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Exogenous gamma-aminobutyric acid increases salt tolerance of wheat by improving photosynthesis and enhancing activities of antioxidant enzymes

M.F. LI[1], S.J. GUO[1]*, X.H. YANG[2], Q.W. MENG[2], and X.J. WEI[1]

College of Life Science, Liaocheng University, Liaocheng 252000, P.R. China[1]
College of Life Science, Shandong Agricultural University, Taian 211018, P.R. China[2]

Abstract

Gamma-aminobutyric acid (GABA) is a non-protein amino acid that accumulates in a number of plant species under various environmental stresses. In this paper, the ability of applied GABA for the alleviation of NaCl stress was investigated in view of growth parameters, gas exchange, photosynthetic pigments, chlorophyll fluorescence, activities of antioxidant enzymes, malondialdehyde (MDA) content, and electrolyte conductivity (REC) in wheat seedlings. Germination rate and shoot dry mass decreased with an increasing NaCl concentration and this decrease was less pronounced when 0.5 mM GABA was applied. In the NaCl-treated seedlings, exogenous GABA partially enhanced photosynthetic capacity and antioxidant enzyme activities and decreased MDA content and REC. Therefore, GABA reduced the impact of salinity on the wheat seedlings.

Additional key words: catalase, electrolyte leakage; lipid peroxidation, malondialdehyde, superoxide dismutase, *Triticum aestivum*.

Introduction

Approximately 7 % of the world's land area and 20 % of the world's irrigated land areas are affected by soil salinity, which results in the inhibition of plant growth and yield (Yamaguchi *et al.* 2005, Abogadallah 2011). The growth inhibition of many plants that are subjected to salinity is often associated with a decrease in their photosynthesis which may be due to stomatal limitations and/or non-stomatal limitations (Debez *et al.* 2008). Salinity strongly limits photosynthetic CO_2 fixation, and the absorption rate of light energy by photosynthetic pigments may exceed the rate of energy consumption in chloroplasts (Foyer *et al.* 2005, Liu *et al.* 2012). This excess energy may accelerate photodamage to photosystem (PS) II *via* the generation of reactive oxygen species (ROS; Yang *et al.* 2007). A number of pathways (photorespiration, xanthophyll cycle-dependent energy dissipation, cyclic electron flows through either PS I or PS II, and the antioxidant system) are proposed to help protection of the photosynthetic apparatus from photo-oxidative stress (Asada 2006). The antioxidative system in plants includes low-molecular-mass antioxidants and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase, ascorbate peroxidase, glutathione reductase, *etc*. (Lu *et al.* 2013). Salinity may cause oxidative stress by inducing an imbalance between the production of ROS and antioxidant defense (Mittler 2002, Apel *et al.* 2004).

Interestingly, a potential compatible solute (γ-amino butyric acid, GABA) is largely and rapidly produced in response to biotic and abiotic stresses including salt stress (Kinnersley 2000, Kato-Noguchi *et al.* 2005, Fait *et al.* 2007, Kim *et al.* 2007, Allan *et al.* 2008). Gamma-amino butyric acid is a four-carbon non-protein amino acid that is produced by glutamate decarboxylase from glutamic acid (Breitkreuz and shelp 1995). However, Su *et al.* (2007) suggested that a higher GABA accumulation

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Abbreviations: Φ_{PSII} – actual photosystem II efficiency; CAT - catalase, Chl - chlorophyll; c_i - intercellular CO_2 concentration; E - transpiration rate; EDTA - ethylenediaminetetraacetic acid; F_m - maximum fluorescence induction; F_v - variable fluorecence; GABA - γ-aminobutyric acid; g_S - stomatal conductance; MDA - malondialdehyde; NPQ - non-photochemical quenching; P_N - net photosynthesis rate; PS - photosystem; Rc - electric conductivity; REC - relative electrolyte conductivity; ROS - reactive oxygen species; SOD - superoxide dismutase; WUE - water use efficiency.

