

FOUR FOLIAR APPLICATIONS OF GLYCINEBETAINE DID NOT ALLEVIATE ADVERSE EFFECTS OF SALT STRESS ON GROWTH OF SUNFLOWER

MUHAMMAD IBRAHIM, AMBREEN ANJUM, NABEELA KHALIQ,
MUHAMMAD IQBAL AND HABIB-UR-REHMAN ATHAR*

Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, Pakistan

Abstract

In response to salt stress, plants have evolved some adaptations such as osmotic adjustment to acclimatize salt stress. Glycinebetaine (GB) is known to have a role in osmotic adjustment. The present investigation was focused to understand the role of fortnightly exogenous foliar application of GB in inducing salt tolerance in sunflower through osmotic adjustment or by modulating plant water relations. Three levels of GB solutions (0, 0.1% Tween 20 solution; 50 and 100 mM GB in 0.1% Tween 20 solution) were foliarly applied to three-week old plants of sunflower grown at 0, 60, and 120 mM NaCl. Salt stress reduced the growth of sunflower plants. However, four foliar applications of 50 mM GB on weekly basis improved the growth of sunflower plants at intermediate level of salt stress, whereas higher level of GB did not improve the growth or even reduced the growth. Although exogenously applied GB reduced the leaf water potential and osmotic potential that resulted in enhanced leaf turgor potential, it did not improve the growth. Salt induced reduction in photosynthetic rate was partially improved by four applications of GB at intermediate level of salt stress. Furthermore, changes in photosynthetic capacity mainly occurred due to stomatal limitations. Finally, it was concluded that four applications of GB partially alleviated adverse effects of salt stress, which was associated with stomatal factors.

Keywords: osmotic adjustment, photosynthetic capacity, glycinebetaine, salt stress

Introduction

A variety of protective mechanisms have evolved in plants to acclimatize unfavorable environmental conditions for survival and growth. The accumulation of compatible solutes increases the ability of cells to retain water without affecting the normal metabolism (Hamilton & Heckathorn, 2001). Among them, glycinebetaine an amino acid derivative is known for its protective effects in higher plants against salt/osmotic stresses, not only by maintaining osmotic adjustment (Ashraf & Foolad, 2007), but by stabilizing many functional units, like oxygen-evolving PS-II complex (Harinasut *et al.*, 1996), membranes, quaternary structures of complex proteins (Murata *et al.*, 1992), and enzymes such a rubisco. Therefore, plants can protect themselves against abiotic stresses by enhanced synthesis and accumulation of GB.

Exogenous foliar application of glycinebetaine is the suggested way as a shotgun approach to induce stress tolerance in crops with poor or no solute accumulating ability (Ashraf & Foolad, 2007). There are contradictory reports on foliar application of glycinebetaine in inducing abiotic stress tolerance in crops. For example, foliar application of GB improved salt and drought tolerance in rice (Harinasut *et al.*, 1996), maize (Agboma *et al.*, 1997), tomato (Makela *et al.*, 1998; Heuer, 2003), and wheat

* Corresponding Author: Habib-ur-Rehman Athar; Ph 092-061-9210053 e-mail: habibathar@yahoo.com

(Raza *et al.*, 2006; 2007). In contrast, no significant improvement in growth has been observed in cotton (Meek & Oesterhiues, 2003). Recently, Ashraf & Foolad (2007) suggested that effectiveness of foliarly applied glycinebetaine depends on a number of factors including type of species, plant developmental stage at which applied, concentration of GB, and number of applications. More research needs to be conducted to assess the effects of different doses of exogenously applied glycinebetaine on growth of crop plants. Thus, the aim of the present study was to assess as to whether number of exogenous foliar applications of glycinebetaine was effective in inducing salt stress tolerance in sunflower plants.

