuer, 2003). In view of the above-mentioned reports, the present study was conducted to assess whether exogenous application of GB can ameliorate the adverse effects of salt stress on photosynthetic capacity of wheat, a moderate GB-accumulator.

## Materials and Methods

Seeds of two spring wheat cultivars, a moderately salt sensitive MH-97 (Ashraf & Iqbal, 2005) and a salt tolerant S-24 (Ashraf, 2002) was obtained from the Ayub Agricultural Research Institute, Faisalabad, Pakistan and Department of Botany, University of Agriculture, Faisalabad, Pakistan, respectively. Field experiments were conducted during the winter 2003 and 2004 in the Botanic Gardens of the University of Agriculture, Faisalabad (latitude 31°30 N, longitude 73°10 E and altitude 213 m), where the average *PAR* of the entire growth period was 1098 µmol $m^{-2}$ $s^{-1}$, average rainfall 9.52±3.62 mm and the maximum and minimum relative humidity values were 79 and 32%, respectively. The average maximum and minimum temperatures were 28±4 and 12±3 °C, respectively. The experiment was laid out in a split-split plot design. Main plots were subdivided into two sub-plots i.e., control and saline. Sub plots were further split into three sub-sub-plots that were considered as replicates. Within sub-sub-plots GB treatments and cultivars were completely randomized. Each GB treatment was applied to three rows of wheat plants. Six sub-plots (formed in the field by digging soil) having a length, width and depth of 152, 137 and 45 cm, respectively, and lined with polythene sheets. They were filled with thoroughly mixed sandy loam soil (pH = 7.56; electrical conductivity of the saturation paste = 2.84; saturation percentage = 25.5). When the soil moisture contents were suitable for germination, seed of each cultivar was sown in rows keeping row to row spacing of 15 cm. In order to develop 15 dS $m^{-1}$ NaCl salinity, 9 days after emergence, the calculated amount of NaCl was dissolved in water required for complete saturation of the soil in three sub-plots (replicates). Thus, the soil was completely saturated with salt solution so as to homogenize soil salinity. The other three sub-plots served as control (2.84 dS $m^{-1}$).

Glycinebetaine treatments (0, no spray; 0.1% Tween-20 solution; 50 and 100 m*M* GB in 0.1% Tween-20 solution) were applied as a foliar spray at the vegetative stage when wheat plants were 56-day old. At the time of GB application, root zone $EC_e$ was 2.17 and 14.67 in control and salinized soil, respectively. Forty days after the exogenous application of GB (when plants were of 96-day old i.e., at the initiation of boot stage), 9 plants from each treatment (3 plants $replicate^{-1}$) were uprooted and washed with distilled water. Before harvest, water relations, proline, leaf chlorophyll, carotenoids, and gas exchange were measured in the 2nd leaf from top of each plant.

**Water relations:** The 2nd leaf from each plant was excised at 7.00 a.m, and the leaf water potential measurements were made with a Scholander type pressure chamber (Arimad, UK). A proportion of the same leaf used for water potential measurements, was frozen into 2 $cm^3$ polypropylene tubes at –40°C in an ultra-low freezer for two weeks, after which time plant material was thawed and the frozen sap was extracted by crushing the material with a glass rod. After centrifugation (8000 x *g*) for four minutes, the sap was directly used for osmotic potential determination using a vapor pressure osmometer (Wescor 5500). Leaf turgor pressure was calculated as the difference between leaf water potential and leaf osmotic potential values.

**Glycinebetaine:** Leaf glycinebetaine was determined following Grieve & Gratan (1983). Leaf glycinebetaine was extracted from the dry leaf material with warm distilled water (70°C). The extract (0.25 ml) was mixed with 0.25 ml of 2*N* HCl and 0.2 ml of Potassium tri-iodide solution. The contents were shaken and cooled in an ice bath for 90 min. Then 2.0 ml of ice cooled distilled water and 20 ml of 1-2 dichloromethane (cooled at -10°C) were added to the mixture. The two layers were formed in the mixture. The upper aqueous layer was discarded and optical density of the organic layer was measured at 365 nm. The concentrations of betaine were calculated on dry weight basis and then converted into fresh weight basis.

**Proline:** Proline in the leaves was measured according to the method of Bates *et al*., (1973) after extraction at room temperature with 3% 5-sulfosalicylic acid solution. The proline concentration was determined from a standard curve and calculated on fresh weight basis.