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* Corresponding author; fax: (+86) 0635 8239680, e-mail: guoshangjing@lcu.edu.cn

(approximately 39 % of total GABA) induced by salt stress can be obtained from polyamine degradation indicating that polyamine can perform its functions through GABA formation under salt stress. Although the role of GABA as neurotransmitter in mammals is clearly established, its exact role in plants is still vague even with an increasing evidence of its importance in regulating pollen tube development (Palanivelu *et al.* 2003, Yu *et al.* 2007) and in responding to abiotic stresses (Kinnersley *et al.* 2000, Cho *et al.* 2008). Exogenous GABA can remove the ROS as demonstrated *in vitro* experiments of Smirnoff *et al.* (1989). Gamma-aminobutyric acid can be also used as source of carbon and nitrogen (Breitkreuz *et al.* 1999, Reyes-García *et al.* 2012). The application of exogenous GABA can improve stress tolerance (Song *et al.* 2010, Shang *et al.* 2011, Cao *et al.* 2012). Gamma-aminobutyric acid has potentially a dual role as signalling molecule and as metabolite (Bouché *et al.* 2004, Roberts 2007). Gamma-aminobutyric acid acts as signal that triggers gene expression in *Agrobacterium. tumefaciens* (Sara *et al.* 2010). All genes composing the GABA shunt are upregulated in the cDNA library of *Fusarium graminearum* grown on a plant cell wall (Carapito *et al.* 2008). The majority of recent work on the GABA shunt in plants has examined its association with stress and its signalling roles (Shelp *et al.* 2006, 2012, Fait *et al.* 2007). Several researchers have suggested that the GABA shunt may be linked to various physiological responses including the regulation of cytosolic pH, carbon fluxes in the tricarboxylic acid cycle, nitrogen metabolism (Sulieman 2011), deterrence of insects (Bouché *et al.* 2004, Bown *et al.* 2006, Kazuhito *et al.* 2007), protection against oxidative stress (Shi 2010), osmoregulation, signalling, and response to a fungal elicitor (Takahashi *et al.* 2008). The GABA metabolism is upregulated in response to NaCl (Renault *et al.* 2010). Thus, GABA roles in salt tolerance must be examined further.

Wheat, an important crop worldwide, is globally threatened by salt stress especially in many arid or semi-arid regions. However, few studies have been published regarding the important function of GABA in crops under salt stress. The application of GABA may be a useful method to reduce salt stress. Therefore, in the present study, we examined the effects of exogenous GABA under salt stress. Growth parameters, gas exchange, chlorophyll fluorescence, antioxidant enzymes, malondialdehyde (MDA) content, and electrolyte leakage were determined.

Materials and methods

The study was conducted using winter wheat (*Triticum aestivum* L. cv. Jingdu40). For germination rate measurements, the seeds were divided into 28 groups and provided with 0, 50, 100, 150, 200, 250, and 300 mM NaCl, and/or 0, 0.25, 0.5, and 1 mM GABA added in Hoagland's solution (Arnon and Hoagland 1940), respectively. Ten replicates of 100 seeds per plate were placed, embryo up, in covered plastic Petri dishes (50 × 90 mm) with *Whatman No. 1* filter paper. Germination rate data were recorded after 72 h.

For dry mass measurements, the germinated seeds were transferred into holes in *Styrofoam* boards and grown hydroponically in the corresponding treatment solutions in a growth chamber at day/night temperatures of 25/16 °C, a relative humidity of 70 %, a 16-h photoperiod, and a photosynthetic photon flux density of 300 - 400 μmol m^{-2} s^{-1} provided by six fluorescent lamp (40 W). After 21 d, 10 plants from each treatment were collected randomly and shoots and roots were segregated. The samples were oven-dried at 70 °C for 48 h and then weighed.

In another experiment investigating a short-term salinity effect, we grew plants in Hoagland's solution in the growth chamber for 24 d and then the plants were divided into 10 groups with different solutions (0, 100, 200, 300, or 400 mM NaCl alone or with 0.5 mM GABA). After 24 h, three plants were randomly selected from each treatment and gas exchange was measured on recent fully expanded leaves using an open gas exchange system (*Ciras-2*, *PP Systems*, Norfolk, UK). Net photosynthesis rate (P_N), stomatal conductance (g_s), intercellular CO_2 concentration (c_i), and transpiration rate (E) were measured between 10:00 and 12:00 at a photosynthetic photon flux density of 800 μmol m^{-2} s^{-1} provided by a red and blue LED source inside the leaf chamber. During measurement, a relative humidity was maintained at 70 % and a leaf temperature at 24 ± 0.5 °C. Water use efficiency was calculated as P_N/E (He *et al.* 2009).

Chlorophyll (Chl) *a* fluorescence was measured at room temperature between 10:00 and 12:00 using a portable *FMS-2* plant efficiency analyser (*PEA, Hansatech*, King's Lynn, UK) after attached leaves had been dark-adapted for 15 min. Basal Chl fluorescence (F_0), maximum fluorescence induction (F_m), maximum fluorescence after light-adaption (F'_m), and steady-state fluorescence (F^s) were determined according to the experimental protocol of Yang *et al.* (2008). Using the above-mentioned fluorescence parameters, we calculated an actual PS II efficiency (Φ_{PSII}) = $(F'_m - F_s)/F'_m$ and non-photochemical quenching (NPQ) = $(F_m - F'_m)/F'_m$ (Yang *et al.* 2008).

