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The role of chitosan priming in induction of GABA shunt pathway during wheat seed germination under salt stress

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Abstract

Soil salinity leads to a reduction in plant growth, germination, relative water content, and production of wheat plants worldwide. Chitosan showed a positive effect on plant growth and development and improved plant stress tolerance. The current study aimed to examine the effect of different chitosan concentrations on the gamma-aminobutyric acid (GABA) shunt pathway in germinating seeds of wheat (*Triticum durum* L.) under salt stress (25 - 200 mM NaCl). We determined the seed germination pattern, seed moisture content, GABA shunt metabolites (GABA, glutamate, and alanine), oxidative damage in terms of malondialdehyde (MDA) accumulation, and the glutamate decarboxylase (*GAD*) mRNA transcription. Pre-treatment of wheat seeds with chitosan improved germination by enhancing germination percentage, seedling length, and seedling fresh and dry masses under salt stress. Data showed an increase in GABA shunt and their metabolites (alanine and glutamate), MDA content, and *GAD* mRNA transcription, and a decrease in germination percentage, seedling length, seedling fresh and dry masses for both untreated and chitosan-treated seeds under salt stress. Our results suggest that the elevation of GABA in chitosan-treated seeds was able to maintain metabolic stability under salt stress. The MDA content increased in chitosan-treated seeds as NaCl concentration increased, however, the increase was slightly lower than the MDA content in untreated seeds which confirmed that chitosan activates *GAD* mRNA expression that leads to activate GABA shunt to involve in the reduction of membrane damage and activation of reactive oxygen species scavenging systems under salt stress. Consequently, this study demonstrated that chitosan significantly enhanced the accumulation of GABA and amino acids metabolism to maintain the C:N balance and improved salt tolerance in wheat seeds during seed germination.

Keywords: chitosan, GABA, salt stress; seed germination, *Triticum durum*, wheat.

Introduction

Plant exposure to stress is the reason why field crops hardly ever reach their yield potential, as evidenced by huge yield gaps when comparing experimental plots with farm data (van Ittersum *et al.* 2013). Wheat (*Triticum durum* L.) is the most important cereal crop grown in temperate regions around the world (Tunio *et al.* 2006, Shewry 2009). Many environmental factors had a negative effect on wheat quality and production worldwide (Rajaram and Van Ginkel 2001). Biotic stress is an additional challenge inducing a strong pressure on plants and adding extra damage through attacks of pathogens or herbivores (Strauss and Zangerl 2002, Maron and Crone

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Abbreviations: GABA - gamma-aminobutyric acid; GAD - glutamate decarboxylase; NADH - nicotinamide adenine dinucleotide; NADPH - nicotinamide adenine dinucleotide phosphate; MDA - malondialdehyde; ROS - reactive oxygen species; TCA - tricarboxylic acid.
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2006, Mordecai 2011). Among several abiotic stresses, drought and salinity are the most serious threats to wheat cultivation globally (Altman 2003). Osmotic stress as an indirect response to salinity caused a decrease in cell water potential in many wheat genotypes that are tolerant and moderately tolerant to salt stress (Sairam *et al.* 2002). Abiotic stresses such as salinity, drought, high or low temperatures, chemical toxicity, and oxidative stress are serious threats to agriculture and cultivation leading to a series of physiological, morphological, molecular, and biochemical changes that adversely affect plant growth and productivity (Wang *et al.* 2003).

Salinity is one of the main factors that affect growth, development, and crop yield in plants (Bano and Fatima 2009). Soil salinity has increased due to poor irrigation practices and the accumulation of organic fertilizers and pesticides (Hahm *et al.* 2017). Salt stress induces osmotic, ionic, and oxidative stress in plants (Yang and Guo 2018). Salinity has multiple physiological impacts on plants including growth inhibition, accelerated development, senescence, and death after prolonged exposure (Zhu 2001). In general, the inhibition of wheat seed germination in response to salt stress might be explained by the reduction of the seed's ability to absorb water, which leads to a decrease in seed osmotic potential and ability to successfully germinate under NaCl treatments (Cramer *et al.* 2007, Jaleel *et al.* 2007, Zhang *et al.* 2010, Al-Quraan *et al*. 2013, Ibrahim 2016). Therefore, in response to salt stress plants have to adjust their physiological and biochemical processes that involved osmotic homeostasis, ion uptake as well as control cellular damage repair (Zhu 2002). Al-Quraan *et al.* (2013) showed that five wheat cultivars exposed to salt stress suffered from reduced germination percentage, increased malondialdehyde (MDA) content, and reduction in chlorophyll *a* and *b* content. Additionally, Al-Quraan *et al.* (2019) observed a decrease in seed germination under salt stress in wheat and barley.

Chitin is the most naturally occurring polysaccharide present in cell walls of fungi, crabs, shrimps, parasitic nematode eggs, insect exoskeletons, and parasitic nematode gut linings (Al-Tawaha *et al.* 2018). Chitosan [(1,4)-linked 2-amino-deoxy-β-D-glucan] is a long-chain polysaccharide polymer obtained by deacetylation of chitin. Chitosan can be used as a preservative coating for fresh fruit because of its stable biochemical properties (Muzzarelli *et al.* 1986). Both chitin and chitosan had been verified as antibacterial, antiviral, and antifungal agents and explored in many agricultural applications because they can elicit a positive response in crops, typically a defense-related response, such as the buildup of bioflavonoids (Al-Tawaha *et al.* 2006, El Hadrami *et al.* 2010).