**Chlorophyll contents:** The chlorophyll 'a' and 'b' contents were determined according to the method of Arnon (1949). Fresh leaves (0.2 g) were cut and extracted overnight with 80% acetone at 0-4°C. The extracts were centrifuged at 10,000 x *g* for 5 minutes. Absorbance of the supernatant was read at 645, 663 and 480 nm using a spectrophotometer (Hitachi-U2001, Tokyo, Japan).

**Chlorophyll fluorescence:** The polyphasic rise of fluorescence transients was measured by a plant Efficiency Analyzer (PEA, Handsatech Instruments Ltd., King's Lynn, UK) according to Strasser *et al*., (1995). The transients were induced by red light of 3000 µmol $m^{-2}$ $s^{-1}$ provided by an array of six light emitting diodes (peak 650 nm), which focussed on the sample surface to give homogenous illumination over exposed area of sample surface. All the samples were dark adapted for 30 minutes prior to fluorescence measurements.

**Gas exchange parameters:** Measurements of gas exchange attributes were made on the 2nd intact leaf from top of each plant using an ADC LCA-4 portable infrared gas analyzer (Analytical Development, Hoddesdon, UK). These measurements were made from 10.30 a.m. to 12.30 p.m. with the following specifications/adjustments: leaf surface area, 11.25 $cm^2$; water vapor pressure into chamber ranged from 0.0006.0 to 0.00089 MPa, ambient $CO_2$ concentration, 352 µmol $mol^{-1}$; temperature of leaf chamber varied from 28.4 to 32.4 °C; leaf chamber gas flow rate (U), 251 µmol $s^{-1}$; molar flow of air per unit leaf area (Us) 221.06 mol $m^{-2}$ $s^{-1}$; RH of the chamber ranged from 25.4 to 41.2 %; PAR ($Q_{leaf}$) at leaf surface during noon was maximum up to 918 µmol $m^{-2}$ $s^{-1}$; and ambient pressure 98.8 kPa.

**Statistical analysis of data:** The data were subjected to analysis of variance using a COSTAT computer package (Cohort Software, Berkeley, California). The mean values were compared with the least significance difference test following Snedecor & Cochran (1980).

## Results

Analysis of GB content in the leaves of two cultivars showed that cv. S-24 accumulated significantly higher GB in the leaves than MH-97 under saline conditions (Fig. 1). Exogenous application of GB caused a significant increase in endogenous level of GB in both cultivars under non-saline or saline conditions. Salt stress significantly increased proline accumulation in shoots of MH-97, whereas that in S-24 it remained unaffected (Fig. 1). However, cv. S-24 was superior to MH-97 in accumulating proline under normal or saline conditions. Application of GB had a non-significant effect on accumulation of proline in the leaves of both cultivars both under non-saline and saline conditions, except in salinized S-24 plants where the pattern of accumulation of proline in the leaves was not consistent.

Salt stress caused a significant reduction in all leaf water relation parameters such as water potential, osmotic potential, and turgor potential of both cultivars (Fig. 2). Leaf water potential of salinized cv. S-24 plants was slightly improved (less –ve) due to GB application. Leaf osmotic potential of both cultivars decreased which was found to be one of the major factors for increasing turgor potential under saline conditions and exogenous GB application. Furthermore, cv. S-24 was superior to MH-97 in both these variables under saline conditions (Fig. 2).

Salt stress significantly reduced chlorophyll ‘a’, ‘b’ and total chlorophyll (Fig. 3). However, exogenous application of GB increased the chlorophyll ‘b’ and total chlorophyll of both cultivars under saline conditions. Chlorophyll ‘a’ of both cultivars did not increase due to GB application. Furthermore, quantum yield of photosystem II (measured as $F_v/F_m$) was not affected either by salt stress or GB application (Fig. 3).

All gas exchange parameters of both cultivars were adversely affected due to salt stress (Fig. 4). Exogenous application of GB improved the net $CO_2$ assimilation rate ($A$) of both cultivars. However, salinized plants of cv. S-24 exhibited higher photosynthetic rate when GB was applied foliarly. Similarly, sub-stomatal $CO_2$ ($C_i$) along with stomatal conductance ($g_s$) was also improved due to GB application under saline conditions (Fig. 4). However, transpiration rate ($E$) of salinized plants of both cultivars was significantly reduced due to GB application. Furthermore, water use efficiency (WUE) of both cultivars was improved when GB was applied as a foliar spray under saline conditions. However, application of GB improved WUE of cv. S-24 more than that in MH-97 under saline conditions.