Approximately 0.5 g of leaf samples from each group were homogenized with 80 % (v/v) acetone. The homogenate was then filtered through filter paper. The absorbance of the resulting solution was measured at 663, 645, and 480 nm for Chl *a*, Chl *b*, and carotenoids, respectively, (Arnon 1949) using a spectrophotometer

(*UV-2450, Shimadzu*, Kyoto, Japan).

Approximately 0.5 g of leaf samples was ground and dissolved in 5 cm^3 of an ice-cold 25 mM HEPES buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM ascorbate, and 2 % (m/v) polyvinylpyrrolidone. The homogenate was centrifuged at 12 000 *g* and 4 °C for 20 min and 2 cm^3 of the supernatant was used for the identification of enzymatic activity. Activity of SOD was assayed by measuring its ability to inhibit photochemical reduction of nitrobluetetrazolium. One SOD unit was defined as the amount of enzyme that produced a 50% inhibition of NBT reduction under the assay conditions. Activity of CAT was measured as decline in absorbance at 240 nm due to a decrease in decomposition of H_2O_2. Each measurement of antioxidant enzymes was conducted in three technical replicates (He *et al.* 2009).

Leaf samples of each group were first shaken in 5 cm^3 of double-distilled water at 170 rpm at 25 °C for 2 h and electric conductivity (Rc) was measured. Then the samples were boiled for 30 min and conductivity was again measured (Rc0). Relative conductivity (REC) was calculated by Rc/Rc0 × 100 (Tang 1999).

For measurement of lipid peroxidation, the thiobarbituric acid test which determines the amount of MDA, a decomposition product of the oxidation of polyunsaturated fatty acids, was conducted as described by Havaux *et al.* (2003).

All data obtained were subjected to one-way analysis of variance (*ANOVA*) using the statistical software *SPSS v. 16.0*. Prior to analysis, germination rate data were log-transformed (lg[x]) to avoid differences in scale within and between traits. Treatment means were compared using Duncan's test with a significance level set at $\alpha = 0.05$. Each data point represents the mean of three replicates ($n = 3$) except for the germination data ($n = 10$) and was expressed as the mean ± standard error (SE).

Results

The NaCl treatments negatively affected germination of the wheat seeds, and GABA improved germination to a certain extent (Table 1). Germination increased by about 50 % in 0.5 mM GABA at 300 mM NaCl compared with

Table 1. The effect of γ-aminobutyric acid (GABA) on the germination rate [%] of wheat seeds under different NaCl concentrations alone or with addition of GABA. Means ± SEs, $n = 10$. Different *lowercase letters* in the same column denote significant differences among four concentrations of GABA at $P < 0.05$ according to Duncan's test.

GABA [mM]	NaCl [mM]						
	0	50	100	150	200	250	300
0	85 ± 0.80a	80 ± 0.36b	78 ± 0.33c	74 ± 0.36d	63 ± 0.32e	57 ± 0.36f	38 ± 0.63g
0.25	84 ± 0.36a	84 ± 0.58a	79 ± 0.42b	72 ± 0.36c	63 ± 0.36d	60 ± 0.42e	47 ± 0.47f
0.50	85 ± 0.36a	85 ± 0.42a	84 ± 0.42a	80 ± 0.42b	76 ± 0.36c	65 ± 0.36d	62 ± 0.39e
1.00	83 ± 0.52a	83 ± 0.30a	77 ± 0.30b	75 ± 0.33c	65 ± 0.36d	58 ± 0.39e	45 ± 0.42f