There are various effects of chitosan on different plant species including promoting plant growth (Guan *et al.* 2009), enhancing the production of secondary metabolites (Sathiyabama and Manikandan 2016), affecting photosynthesis (Zong *et al.* 2017), influencing activities of different critical enzymes such as catalase and ascorbate peroxidase (Lin *et al.* 2005), and improving plant resistance against low-temperature stress (Guan *et al.* 2009). Biochemical and molecular changes were observed in plants nourished with chitosan including callose apposition (Franco and Iriti 2007) and synthesis of alkaloids (Orlita *et al.* 2008). A study conducted by Al-Tawaha and Al-Ghzawi (2013) inferred that priming lentil seeds with chitosan resulted in increasing the germination percentage, hypocotyl length, radicle length, hypocotyl dry mass, and radicle dry mass. Guan *et al.* (2009) found that seeds of maize primed with chitosan improved the length and dry mass of shoots and roots. Similar results were reported by Zhao *et al.* (2005) who indicated that chitosan exhibited elicitor activity in different plant species and induced phytoalexins production in various plant tissues.

Chitosan was shown to enhance seedling growth and plant tolerance to oxidative stress of safflower and sunflower under salt stress (Jabeen and Ahmad 2013). Pretreating *Carum copticum* seeds with chitosan enhanced germination, growth, and increased tolerance to salt stress (Mahdavi and Rahimi 2013). Studies showed that chitosan priming enhanced seedling growth, and seed germination, and increased the activities of antioxidative enzymes of maize plants under salt stress and low-temperature treatment (Guan *et al.* 2009, Al-Tawaha *et al.* 2018).

Gamma-aminobutyric acid (GABA) is a four-carbon non-protein amino acid that is well recognized as an endogenous plant signaling molecule. GABA is known to be accumulated in plant tissues in response to various biotic and abiotic stresses, suggesting its roles range from the involvement in central carbon:nitrogen (C:N) metabolism to function as a signaling molecule during microbe interaction (Roberts 2007, Fait *et al.* 2008). In response to stress, Ca^{2+} content was elevated in plant cells which in turn induced calmodulin (*CaM*) gene expression (Shelp *et al.* 1999, Al-Quraan *et al.* 2010). CaM protein and Ca^{2+} make an active complex that binds to the glutamate decarboxylase (GAD) enzyme and stimulates GABA biosynthesis (Bouché and Fromm 2004, Al-Quraan *et al.* 2011). Typically, the metabolic pathway for GABA synthesis is referred to as the GABA shunt pathway. This pathway started with the decarboxylation of glutamate to produce GABA and CO_2 in the cytosol (Fait *et al.* 2008). Then GABA is transported to mitochondria and by GABA transaminase (GABA-T) in a reversible transamination produced succinic semialdehyde (SAA) that is oxidized to succinate by succinic semialdehyde dehydrogenase (SSADH) (Shelp *et al.* 1999). The resulting succinate is fed into the tricarboxylic acid (TCA) cycle (Hijaz *et al.* 2018). A rapid accumulation of GABA in response to salinity has been demonstrated as a result of increased activity of the GAD enzyme (Renault *et al.* 2010).

Due to its strong effect on plant growth and development, chitosan showed a significant result in the induction of plant responses under various stresses. The present study was performed to investigate the effect of chitosan treatments on the GABA shunt pathway in germinating seeds of wheat (*Triticum durum* L.) under salt stress (characterization of seed germination pattern, seedling growth, GABA shunt metabolites, oxidative damage, and the expression of *GAD* gene).

Materials and methods

Plants and cultivation: Freshly harvested durum wheat seeds (*Triticum durum* L. cv. Um Qais) were obtained from Jordan National Agricultural Research Center, Amman, Jordan. Surface sterilization of the seeds was performed by suspending seeds in 100% bleach (v/v, 6% sodium hypochlorite) for 5 min followed by five times washing with sterile distilled water (Lindsey *et al.* 2017).

The chitosan solutions were prepared by dissolving chitosan (*Sigma*, St. Louis, USA) in 1% acetic acid (*Medex*, Rugby, UK). The solution pH was adjusted to 5.6 using 1 M NaOH solution. Surface sterilized seeds (20 000 seeds) were treated by submerging in 0.01, 0.05, 0.1, 0.3, and 0.5% chitosan, 1% acetic acid, and distilled water (hydro-priming), separately for 24 h at 25°C. After soaking, the seeds were allowed to air dry to return to their original moisture at room temperature for 3 d. Untreated dry seeds were used as a control group.

The sterilized, hydro-primed and chitosan-treated seeds were grown on filter paper in Petri dishes supplemented with different concentrations of sodium chloride (NaCl): 0, 25, 50, 75, 100, and 200 mM (3 mL in each Petri dish), separately. All experiments were conducted in the laboratory by incubating the treated seeds at 25°C for 8 d.

Seed moisture content was measured for three replicates of 20 seeds each immediately after imposing seed treatments with chitosan and after drying the seeds back to their original moisture content. Seed moisture content was measured according to the International Seed Testing Association (ISTA) by calculating the difference in seed fresh mass before and after drying them in an oven at 80°C for 72 h (oven-dry mass). This difference was then divided by seed fresh mass and was expressed as a percentage [%] of the fresh mass.

Seed germinations and seedling growth: Twenty seeds from each chitosan treatment were planted on two filter papers supplemented with 0, 25, 50, 75, 100, and 200 mM NaCl, separately. The seeds were incubated at 25°C for 8 d. Seeds with radicle protrusion were counted on day 8. The effect of NaCl on seed germination was calculated as the germination percentage: G [%] = (number of seeds germinated/total number of seeds planted) × 100 and was compared to the untreated seeds (control group). An average of three replicate plates was used for each treatment. After 8 d post-germination, only germinated seeds were used for further experiments (GABA metabolites extraction, MDA analysis, and *GAD* expression).

Seedling length, fresh mass, and dry mass were determined for each treatment on the 8th day post-germination. The seedling length was measured from the seed emerging radicle to the shoot tip using a ruler. Seedling fresh mass was determined by collecting the seedling sample separately and weighing them directly. Seedling dry mass was determined after oven drying at 70°C of each seedling sample for 72 h.