## Discussion

In the present study, endogenous level of GB was significantly enhanced in both stressed and non-stressed plants of both cultivars with increase in the level of GB applied. In the present study, the moderately salt tolerant cultivar S-24 (Ashraf, 2002) accumulated more GB than moderately salt sensitive MH-97 (Iqbal & Ashraf, 2005) under salt stress. It is well evident that most of salt tolerant cultivars accumulate more GB than salt sensitive cultivars of different crops, e.g., sugar beet (*Beta vulgaris*), spinach (*Spinacia oleracea*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), and sorghum (*Sorghum bicolor*) (Weimberg *et al*., 1984; Fallon & Phillips, 1989; McCue & Hanson, 1990; Rhodes & Hanson, 1993; Yang *et al*., 2003). Thus, higher endogenous level of GB of S-24, due to exogenous application of GB, can be related to its enhanced salt tolerance.

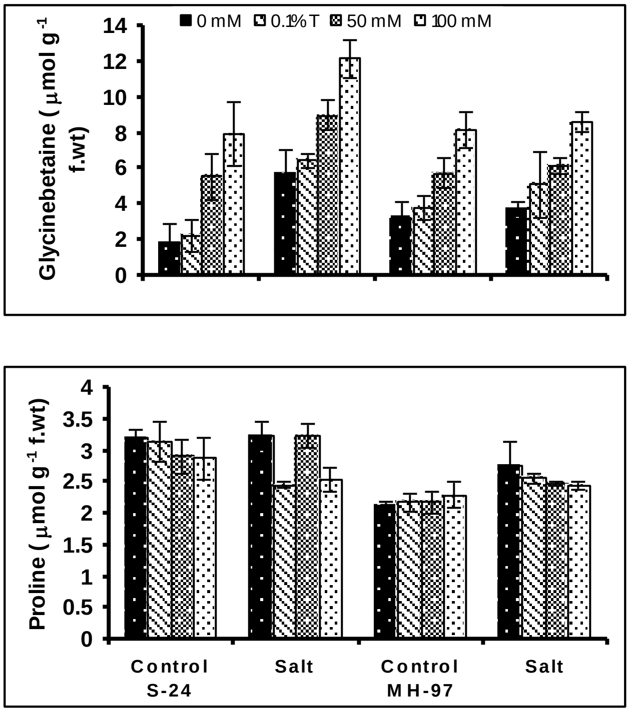

Fig. 1. Leaf glycinebetaine and proline of two spring wheat (*Triticum aestivum* L.) cultivars differing in salinity tolerance when different levels of GB were exogenously applied to salt-stressed or non-stressed plants (No spray; 0.1% Tween20 solution; 50 mM GB; and 100 mM GB in 0.1% Tween 20).

In view of some previous reports (Makela *et al*., 1998; 1999; Yang & Lu, 2005) it is suggested that GB-induced increase in salt tolerance was associated with improved photosynthetic capacity of both wheat cultivars under saline conditions (Fig. 4). Glycinebetaine induced increase in photosynthetic capacity of both cultivars might have been due to stomatal or non-stomatal limitations, major controlling factors of photosynthetic rate (Brugnoli & Bjorkman, 1992; Athar & Ashraf, 2005; Dubey, 2005). In this study, an increase in stomatal conductance and sub-stomatal $CO_2$ with an increase in net $CO_2$ assimilation rate due to GB application in salt stressed wheat plants of both cultivars suggests that the increase in photosynthesis is primarily due to increase in stomatal conductance which caused higher $CO_2$ diffusion inside the leaf thus favoring higher photosynthetic rate (Taize & Zeiger, 2002). These results are similar to those of Makela *et al*., (1998, 1999) in which an increase in salt tolerance of field grown tomato due to GB application was linked with increased net $CO_2$ assimilation rate and stomatal conductance under salt or water stress. The positive effect of GB on the stomatal conductance under salt stress could be simply mechanical, as GB may increase the proportion of bound water in the cell structure (Timasheff, 1992) thereby increasing the turgor potential in the stomatal guard cells with a subsequent increase in stomatal conductance. The mechanism by which GB application reversed to some extent salt induced injurious effects on photosynthesis through stomatal conductance was not clearly known (Makela *et al*., 1998; 1999; Yang & Lu, 2005). More importantly, rate of transpiration was decreased with GB application in both wheat cultivars under saline conditions. However, water use efficiency (WUE) calculated as *A*/*E* was improved with GB application under saline conditions, particularly in S-24. This improvement in WUE with GB application in both cultivars under saline conditions was due to an increase in *A* and decrease in *E*.