Materials and Methods

Achenes of sunflower (*Helianthus annuus* L.) line Hisun 33 were obtained from Seed Distribution Center of ICI, Industrial Estate, Multan, Pakistan. The experiment was conducted in the Botanic Gardens of the Bahauddin Zakariya University, Multan, Pakistan (30°11N and 71°28E). The average day and night temperatures were 34±6°C and 25±4°C, respectively. The relative humidity ranged from 55-76 percent, and day length from 11-12 h. Ordinary river sand was washed thoroughly with tap water, distilled water, and finally with full strength Hoagland solution. Plastic pots of 36 cm diameter were filled with that sand. Achenes of sunflower were surface sterilized in 5% sodium hypochlorite solution for 5 minutes before further experimentation. The experiment was arranged in the glasshouse in a randomized complete block design with four replicates, three NaCl treatments (0, 60, 120 mM NaCl) and three treatments (0, 0.1% Tween 20 solution; 50 and 100 mM GB in 0.1% Tween 20 solution) of glycinebetaine were applied weekly. Five to six randomly chosen pre-germinated seeds of 4-7 days old of sunflower transplanted into each mosaic-cemented pots having sandy loam soil. The NaCl and glycinebetaine treatments were begun 21 days after emergence. The NaCl treatments were increased stepwise in aliquots of 30 mM every day until the appropriate treatment concentration was attained. Every time treatment solutions were applied in the evening and nutrient solutions were given every week. Different concentrations of GB were also applied every week and four times during whole experiment.

Relative water contents: A fully developed and young leaf from each plant was taken and fresh weight of each leaf was recorded. All the samples were immersed in distilled water for 10 h and then turgid weight of each leaf was taken. Then all the samples were oven dried at 70 °C and dried weights were measured. Then RWC (Relative water content) was calculated as given below:

$$\text{Relative water content \%} = \frac{\text{Leaf fresh weight} - \text{Leaf dry weight}}{\text{Leaf turgid weight} - \text{Leaf dry weight}} \times 100$$

Water relations The third leaf from top of each plant was used for measurement of water relations. The leaf from each plant was excised at 7.00 am, and leaf water potential measurements were made with a Scholander type pressure chamber (Chas W. Cook and Sons, Birmingham, UK). A proportion of the leaf used for water potential measurements, was frozen into 2 cm³ polypropylene tubes for two weeks at -40°C in ultra-low freezer,

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 used directly for osmotic potential determination using a vapor pressure osmometer (Wescor 5200). Turgor pressure was calculated by subtracting the leaf water potential from leaf osmotic potential. Leaf osmotic potential values were corrected for the dilution of the symplastic sap by apoplastic water, which occurs when sap is expressed. Apoplastic water was considered 10% following Wilson *et al.*, (1980).

Gas exchange parameters: Measurements of gas exchange attributes were made on the intact third young leaf from top of each plant using an ADC LCA-4 portable infrared gas analyzer (Analytical Development, Hoddesdon, UK). These measurements were made from 9.30 to 11.30 a.m. with the following specifications/adjustments: leaf surface area, 6.25 cm²; ambient temperature, 45±3°C; ambient CO₂ concentration, 352 μmol mol⁻¹; temperature of leaf chamber varied from 41.0 to 49.0°C; leaf chamber gas flow rate, 410 ml min⁻¹; RH of the chamber ranged from 25.4 to 41.2 %; PAR (Q_{leaf}) at leaf surface during noon was maximum up to 1200 μmol m⁻² s⁻¹; ambient pressure 98.8 kPa.

After five weeks growth, plants were harvested. Plant roots were removed from the culture solution and washed in cold LiNO₃ solution isotonic with the corresponding treatment in which plants were growing. 1mM Ca (NO₃)₂.4H₂O was added in LiNO₃ solution to maintain membrane integrity. Plants were separated into shoots and roots and then blotted dry before recording their fresh weights. After harvesting, leaf area was measured with a leaf area meter (Delta T-Devices). All plant parts were dried at 65°C until constant dry weight, and their dry weights measured.

Statistical analysis of the 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 (LSD) following Snedecor & Cochran (1980).

Results

Shoot fresh and dry weight of sunflower plants significantly reduced due to imposition of salt stress, particularly at the highest external NaCl regime. However, four applications of 50 mM GB improved shoot fresh and dry weight of non-stressed sunflower plants, whereas these were reduced due to four 100 mM GB applications. Similarly, lower level of GB improved shoot fresh and dry weight of sunflower plants when grown at 60 mM NaCl. However, at the highest NaCl salinity stress exogenous application of 50 mM GB did not change shoot fresh and dry weight whereas it reduced at highest level of salinity. Likewise, shoot length of non-stressed plants increased with 50 mM GB application, whereas at the highest level of salt stress it did not change the shoot length. Exogenous application of 100 mM GB caused a reduction in shoot length at the highest level of salt stress (Fig 1). Similarly, 50 mM GB was only effective in improving leaf area of sunflower plants either under normal growth conditions or at intermediate level of salt stress (Fig. 1). However, foliar application of 100 mM GB reduced leaf area under control conditions or at the intermediate level of salt stress, while at the highest level of salt stress it did not change the leaf area. Furthermore, response of leaf area to GB appears to be dose dependent at either salt treatment.