GABA-metabolite extraction: GABA metabolites were extracted according to Zhang and Bown (1997) with the following modification: 500 mg of germinating seeds (seeds and the emerged seedlings) at 1st, 4th, and 8th day post-germination for each NaCl treatment (0, 25, 50, 75, 100, and 200 mM) separately were grounded with mini pestle and mortar and placed in 1.5-mL microcentrifuge tubes. To each tube, 0.4 mL of methanol was added and the samples were mixed for 10 min. Liquid from the samples was removed by regular evaporation overnight (tubes were kept open to allow methanol evaporation). Then 0.5 mL of 70 mM lanthanum chloride was added to each tube. The tubes were mixed for 15 min and subsequently centrifuged at 10 000× *g* for 5 min. The supernatant was removed to new tubes and mixed with 0.16 mL of 1 M KOH. The tubes were mixed for 10 min and then centrifuged at 10 000× *g* for 5 min. The supernatant containing metabolites was transformed into a new tube and was used for GABA, alanine, and glutamate determination.

GABA content was measured according to Zhang and Bown (1997) with the following modifications: the reaction mixture contained 50 µL of sample extract, 14 µL of 4 mM $NADP^+$, 19 µL of 0.5 M potassium pyrophosphate at pH 8.6, 10 µL of GABASE (2 U μL^{-1}) (GABASE enzyme powder was suspended in 0.1 M potassium pyrophosphate at pH 7.2 containing 12.5% glycerol and 5 mM β-mercaptoethanol) and 10 µL of α-ketoglutarate. Change in absorbance at 340 nm was recorded after 90 min incubation at 25°C using the microplate reader (*Multiskan FC*, *Thermo-Fisher Scientific*, Ratastie, Finland). The content of GABA was determined using the NADPH standard curve (range from 0 to 10 nM).

Alanine content was measured according to Bergmeyer (1983) with the following modifications: the reaction mixture contained 180 µL of 0.05 M Na-carbonate buffer (pH 10), 7 µL of 30 mM β-NAD^+, 50 µL of sample extract, and 5 µL of (0.3 U μL^{-1}) alanine dehydrogenase (*Sigma-Aldrich*, St. Louis, USA) suspension. Change in absorbance at 340 nm after the addition of alanine dehydrogenase was recorded after 60 min incubation at 25°C using the microplate reader (*Multiskan FC*). The content of alanine was determined using NADH standard curve (range from 0 to 5 nM).

Glutamate content was measured according to Bergmeyer (1983) with the following modifications: the deamination reaction mixture contained 180 µL of 0.1 M Tris-HCl (pH 8.3), 8 µL of 7.5 mM β-NAD^+, 50 µL of sample extract, and 5 µL of (0.8 U mL^{-1}) glutamate dehydrogenase suspension (*Sigma-Aldrich*). Change in absorbance at 340 nm after the addition of glutamate dehydrogenase was recorded after 60 min incubation at 25°C using the microplate reader (*Multiskan FC*). The content of glutamate was determined using NADH standard curve (range from 0 to 5 nM).

The content of malondialdehyde (MDA) as a reference for lipid peroxidation caused by reactive oxygen species in germinating seeds: 100 mg tissue was grounded using a mini pestle and mortar, then placed in 1.5-mL microcentrifuge tubes at 1^{st}, 4^{th}, and 8^{th} day post-germination for each NaCl treatment (0, 25, 50, 75, 100, and 200 mM). The MDA assay kit (colorimetric) (*ab118970*, *Abcam*, Waltham, USA) was used according to the manufacturer's instructions. In this kit, lipid peroxidation was determined by the reaction of free MDA (present in the sample) with thiobarbituric acid (TBA) to generate an MDA-TBA adduct that formed a colorimetric (532 nm) product, proportional to the MDA content. The absorbance was measured spectrophotometrically at 532 nm using the microplate reader (*Multiskan FC*). The content of MDA was determined from a standard curve of MDA (range from 0 to 5 nM). An average of three replicate plates was used for each treatment.

***GAD* mRNA expression:** Total RNA from fresh samples was extracted by using the *IQeasy™ plus* plant RNA extraction kit from *Intron Biotechnology* (Seongnam, South Korea) according to the manufacturer's instructions. Total RNA was extracted from germinating seeds on the 8^{th} day post-germination for each NaCl concentration (0, 25, 50, 75, 100, and 200 mM) which were treated by different chitosan concentrations (0.01, 0.05, 0.1, 0.3, and 0.5%) in addition to germinating seeds treated with 1% acetic acid and non-treated germinating seeds as a control group, separately suspended in RNase-free water. RNA concentrations were determined by their absorbance A_{260} using a nanodrop spectrophotometer (*ND-100*, *NanoDrop Technologies*, Wilmington, USA). The integrity of RNA was determined after the separation of RNA on a 1.5% (m/v) agarose gel after electrophoresis and staining with *RedSafe* nucleic acid staining solution and was visualized using a UV trans-illuminator and detection system.

Gene-specific primers for the wheat *GAD* (glutamic acid decarboxylase) gene (forward primer 5'-TGC CGG AGA ACT CGA TCC CCA AG-3') (reverse primer 5'-CGG TTC TGG AGC TCG GTG GTG AC-3') (Mazzucotelli *et al.* 2006) were used for RT-PCR analysis of steady-state mRNA content in wheat seeds. A one-step reverse transcriptase-PCR (RT-PCR) reaction was performed using primer pairs specific for the wheat *GAD* gene (Mazzucotelli *et al.* 2006), *SuperScript™ III* one-step RT-PCR system with platinum® Taq DNA polymerase according to the manufacturer's instructions (*Intron Biotechnology*) as the following: one cycle of reverse transcription reaction (45°C for 30 min) and denaturation of RNA:cDNA hybrid (94°C for 5 min) followed by three step cycling (denaturation (94°C for 30 s), annealing (56°C for 40 s), extension (72°C for 1 min) for 40 cycles then final extension (72°C for 5 min) for one cycle. RT-PCR amplification products were separated on 2% agarose gels and stained with *RedSafe* nucleic acid staining solution. Transcript abundance of *GAD* was calculated according to Al-Quraan *et al.* (2010). Transcript abundance was measured using the amount of fluorescence in the cDNA amplicon. The fluorescence of the 18S RNA (forward primer 5'-CCA CCC ATA GAA TCA AGA AAG AG-3' and reverse primer 5'-GCA AAT TAC CCA ATC CTG AC-3') as an internal control in each tube was used to normalize the abundance of transcribed *GAD* RNA. The background fluorescence was subtracted from the fluorescence value of each DNA band. Data was calculated as Log_2 fold in the abundance of *GAD* gene expression on day 8 post-germination in chitosan treated seeds after each NaCl treatment.