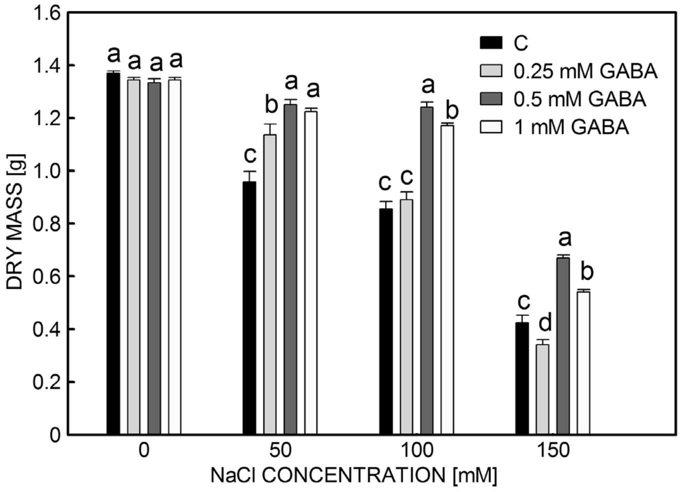

Fig. 1. Changes of shoot dry mass in wheat plants exposed to different concentrations of NaCl (0, 50, 100, 150 mM) and/or different concentrations of γ-aminobutyric acid (GABA) (0 = C, 0.25, 0.5 and 1 mM) in a nutrient solution for 21 d. Means ± SE, $n = 3$, different letters indicate significant differences according to Duncan's test ($P < 0.05$).

300 mM NaCl only.

After 21 d of treatment, shoot dry masses gradually decreased as the NaCl concentration increased. The exogenous GABA demonstrated beneficial effects on shoot dry mass under the salt stress, whereas no effects were observed without NaCl (Fig. 1). The seedlings treated with 0.5 mM GABA grew better than those treated with 0.25 mM GABA. However, the seedlings treated with 1 mM GABA did not exhibit obvious increases compared with those treated with 0.5 mM GABA. The applied 0.5 mM GABA strongly increased shoot dry mass by 19 - 36 % under the different salinities (Fig. 1). However, the application of GABA did not have an apparent effect on the root dry mass under the salt