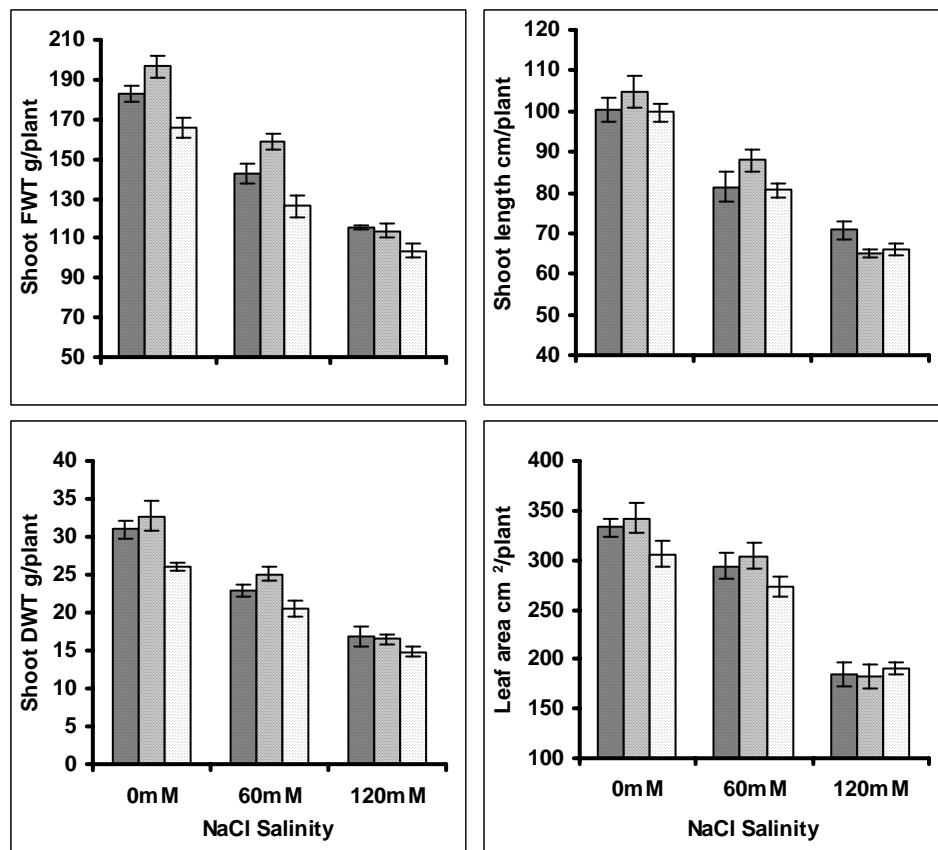


Fig. 1. Fresh and dry weight of shoot and root (g/plant) of sunflower (*Helianthus annuus* L.) when 21 day-old plants were subjected to exogenous foliar application of glycinebetaine for 30 days under non-saline and saline conditions.

Leaf water potential, osmotic potential, turgor potential and relative water content (RWC) of sunflower plant (Fig.2) decreased significantly due to growth medium salinity. However, salt induced reduction was the highest in all these water relation parameters at the highest level of salt stress. Application of GB further decreased all these leaf water potential and leaf osmotic potential (Fig 2). However, leaf turgor potential was increased with GB application, particularly at 100 mM GB. In contrast, at 60 mM NaCl RWC was improved by 50 mM GB. However, at the highest level of salt stress 50 mM or 100 mM GB did not change RWC (Fig 2).

All gas exchange characteristics net CO₂ assimilation rate (*A*), transpiration rate (*E*), sub-stomatal CO₂ (*C_i*) and water use efficiency (WUE) were adversely affected due to increasing level of salt stress (Fig 3). However, exogenous application of 50 or 100 mM GB improved *A*, *E*, and *C_i* only at intermediate level of salt stress, whereas GB applied foliarly four times did not change these gas exchange attributes (Fig 3). However, WUE was reduced due to GB application at 60 or 120 mM NaCl stress. Furthermore, a positive association has been found among each of *A*, *E* and *C_i*.