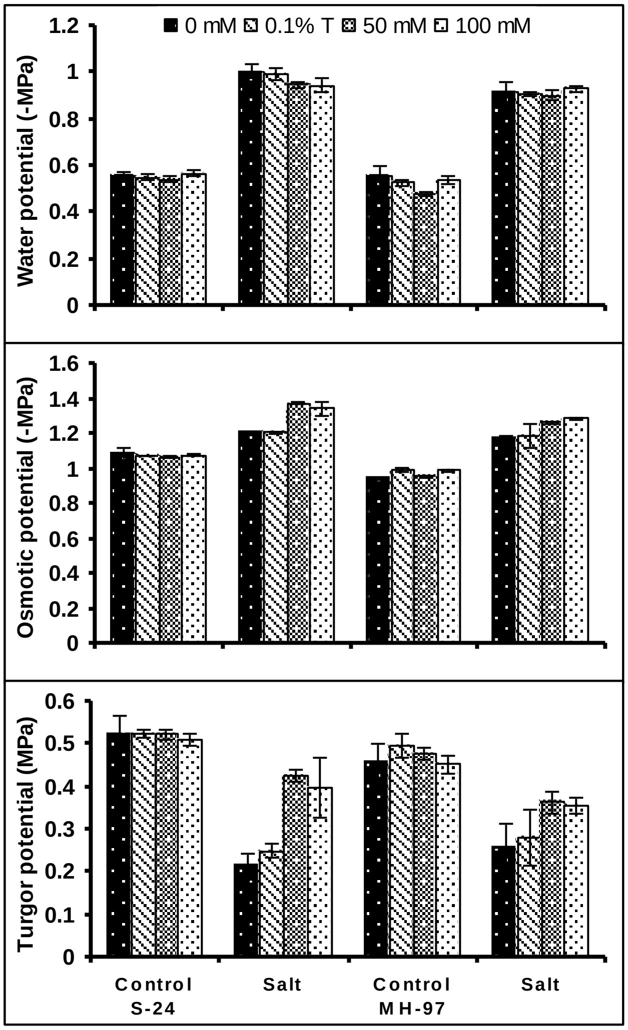

Fig. 2 Leaf water potential, osmotic potential and turgor potential of two spring wheat (*Triticum aestivum* L.) cultivars differing in salinity tolerance when different levels of GB were exogenously applied to salt-stressed or non-stressed plants (No spray; 0.1% Tween20 solution; 50 mM GB; and 100 mM GB in 0.1% Tween 20).