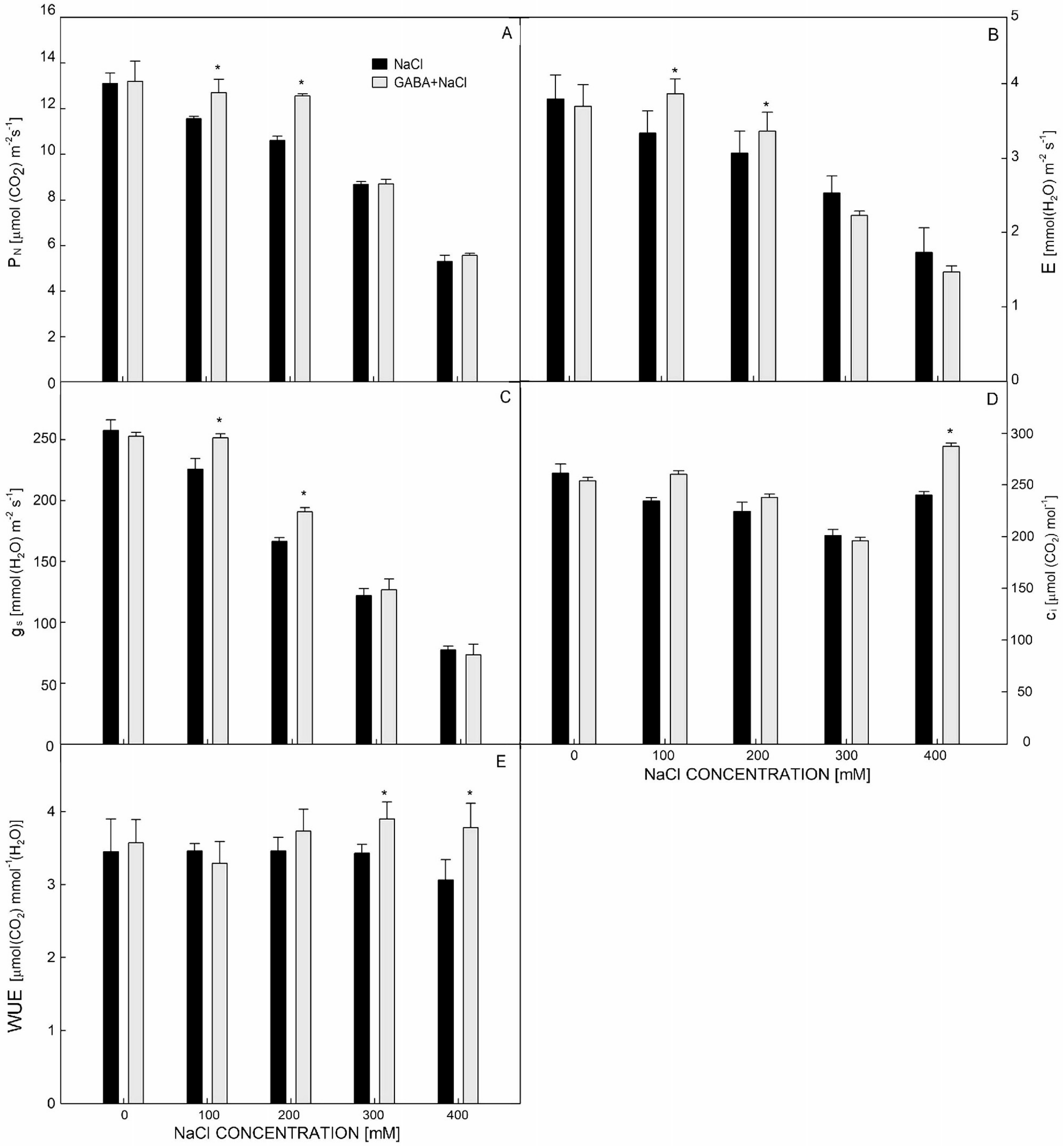

Fig. 2. Effects of 0.5 mM γ-aminobutyric acid (GABA) on gas echange of wheat leaves under different NaCl concentrations (0 - 400 mM). Treatments of 24-d-old seedlings lasted 24 h. *A* - net photosynthesis rate (P_N); *B* - transpiration rate (E); *C* - stomatal conductance (g_s); *D* - intercellular CO$_2$ concentration (c_i), and *E* - water use efficiency (WUE). Means ± SE, *n* = 3, * - significant differences in comparison with plants receiving NaCl alone at $P < 0.05$.

stress (data not shown). These results indicate that 0.5 mM GABA was an optimal concentration. Thus, subsequent experiments only included the applications of GABA at a concentration of 0.5 mM to different NaCl solutions.

Net photosynthesis rate in the GABA+NaCl groups was higher (7 - 15 %) than in the NaCl groups without GABA at treatments with 100 and 200 mM NaCl (Fig. 2*A*). Transpiration and g_s of the GABA+NaCl group plants were also obviously higher than those in the NaCl groups under the moderate NaCl stress, but lower at the serious NaCl stress (Fig. 2*B*,*C*). The intercellular CO_2 concentration and water use efficiency of the GABA+NaCl groups significantly increased compared with those of the NaCl groups under the serious stress (Fig. 2*D*,*E*). Actual PS II efficiency and NPQ of the GABA+NaCl plants were also higher compared with those in the NaCl groups (Fig. 3).

The decline of pigment content (Chl *a* and Chl *b*) caused by the NaCl stress was prevented by GABA even if a slight increase was found at a specific concentration (Fig. 4*A*,*B*). Activities of SOD and CAT were considerably higher in the GABA+NaCl groups compared with those in the NaCl groups especially at the NaCl treatment concentration of 200 mM (Fig. 5). Gamma-aminobutyric acid probably protected the photosynthetic pigments by antioxidative defence in the wheat seedlings under the NaCl stress.

Relative electrolyte conductivity in the GABA+NaCl groups was lower than in the NaCl groups (Fig. 6*A*), which demonstrates that less membrane damage occurred in the plants. Compared with those in the NaCl alone group, MDA content in the GABA+NaCl groups decreased by 36.4, 34.2, and 33.1 % at NaCl concentrations of 100, 300, and 400 mM, respectively. The exogenous GABA decreased REC and MDA content due to higher activities of antioxidant enzymes indicating that GABA effectively protected the membrane system.

Discussion

Salt stress is an important abiotic stress that seriously affects crop productivity and survival. Accumulation of GABA is strongly induced by salt stress (Kim *et al.* 2007). The application of GABA is thus a useful method for studying salinity tolerance. Salt in soil water inhibits plant growth due to a water deficit and ion excess (Munns *et al.* 2005). Photosynthesis does not limit plant growth for a short time during the first phase of salt stress. The sustained NaCl stress markedly decreased the rate of wheat seed germination and shoot dry mass, whereas plants to which GABA was applied were less affected (Fig. 1). These findings confirm previous results that GABA significantly affects germination of barley and fava beans (Chung *et al.* 2009, Li *et al.* 2010). Gamma-aminobutyric acid accumulates very quickly in rice during germination and potentially functions as signalling molecule in plants similarly as in animals (Laurent *et al.* 2010). In this study, 0.5 mM GABA significantly promoted germination rate and inhibited decrease in shoot dry mass of wheat under the long-term NaCl stress. However, growth parameters were not improved by 1.0 mM GABA. These results are consistent with those of Kathiresan *et al.* (1998) for *Stellaria*. We thus applied 0.5 mM GABA in subsequent experiments.