Statistical analyses: The experimental design for all studies was a completely randomized design (CRD). Treatments were replicated three times. All assays were conducted in triplicate. Mean and standard deviation (SD) values were determined for all assay parameters. Normality tests of one-way analysis of variance (*ANOVA*) using the least significant difference (LSD) multiple comparison tests on the means were used for data analysis at a 95% confidence level (P-value < 0.05). Pearson correlation coefficient (r) (Tables 1 Suppl., 2 Suppl., and 3 Suppl.) was used to show the trend between the treatment and the means of measured parameters for each chitosan concentration. All statistical analyses were done using the *SPSS version 25.0* software.

Results and discussion

Effects of chitosan on wheat seed germination and seedling growth under salt stress: Wheat seed germination percentage, seedling length, and seedling fresh and dry masses were recorded under salt stress after each chitosan treatment used in this study. Significant ($P \leq 0.05$) differences in germination percentage and seedling length, fresh and dry masses (Table 1 Suppl.) were observed after chitosan treatments irrespective of different NaCl concentrations (Fig. 1). In general, seed germination percentage (Fig. 1*A*) was negatively affected by salt stress under all chitosan treatments. Treatment with 0.05% chitosan significantly enhanced wheat seed germination percentage even under high NaCl concentrations. In addition, at 0 mM NaCl, 0.01 and 0.1% chitosan-treated seeds showed enhancement in seed germination percentage compared to untreated seeds. Whereas treatments with 0.01, 0.1, 0.3, 0.5% chitosan, distilled water, and 1% acetic acid showed a reduction in seed germination percentage under all NaCl concentrations compared to untreated seeds. Hydro-primed seeds showed the highest seed germination enhancement in response at 0 mM NaCl in addition to all other NaCl treatments.

The seedling length was significantly reduced when NaCl concentration increased among all chitosan treatments, 1% acetic acid in addition to hydro-primed and untreated seeds (Fig. 1*B*). All chitosan treatments showed a decrease in seedling length at 0 mM NaCl compared to untreated seeds. However, at 200 mM NaCl treatment, all chitosan treatments except at 0.1% chitosan showed an increase in seedling length compared to untreated seeds.

Seedling fresh mass (Fig. 1*C*) and dry mass (Fig. 1*D*) were significantly inhibited (Table 1 Suppl.) when NaCl