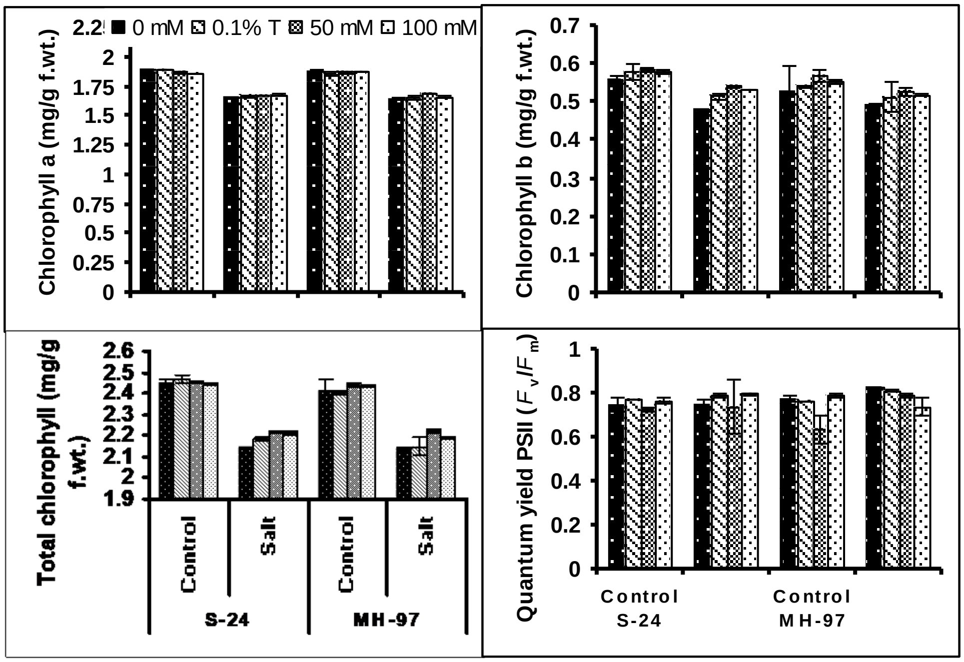

Fig. 3. Leaf chlorophyll 'a', 'b', total chlorophyll and quantum yield of PSII ($F_v/F_m$) of two spring wheat (*Triticum aestivum* L.) cultivars differing in salinity tolerance when different levels of GB were exogenously applied to salt-stressed or non-stressed plants (No spray; 0.1% Tween20 solution; 50 mM GB; and 100 mM GB in 0.1% Tween 20).

The reduction in photosynthesis under salt stress can also be attributed to a decrease in chlorophyll content (Delfine *et al.*, 1999). In the present study, photosynthetic pigments like chlorophyll ‘a’ or ‘b’ decreased in both wheat cultivars due to salt stress (Fig. 3), which is in agreement with some previous studies on different crops e.g., alfalfa (Winicov & Seemann, 1990), sunflower (Ashraf & Sultana, 2000), and wheat (El-Hendawy *et al.*, 2005). Application of GB increased the leaf chlorophyll ‘b’ and total leaf chlorophyll under saline conditions. Thus, higher leaf chlorophyll content is one of the additional factors (other than stomatal limitations) that may have contributed to a higher photosynthetic capacity of wheat cultivars under saline conditions, particularly in cv. S-24. This type of relationship between net $CO_2$ assimilation rate and photosynthetic pigments has already been observed in maize (Ashraf & Rehman, 1999), and some trees (Kozlowski, 1982).

Many studies have demonstrated that the improvement of photosynthesis by GB in salt stressed plants strongly correlates with the enhanced PSII photochemical performance (Sakamoto *et al.*, 1998; Holmstrom *et al.*, 2000), because GB protects the PSII complex by stabilizing the association of the extrinsic PSII complex proteins under salt stress (Sakamoto & Murata, 2002). However, in the present study quantum yield of PSII as measured by $F_v/F_m$ was not affected either due to salt stress or foliar application of GB (Fig. 3). These results can be related to some earlier findings in which it has been observed that salt stress had no effect on PSII photochemical activity e.g., in cotton (Brugnoli & Bjorkman, 1992), and barley (Morales *et al.*, 1992).