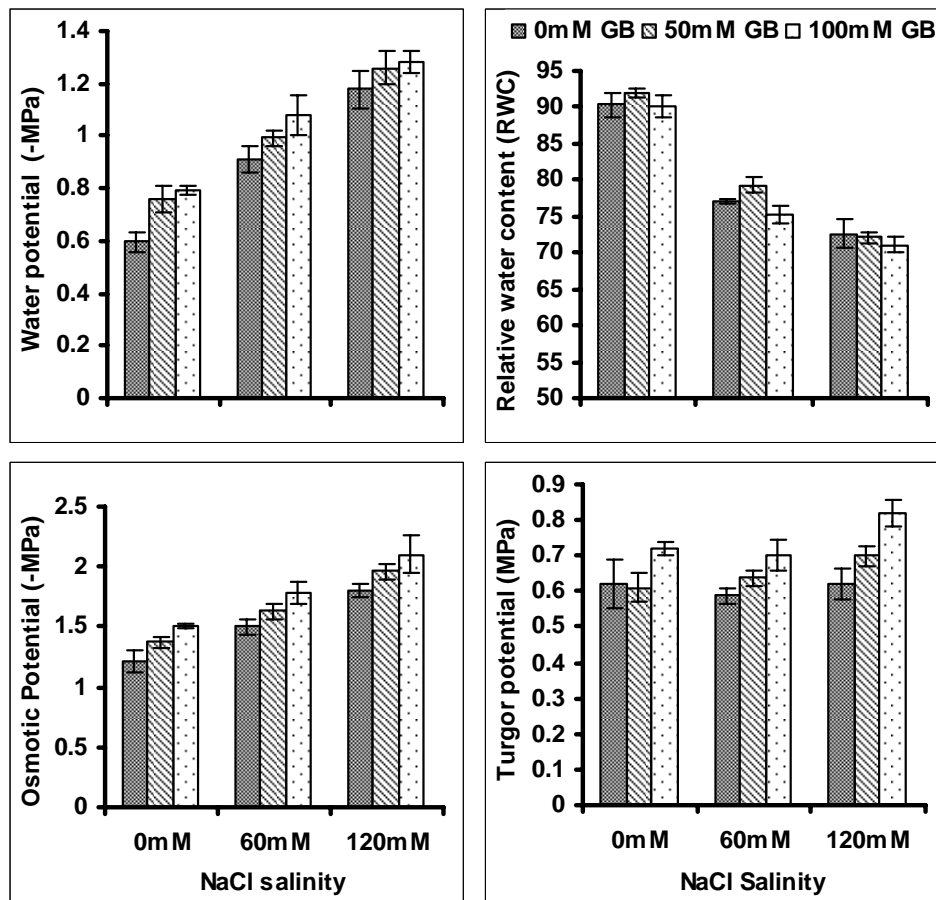


Fig. 2. Water potential, osmotic potential and turgor potential (MPa) of sunflower (*Helianthus annuus* L.) when 21 day-old plants were subjected to exogenous foliar application of glycinebetaine for 30 days under non-saline and saline conditions.

Discussion

In the present study, salt stress caused a decrease in growth of sunflower plants. Under normal growth conditions or at intermediate level of salt stress only 50 mM GB applied four times as a foliar spray improved the growth. Thus, exogenous foliar application of GB did not completely alleviate salt induced adverse effects on growth of sunflower plants. These results are inconsistent with earlier findings in which different scientists reported that exogenous foliar application of GB resulted in improvement of different crops, e.g., wheat (Raza *et al.*, 2006; Ashraf & Foolad, 2007 and references therein). Furthermore, highest level of GB applied four times caused reduction in growth.