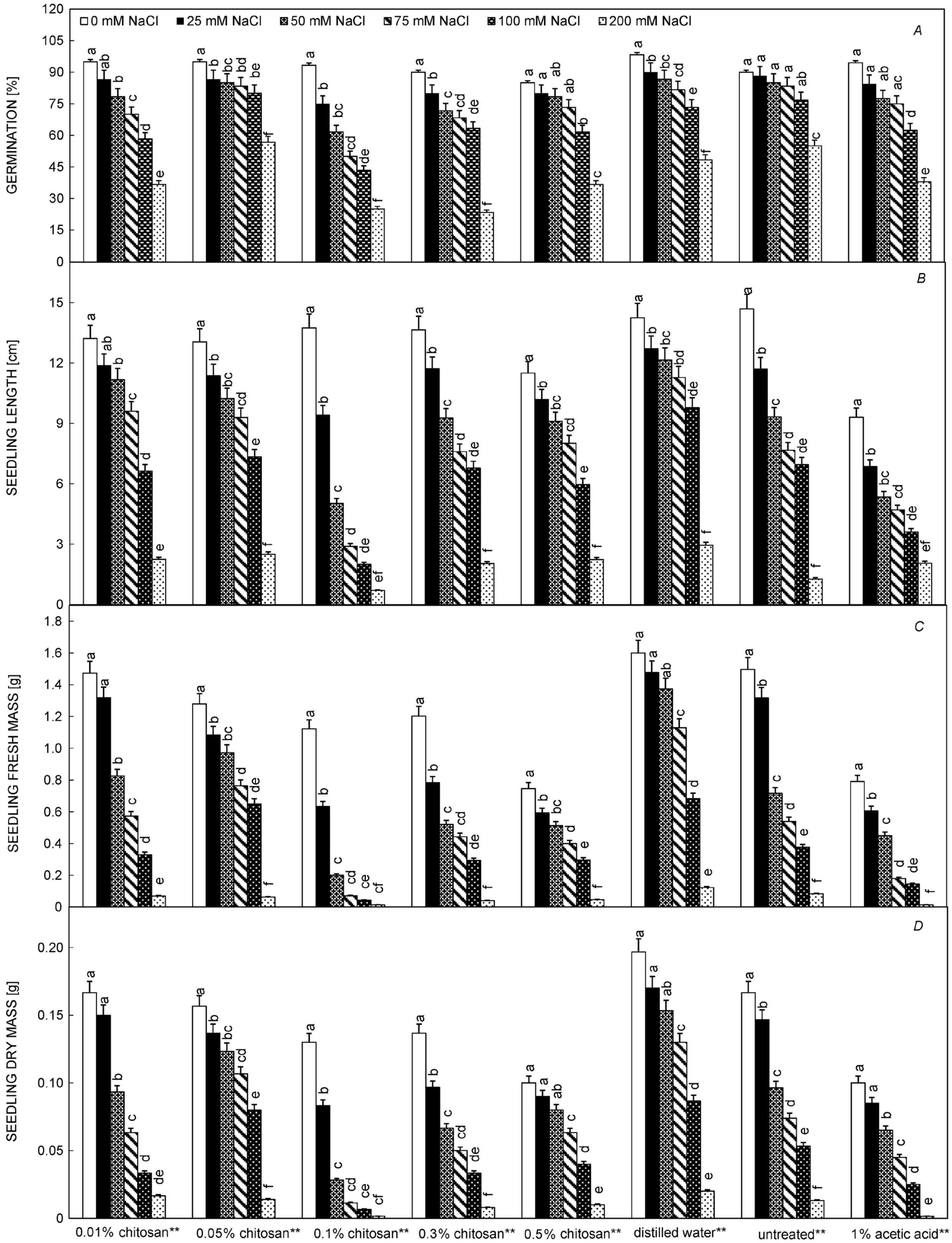

Fig. 1. Germination percentage (*A*), seedling length (*B*), seedling fresh mass (*C*), and seedling dry mass (*D*) in wheat after seed exposed to eight treatments (0.01, 0.05, 0.1, 0.3, 0.5% chitosan, distilled water, untreated, and 1% acetic acid) and supplemented with 0, 25, 50, 75, 100, and 200 mM of NaCl measured at 8th day post-germination. Columns with *different letters* show statistically different ($P \leq 0.05$) values as determined by LSD test. $^{**}P$ value ≤ 0.01.

concentration increased among all chitosan treatments in addition to hydro-primed, 1% acetic acid, and untreated seeds. Seeds treated with 0.05, 0.1, 0.3, and 0.5% chitosan showed a decrease in seedling fresh mass among all NaCl concentrations compared to untreated seeds. Hydro-primed seeds showed higher seedling fresh mass whereas seeds treated with 0.01% chitosan showed no change in seedling fresh mass compared to untreated seeds. Furthermore, seeds treated with 0.1, 0.3, and 0.5% chitosan showed a decrease in seedling dry mass compared to untreated seeds. Seeds treated with 0.05% chitosan and hydro-primed seeds showed enhancement in seedling dry mass among all NaCl concentrations compared to untreated seeds. Whereas, seeds treated with 0.01% chitosan showed no change in seedling dry mass among all NaCl concentrations compared to untreated seeds.

Seed germination and early seedling growth are critical stages for plant survival which are affected by many environmental factors (Hampson and Simpson 1990, Miransari and Smith 2014). Chitosan acts as a natural elicitor that has the potential to control plant stresses. By using chitosan and chitosan nanoparticles, Li *et al.* (2019) found that wheat seed germination percentage, seedling length, adventitious root number, and vegetative biomass were enhanced by treating the seeds with chitosan concentration ranging between 1 to 100 µg mL^{-1}. Guan *et al.* (2009) found that priming maize seeds with 0.5% chitosan had no difference in germination percentage but had the best germination index and the least mean germination time in maize chilling-sensitive inbred lines. In this study, we found that chitosan increased germination percentage under salt stress which is in agreement with results found by Ma *et al.* (2012) who observed that wheat seeds treated with chitosan showed higher shoot and root length than control seedlings under salt stress. Shao *et al.* (2005) suggested that priming maize seeds with chitosan enhanced the vigor of germination, reduced the mean time of germination, and increased shoot height. Similarly, Mahdavi and Rahimi (2013) reported that pretreating seeds with chitosan could enhance the germination percentage, germination rate, and seedling vigor index of *Carum copticum* under salt stress. Our results indicated that seed priming with a low concentration of chitosan could improve the salt tolerance of wheat seeds. Chitosan increased plant growth and yield, and improved physiological processes in radish plants subjected to cadmium stress (Farouk *et al.* 2011). In addition, chitosan treatments enhanced the photosynthetic rate, chlorophyll content, and nutrient uptake in robusta coffee (Van *et al.* 2013). Also, yield components and production quality of four different wheat cultivars were improved by chitosan seed priming and foliar spraying at different growth stages (Wang *et al.* 2015). Plant height and dry mass improved by chitosan treatment under no stress or salt stress in *Plantago ovata* (Mahdavi 2013). In general, salt stress is negatively correlated with wheat seed germination percentage, seedling length, seedling fresh mass, and seedling dry mass (Table 1 Suppl.). Seeds treated with 0.01 and 0.05% chitosan showed an improvement in all measured growth parameters. Our results suggest that treatments with low chitosan concentrations (0.01 and 0.05%) enhanced durum wheat seed germination, seedling length, seedling fresh mass, and seedling dry mass under salt stress.

Seed moisture content in response to chitosan treatment: Seed moisture content was measured immediately after soaking seeds for 24 h with different concentrations of chitosan, 1% acetic acid, and hydro-primed with distilled water. The seed moisture content ranged from 41.25 to 44.29% in all treatments except in untreated seeds (8.56%) (Fig. 2). Data showed a significant ($P \leq 0.05$) difference in seed moisture content between all chitosan-treated seeds, 1% acetic acid, and distilled water in comparison with untreated seeds (Fig. 2).

Studies showed that injury to the root system caused by a reduction in osmotic pressure and a decrease in water content leads to changes in cell wall properties (Garg and Singla 2009, Parvin *et al.* 2019). Seeds coated with chitosan had improved selective permeability characteristics, which can prevent oxygen from entering the seed, prevent loss of CO_2, and maintain high concentrations of CO_2 inside seeds (Furbank *et al.* 2004). Chitosan characteristics for