Growth of wheat seedlings was dramatically hindered by the long-term salt stress (≥ 200 mM, data not shown), which complicated measurement of photosynthetic capacity and other experiments. According to Munns (2005), water deficits and ion excess occur sequentially resulting in a two-phase growth response to a long-term salinity in wheat. The 24-d-old seedlings were treated for 24 h and sampled randomly. The plants that received GABA+NaCl exhibited a higher P_N and g_s than those that received NaCl alone (Fig. 2). A P_N and g_s are usually highly sensitive to salt stress (Netondo *et al.* 2004, Jiang *et al.* 2006). However, g_s has also been reported to be resistant to salt stress (Lu *et al.* 2003, Naumann *et al.* 2007). This discrepancy may reflect differences among plant species or environmental conditions. It seems that the inhibition of P_N in plants that did not receive GABA was mostly due to water deficit, which is consistent with a report by Flexas *et al.* (2002).

A reduction of photochemical activity is one of the non-stomatal factors that limit photosynthesis (Souza *et al.* 2004). A ratio of variable to maximum fluorescence (F_v/F_m) is frequently used as indicator of photoinhibition or stress damage to PS II (Calatayud *et al.* 2004). Conflicting reports in the literature suggest that salt stress may not cause changes in F_v/F_m (Lutts *et al.* 1996) and that Chl fluorescence may not be a reliable index of salt tolerance (Dionisio-Sese and Tobita 2000). The constant F_v/F_m ratio observed in our study may indicate that photoinhibition of PS II was not triggered (data not shown). The exogenous GABA considerably ameliorated the decrease in photosynthetic pigments (Fig. 4*A*,*B*). Compared to F_v/F_m, Φ_{PSII} was more sensitive to the salt stress. The decrease in Φ_{PSII} (Fig. 3*A*) implies a lower electron transport to carbon fixation, which would consequently decrease CO_2 assimilation rate (Maxwell *et al.* 2000). The higher Φ_{PSII} in the GABA+NaCl plants confirms an improved photosynthetic performance under the salt stress. The seedlings were mainly subjected to the first phase of the salt stress (Munns 1993) for a short time. The GABA+NaCl plants had a higher WUE under the severe salinity compared to the plants that received NaCl alone (Fig. 3*B*), which is important for salt tolerance (Karaba *et al.* 2007). Our findings regarding the protection of Chl and photosynthetic function are similar to the observations of Nayyar *et al.* (2014) for rice seedlings treated with GABA under heat stress.

Excessive ROS also frequently accumulate under salinity (Asada 2006). Superoxide dismutase and CAT are assumed to be effective antioxidant enzymes in preventing cellular damage (Scandalios 1993). Zhu *et al.* (2004) reported that CAT activity decreases under salinity, whereas SOD activity increases. In our experiments, the SOD and CAT activities decreased under the salt stress but less in the GABA+NaCl groups than in the plants receiving NaCl alone (Fig. 5). This result is consistent with the findings of Smirnoff *et al.* (1989) and Shi *et al.* (2010) who demonstrated that exogenous GABA can reduce ROS accumulation. Salt stress decreases the stability and integrity of the biological membrane. Electrolyte leakage and MDA content are often used to assess stress-induced damage (Smirnoff 1995, Bajji *et al.* 2001). The REC and MDA as indexes of membrane injury were lower in the