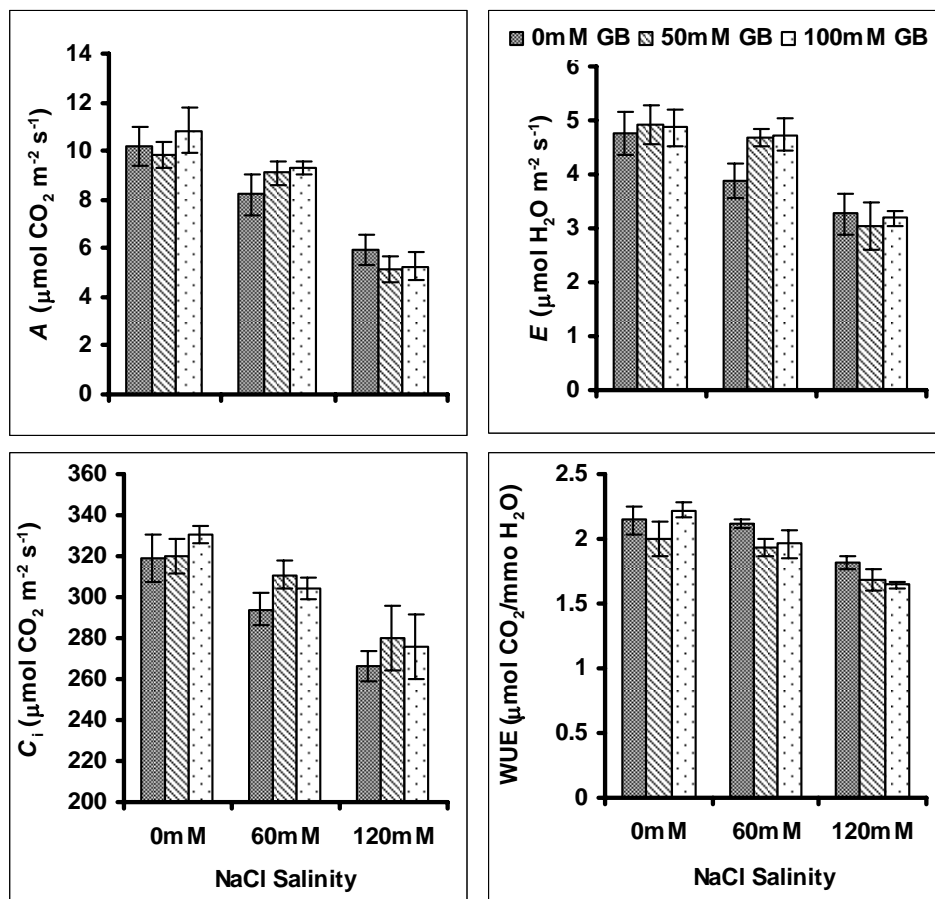


Fig. 3. Net assimilation rate (A), transpiration rate (E), sub-stomatal CO_2 (C_i) and water use efficiency (A/E) of sunflower (*Helianthus annuus* L.) when 21 day-old plants were subjected to exogenous foliar application of glycinebetaine for 30 days under non-saline and saline conditions.

These results are in agreement with the findings of Gibon *et al.* (1997) who found reduction in growth due to toxic effects of GB. Similarly, it has been observed that application of GB in higher quantity or its application more than two times has adverse effects on growth and yield of cotton (Makhdam & Shahabuddin, 2006). In another study, Lopez *et al.* (2002) showed increased leaf area with application of 10 mM GB in kidney beans, but no increase in leaf area with 30 mM GB. Similarly, while working with grapevine Mickelbart *et al.* (2006) found that exogenous application of 50 mM GB reduced the leaf area, growth rate and longer leaf development period. Furthermore, they found that exogenous application of 100 or 200 mM GB completely stopped the growth of plants and leaves exhibited severe phytotoxic symptoms. Wilson (2001) also observed severe phytotoxicity with applications of 25 mM GB to field-grown grapevines. This concentration is lower than the level in our study. While working with spring wheat, and

turnip rape Makela *et al.* (1996) found that GB applied at 15, 50, 100, and 300 mM on spring wheat caused no visible phytotoxicity effects. However, application of 300 mM GB solution caused severe phytotoxicity effects on turnip rape seedlings, whereas 100 mM GB caused slight blotching. However, Naidu *et al.* (2003) found that foliar application of GB (40 mM) at a rate of 200 kg ha⁻¹ increased pasture growth but increasing the application from 40 to 85 mM and 170 mM did not cause any further increase in yield or toxicity symptoms. In view of all these results, it is suggested that GB is not a compatible organic osmoticum for all plants or it cause phytotoxicity when applied either at higher concentration or by increasing the number of applications. It can also be generalized that broadleaf species such as bean, tomato, and grape are more sensitive to high concentrations of GB than are grass species/cereals. Therefore, it is important to determine optimal concentration of GB, number of application, and time of application for each crop species. Since GB applied four times as a foliar spray, higher