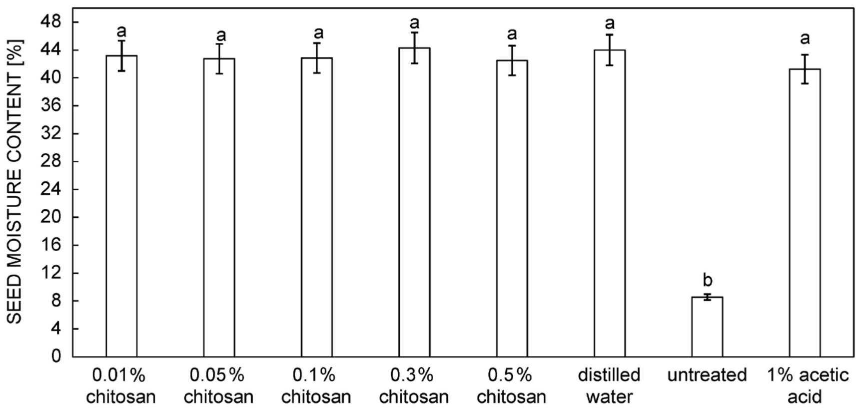

Fig. 2. Wheat seed moisture content immediately after imposing treatments with 0.01, 0.05, 0.1, 0.3, and 0.5% chitosan, distilled water, untreated, and 1% acetic acid, separately for 24 h at 25°C and after drying the treated seeds in oven at 80°C for 72 h. Columns with *different letters* show statistically different ($P \leq 0.05$) values according to LSD test.

semi-permeability suggested its ability to maintain seed moisture content and absorb soil moisture which can aid in promoting seed germination (Sigler and Turco 2002). Seeds treated with different concentrations of chitosan exhibited significant elevation in seed moisture content compared to untreated seeds. These results come in agreement with ALKahtani *et al.* (2020) and Mahdavi and Rahimi (2013) who reported that seeds priming of *Capsicum annuum* and *Carum copticum* with chitosan increased relative water content under salt stress. Our results suggest that chitosan priming promotes plant growth through elevation of water uptake. This data come in agreement with Chookhongkha *et al.* (2012) who found that chitosan coating of *Capsicum frutescens* seeds was an appropriate method for germination enhancement without increasing the seeds moisture content. However, priming of *Capsicum annum* seeds with chitosan (Samarah *et al.* 2020) and nano-chitosan (Samarah *et al.* 2016) induced no change in seeds moisture content after four days post-treatment. Our results indicated that chitosan might assist seeds to absorb more water even when return back into their original moisture and maintain seed moisture content compared to untreated seeds (data not shown).

The effect of chitosan on GABA shunt metabolism under salt stress: GABA shunt pathway is one of the metabolic pathways activated by higher plants in response to salt stress conditions. GABA shunt metabolites of chitosan-treated and untreated durum wheat seeds were measured on the 1st, 4th, and 8th day post-germination at different NaCl concentrations. Our data showed a significant ($P \leq 0.05$) increase with a positive correlation between the abundance of GABA shunt metabolites (GABA, alanine, and glutamate) and NaCl concentrations in all chitosan treatments (Table 2 Suppl.). Seeds treated with 0.01, 0.1, 0.5% chitosan, and 1% acetic acid showed an elevation in GABA content at all NaCl concentrations treatment until the 8th day post-germination compared to untreated seeds (Fig. 3). Wheat seeds treated with 0.3% chitosan and distilled water showed a significant reduction in GABA content under all NaCl treatments until the 8th day post-germination compared to the untreated seeds. Seeds treated with 0.5% chitosan increased GABA content up to 47- and 3-fold at the 1st and the 8th day post-germination among all NaCl concentrations, respectively, and decreased at the 4th day post-germination. Similarly, alanine content was increased up to 6- and 1.5-fold on the 1st and the 8th day post-germination among all NaCl concentrations, respectively, and decreased on the 4th day (Fig. 4). The same trends were observed in glutamate accumulation, where glutamate content was increased up to 2- and 1.6-fold on the 1st and the 8th day of germination under all NaCl treatments, respectively, and decreased at the 4th day (Fig. 5).

The GABA shunt pathway provides a critical crossroad between amino acid metabolism and other organic acid intermediates. It is significantly interconnected with the tricarboxylic acid (TCA) cycle by serving as a source of carbon and nitrogen to supply the C:N deficit in plants under various abiotic stresses (Kishor *et al.* 2005, Batushansky *et al.* 2014). The supply of metabolic intermediates is suggested to be the reason for GABA accumulation, which might be consumed in sugar and amino acid metabolism and used to nourish the TCA cycle under salt stress. The decline in GABA metabolites on the 4th day post-germination under salt stress might occur as a reason for GABA metabolites consumption by durum wheat seeds to deliver a substitute carbon source for mitochondrial respiration (Che-Othman *et al.* 2020). Irrespective of chitosan concentration or any other treatments used in this study, 200 mM NaCl concentration recorded the highest accumulation of GABA metabolites. The elevation in GABA metabolites might occur to overcome the disruption of metabolic stability, imbalance in carbon and nitrogen (C:N) assimilation, and oxidative damage during seed germination under the 200 mM NaCl treatment.

The GABA shunt pathway accumulates GABA in different plant species as an adaptive metabolite in response to various abiotic stresses (Kinnersley and Turano 2000, Fraire-Velázquez *et al.* 2013, Scholz *et al.* 2017, Al-Quraan *et al.* 2019). Kinnersley and Turano (2000) demonstrated that the GABA shunt pathway is an obligatory pathway for proper plant growth in response to salt stress. A significant increase in GABA abundance has been shown in five cultivars of wheat and three cultivars of barley under salt and osmotic stress conditions (Al-Quraan *et al.* 2013, 2019). Furthermore, GABA shunt activation might be associated with various physiological responses, including the control and regulation of cytosolic pH (Fait *et al.* 2008), carbon flux into the TCA cycle, nitrogen metabolism (Li *et al.* 2016, Che-Othman *et al.* 2020), protection against oxidative stress (Zhu *et al.* 2019), and osmoregulation and signaling (Kaplan *et al.* 2004, Akçay *et al.* 2012). Chitosan has been involved in salt stress tolerance by enhancing photosynthetic efficiency, accumulating total sugars and soluble proteins, improving various antioxidant enzyme activities, and increasing cellular metabolic stability (Yang *et al.* 2009, Farouk *et al.* 2011, Jabeen and Ahmad 2013, Tourian *et al.* 2013). Our results came in agreement with Li *et al.* (2017) who confirmed that chitosan treatment enhanced the accumulation and metabolism of amino acids including GABA which could maintain the metabolic balance to improve drought stress resistance in white clover. Overall, our results suggested that chitosan enhances plant response against salt stress by increasing GABA metabolites accumulation. Durum wheat seeds treated with chitosan might tolerate salt treatment by activating GABA shunt pathway through enhanced organic acids metabolism and increasing the capability of seeds to germinate under salt stress.