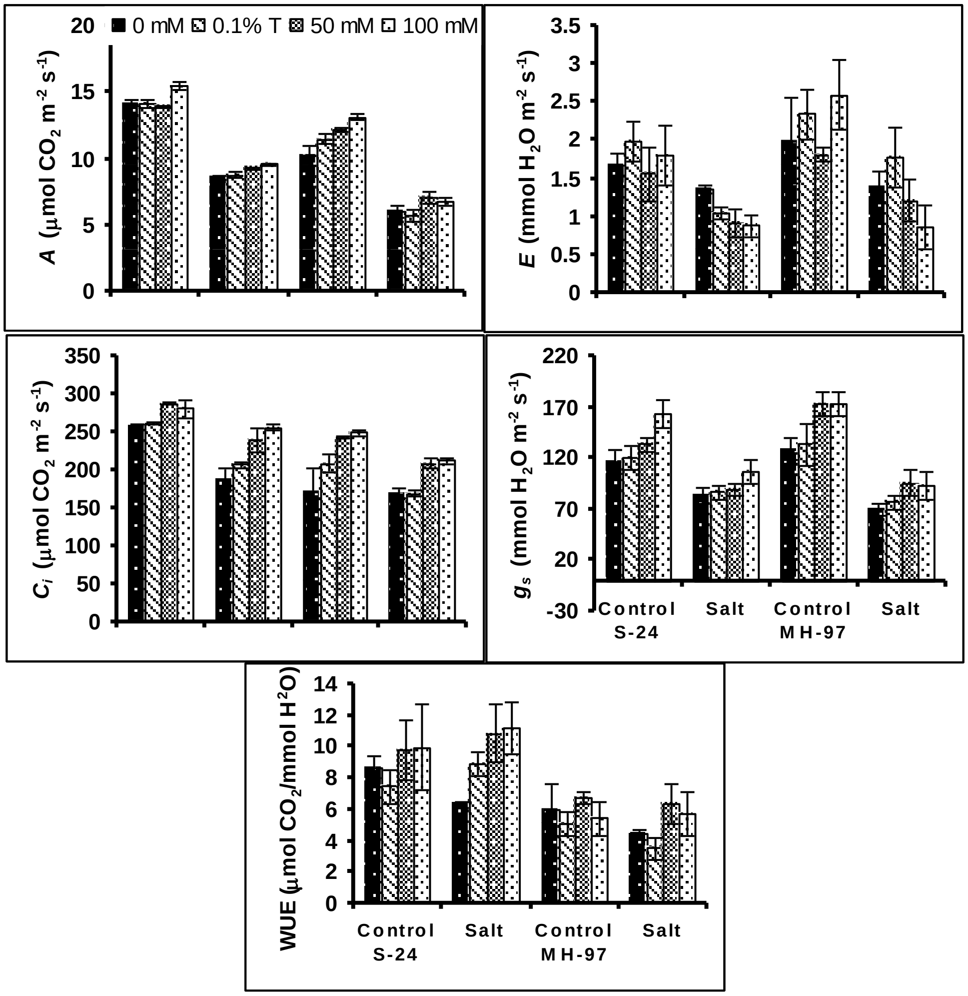

Fig. 4. Net $CO_2$ assimilation rate (*A*), transpiration rate (*E*), sub-stomatal $CO_2$ ($C_i$), stomatal conductance ($g_s$), water use efficiency (WUE measured as *A*/*E*) of two spring wheat (*Triticum aestivum* L.) cultivars differing in salinity tolerance when different levels of GB were exogenously applied to salt-stressed or non-stressed plants (No spray; 0.1% Tween20 solution; 50 mM GB; and 100 mM GB in 0.1% Tween 20).

Increase in stomatal conductance accompanied with a decrease in transpiration rate of wheat cultivars due to GB application under saline conditions may change the plant water relations. This was also supported by the fact that GB application improved the WUE under saline conditions. A decrease in leaf osmotic potential coupled with an increase in leaf turgor, as observed in the present study (Fig. 2), is a phenomenon referred to as osmotic adjustment. Osmotic adjustment (OA) is one of the factors that can enhance salt tolerance (Ashraf, 2004). Osmotic adjustment in plants may have been due to accumulation of inorganic and/or organic solutes but their relative contribution of these solutes varies among species, among cultivars and even among compartments within the same plant (Ashraf, 2004). The organic osmotica generally found in higher plants are low molecular weight sugars, organic acids, polyols and nitrogen containing compounds such as glycinebetaine, proline, free amino acids and their derivatives (Ashraf & Harris, 2004). If parallels are drawn between leaf osmotic potential and each of GB or proline, it is evident that GB contributed more to osmoregulatory process (Figs. 1, 2).

The result of the present study would suggest that salt-induced adverse effects on photosynthetic capacity in wheat can be improved by the exogenous application of GB through enhanced level of endogenous GB. High accumulation of GB mainly contributes to OA, which is one of the factors for improving photosynthetic capacity under salt stress. Better osmotic adjustment or plant water status due to GB application increased the stomatal conductance and thus favored higher $CO_2$ fixation rate. The protective effect of GB on photosynthetic pigments and GB induced reduction in transpiration rate are additional factors which might have contributed to better growth of wheat cultivars under salt stress. However, cv. S-24 was higher in photosynthetic capacity and plant water status or osmotic adjustment than MH-97, which could explain the ability of the salt tolerant S-24 to show higher salt tolerance than the moderately sensitive MH-97.

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