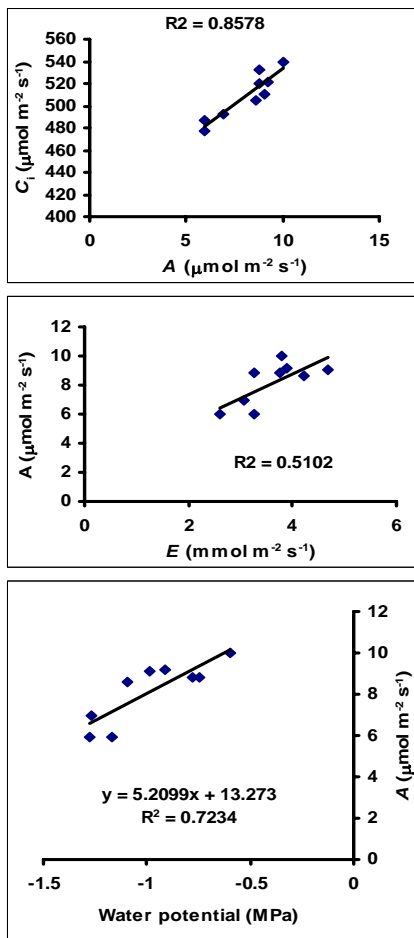


Fig. 4. Linear regression between A and C_i , E , water potential, and C_i of sunflower (*Helianthus annuus* L.) when 21 day-old plants were subjected to exogenous foliar application of glycinebetaine for 30 days under non-saline and saline conditions.

endogenous level of GB is expected which might have caused some phytotoxic effects on plant biochemical or physiological processes such as photosynthesis, activity of enzymes involved in metabolic processes or plant water relations. In present study, GB reduced leaf water potential and leaf osmotic potential of the leaves at all levels of salt stress. Although these changes in leaf water potential and leaf osmotic potential of the leaves due to foliar application of GB were very small, it improved the leaf turgor potential. Furthermore, changes in water relations parameters were mainly due changes in osmotic potential, because changes in RWC due to GB application were almost negligible. The reduction in osmotic potential occurred mostly at the higher GB application rate, which in turn enhanced leaf turgor potential. Similar results for higher GB-induced increase in leaf turgor potential were observed in maize (Quan *et al.*, 2004), wheat (Raza *et al.*, 2006), and grapevine (Mickelbart *et al.* 2006). These results can also be explained in view of earlier findings of Makela *et al.* (1998) who found improved water status in tomato with GB application under saline conditions. Similarly, working with different isolines of maize, Saneoka *et al.* (1995) demonstrated that leaf tissue of maize isolate synthesizing GB maintained higher relative water content (RWC) and turgor when grown under salt stress than an isolate deficient in GB synthesis. However, if we draw the relationship between leaf turgor potential and growth of sunflower plants, it is clear that GB-induced changes in plant water status did not affect the plant growth of sunflower plants. In contrast, though growth is positively associated with RWC, GB-induced changes in growth of sunflower plants were due to some physiological process other than plant water status such as photosynthesis.

In the present study, GB application increased the net CO₂ assimilation rate of sunflower plants growing at intermediate level of salt stress. However, at the highest level of salt stress, GB application did not improve photosynthetic capacity. These results can be related to some extent, to the earlier findings of Meek & Oosterhuis (1999) who found that application of GB to cotton plants do not increased photosynthetic rate. In contrast, Raza *et al.* (2006) found that foliar application of GB enhanced photosynthetic capacity of wheat under saline conditions. Variability in results could be due to several factors such as type of crop, environmental condition, timing and rate of application. The later seems to be more relevant in view of Lopez *et al.*, (2002) who demonstrated that 30 mM GB application had an adverse effect on kidney bean plants. They suggested that GB can be used to reduce the effects of salt stress through improving water status of plants but only to limited salinity level. Salt induced reduction in *A* occurs due to limited CO₂ supply through stomata or mesophyll resistance, or efficiency of photosynthetic enzymes (Dubey, 2005). From the results of present study, *A* was positively associated with *E* and *C_i*, which indicates changes in *A* mainly occurred due to stomatal limitations. A positive relationship between shoot biomass and net CO₂ rates of sunflower was found in the present study. Such positive relationship between the two variables was earlier observed in different crops species e.g., wheat (Raza *et al.*, 2006; Arfan *et al.*, 2007), and *Brassica spp.* (Nazir *et al.*, 2001).

By summarizing the results, it is clear that reduction in growth of sunflower was associated with decreased photosynthetic rate due to limitation of CO₂ supply through stomatal resistance. However, four applications of GB partially ameliorate the adverse effects of salinity. Although four applications of GB improved leaf turgor potential it did not improve growth of sunflower plants under saline conditions. Therefore, it is suggested that its use as a potential means of inducing salt tolerance should be further investigated.

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