Oxidative damage in wheat seedlings from seeds treated with chitosan under salt stress: Malondialdehyde (MDA) is a natural organic compound that can be used as a marker to measure lipid peroxidation in response to abiotic stresses such as salt stress. MDA content of treated and untreated durum wheat seeds was measured at the 1st, 4th, and 8th days post-germination at different NaCl concentrations (Fig. 6). Our results showed a significant

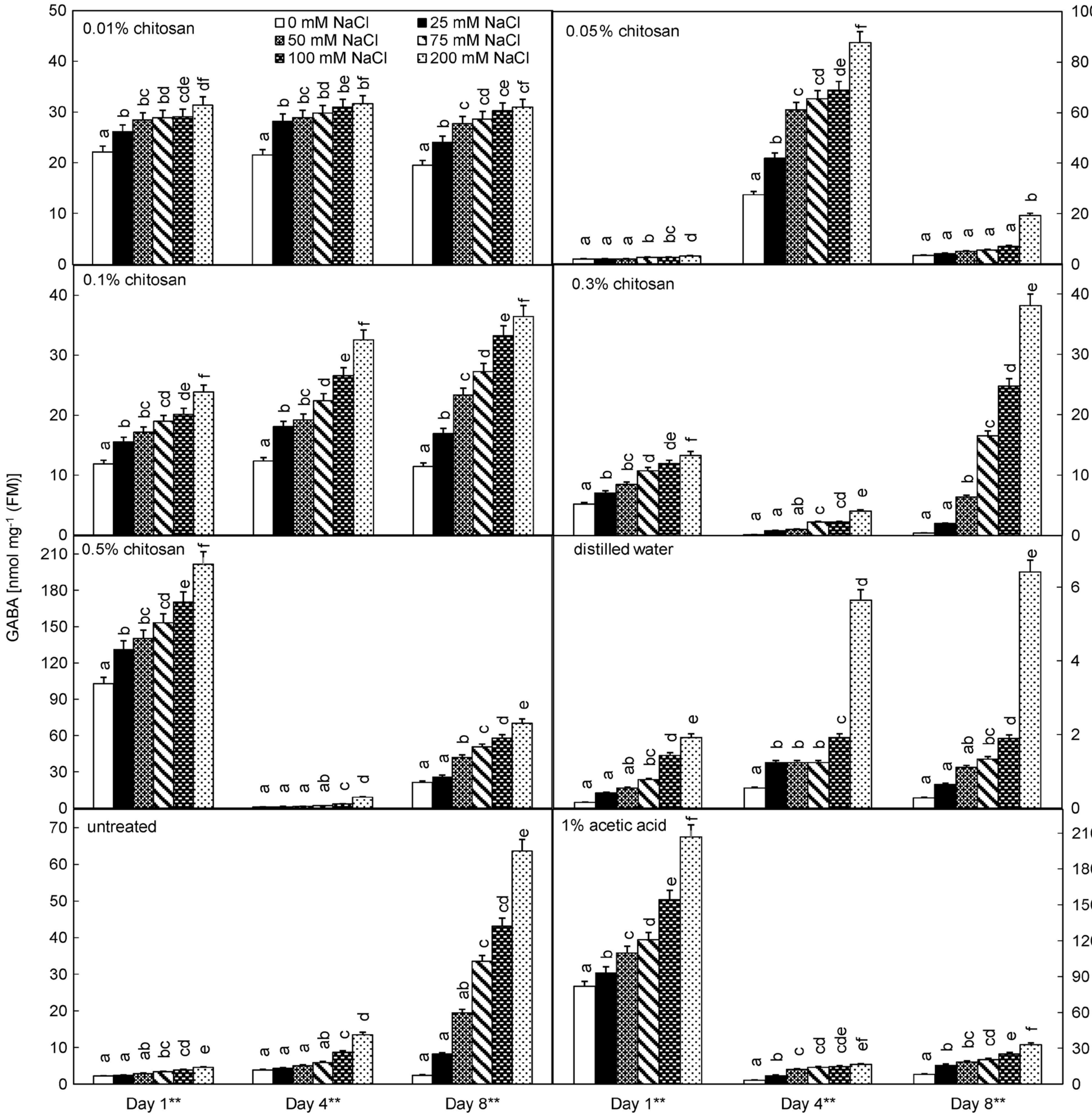

Fig. 3. Content of GABA in wheat seeds exposed to eight treatments (0.01, 0.05, 0.1, 0.3, and 0.5% chitosan, distilled water, untreated, and 1% acetic acid) and supplemented with 0, 25, 50, 75, 100, and 200 mM of NaCl. For each day under different NaCl treatments, columns with *different letters* show statistically different ($P \leq 0.05$) values according to LSD test. $^{**}P$ value ≤ 0.01.