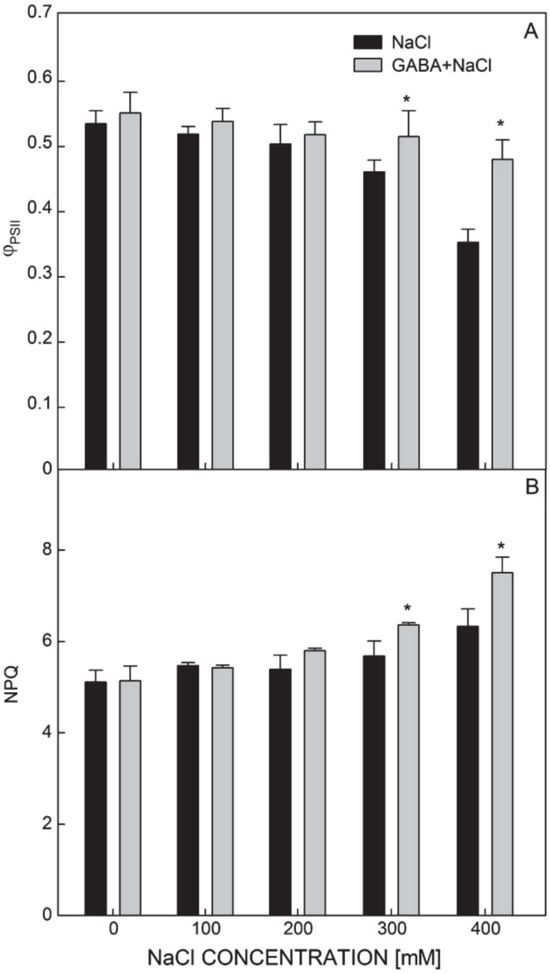

Fig. 3. Effects of 0.5 mM GABA and different NaCl concentrations (0 - 400 mM) on chlorophyll fluorescence parameters measured in dark-adapted leaves. *A* - actual photosystem II efficiency (Φ_{PSII}), *B* - non-photochemical quenching (NPQ). Treatments of 24-d-old seedlings lasted 24 h.Means ± SE, *n* = 3, * - significant differences from plants receiving NaCl alone at $P < 0.05$.

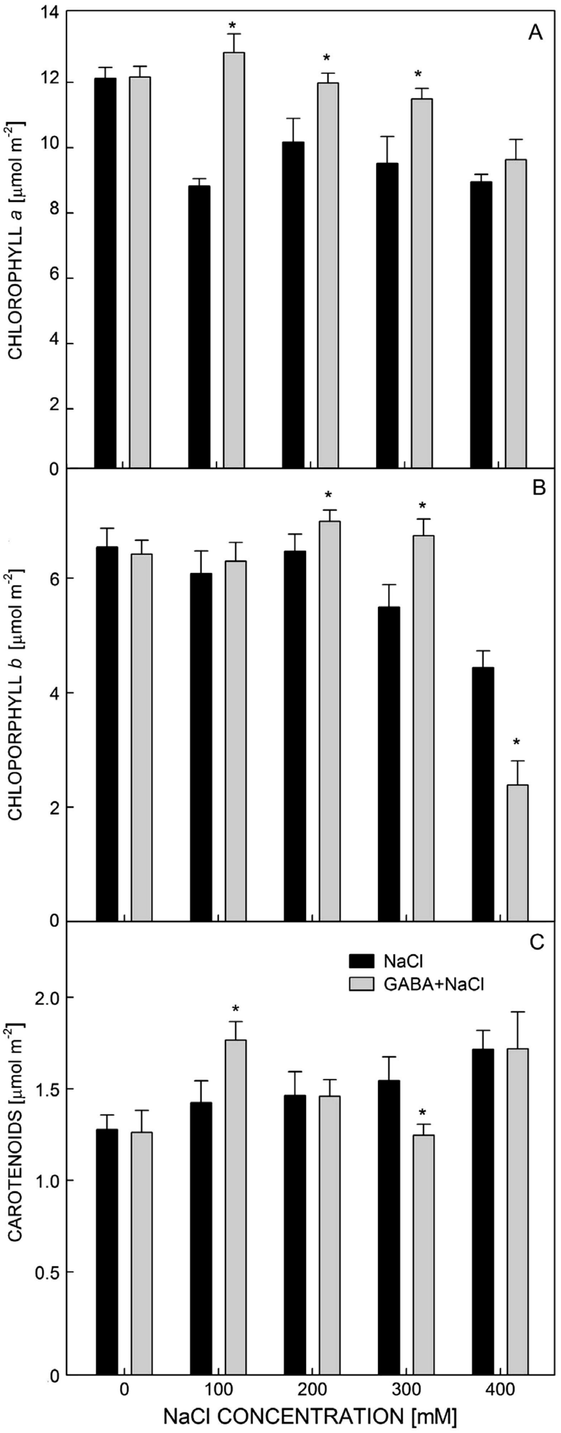

Fig. 4. Effects of 0.5 mM GABA and different NaCl concentrations (0 - 400 mM) on photosynthetic pigments. *A* - Chlorophyll *a*, *B* - chlorophyll *b*, *C* - carotenoids. Treatments of 24-d-old seedlings lasted 24 h. Means ± SE, *n* = 3, * - significant differences from plants receiving NaCl alone at $P < 0.05$.

GABA+NaCl groups than in the plants under NaCl alone (Fig. 6*A*,*B*) in accordance with higher activities of antioxidant enzymes (Fig. 5*A*,*B*). Similar observations were reported for heat-stressed plants by Nayyar *et al.* (2014). Gamma-aminobutyric acid appeared to impart a partial protection to the wheat plants against the salt stress by elevating resistance to oxidative stress.

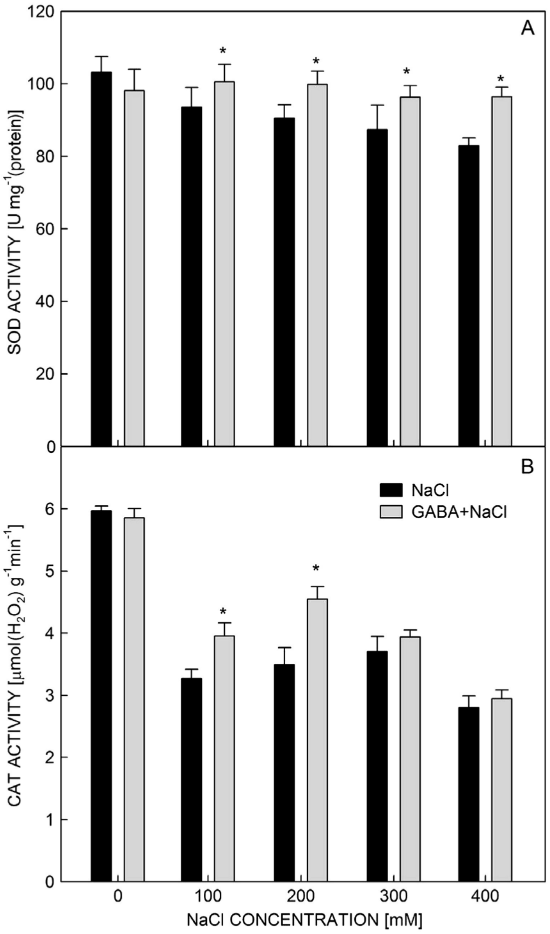

Fig. 5. Effects of 0.5 mM GABA and different NaCl concentrations (0 - 400 mM) on activities of antioxidant enzymes: *A* - SOD, *B* - CAT. Treatments of 24-d-old seedlings lasted 24 h. Means ± SE, *n* = 3, * - significant differences from plants receiving NaCl alone at $P < 0.05$.

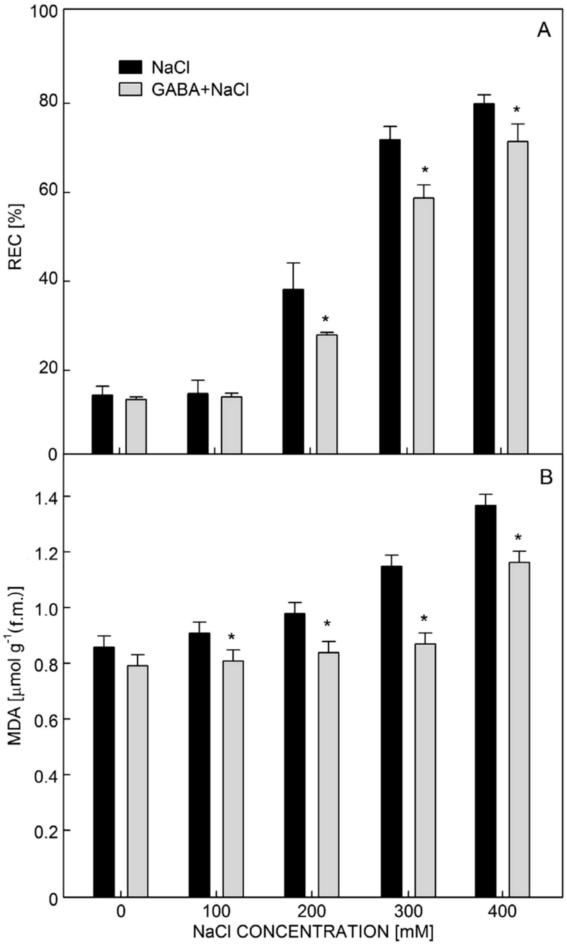

Fig. 6. Effects of 0.5 mM GABA and different NaCl concentrations (0 - 400 mM) on relatice conductivity (REC) and MDA content. *A* - REC, *B* - MDA content. Treatments of 24-d-old seedlings lasted 24 h. Means ± SE, *n* = 3, * - significant differences from plants receiving NaCl alone at $P < 0.05$.

In conclusion, this study demonstrates that the exogenous GABA improved photosynthesis and resistance to the ROS induced stress. Consequently, the growth of the wheat seedlings was promoted. Therefore, the exogenous application of GABA may be a useful method to improve growth and productivity of higher plants under soil salinity.

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