($P \leq 0.5$) increase in MDA content with a positive correlation to increasing NaCl concentration (Table 2 Suppl.). The MDA content in wheat seeds treated with 0.05% chitosan showed a reduction in MDA content on the 1st and 4th day and an elevation on the 8th day post-germination among all NaCl concentrations. On the contrary, wheat seeds treated with distilled water showed an elevation in MDA content on the 1st and 8th days and a reduction in MDA content on the 4th day post-germination under all NaCl concentrations. Furthermore, wheat seeds treated with 0.01, 0.1, 0.3, and 0.5% chitosan and 1% acetic acid showed a reduction in MDA content at the 1st, 4th, and 8th day post-germination under all NaCl treatments compared to untreated seeds. These results indicated that 0.01, 0.1, 0.3, and 0.5% chitosan treatment enhances seeds reactive oxygen species (ROS) scavenging by activating defense mechanisms against salt stress. This outcome might be due to the physiological adaptation of chitosan-treated seeds to NaCl treatments, where chitosan operated as an effective bio-activator to enhance seeds germination under salt stress. Seeds treated with 0.01% chitosan showed the lowest MDA accumulation

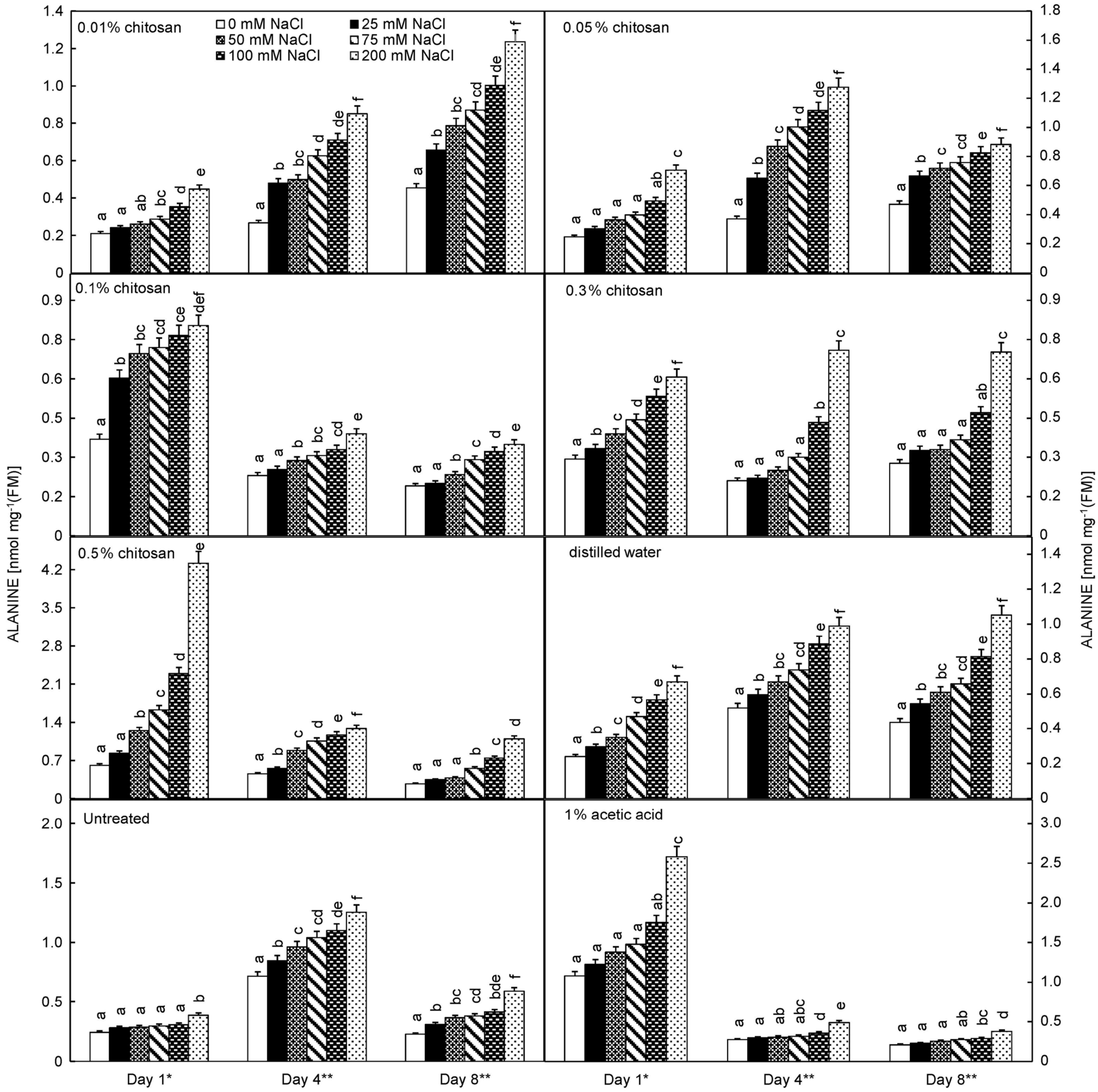

Fig. 4. Content of alanine in wheat seeds exposed to eight treatments (0.01, 0.05, 0.1, 0.3, and 0.5% chitosan, distilled water, untreated, and 1% acetic acid) and supplemented with 0, 25, 50, 75, 100, and 200 mM of NaCl. For each day under different NaCl treatments, columns with *different letters* show statistically different values ($P \leq 0.05$) according to LSD test. *P value ≤ 0.05, **P value ≤ 0.01.

compared to untreated seeds at the 1st, 4th, and 8th day post-germination in response to all NaCl concentrations, which in turn implicated the efficient scavenging of ROS in wheat seeds (Fig. 6).

MDA accumulation occurred in maize seedlings under low-temperature stress due to membrane lipid peroxidation (Guan *et al.* 2009). Al-Quraan *et al.* (2013) found that MDA accumulated in five wheat cultivars under salt and osmotic stress. Our study found that the treatment of seeds with 0.01, 0.1, 0.3, and 0.5% chitosan had a negative effect on MDA content under salt stress. Our study came in agreement with Ma *et al.* (2012) who found that pretreatment of wheat with chitosan reduces MDA content under salt stress. Li *et al.* (2017) suggested that chitosan application may reduce the negative impact of drought stress by increasing the production of stress-protective metabolites. The application of chitosan stimulated plant growth and increased water uptake, thereby enhancing ROS scavenging activities (Guan *et al.* 2009). Bistgani *et al.* (2017) showed that spraying *Thymus daenensis* leaves with chitosan reduced cell membrane damage under drought stress. MDA content was reduced

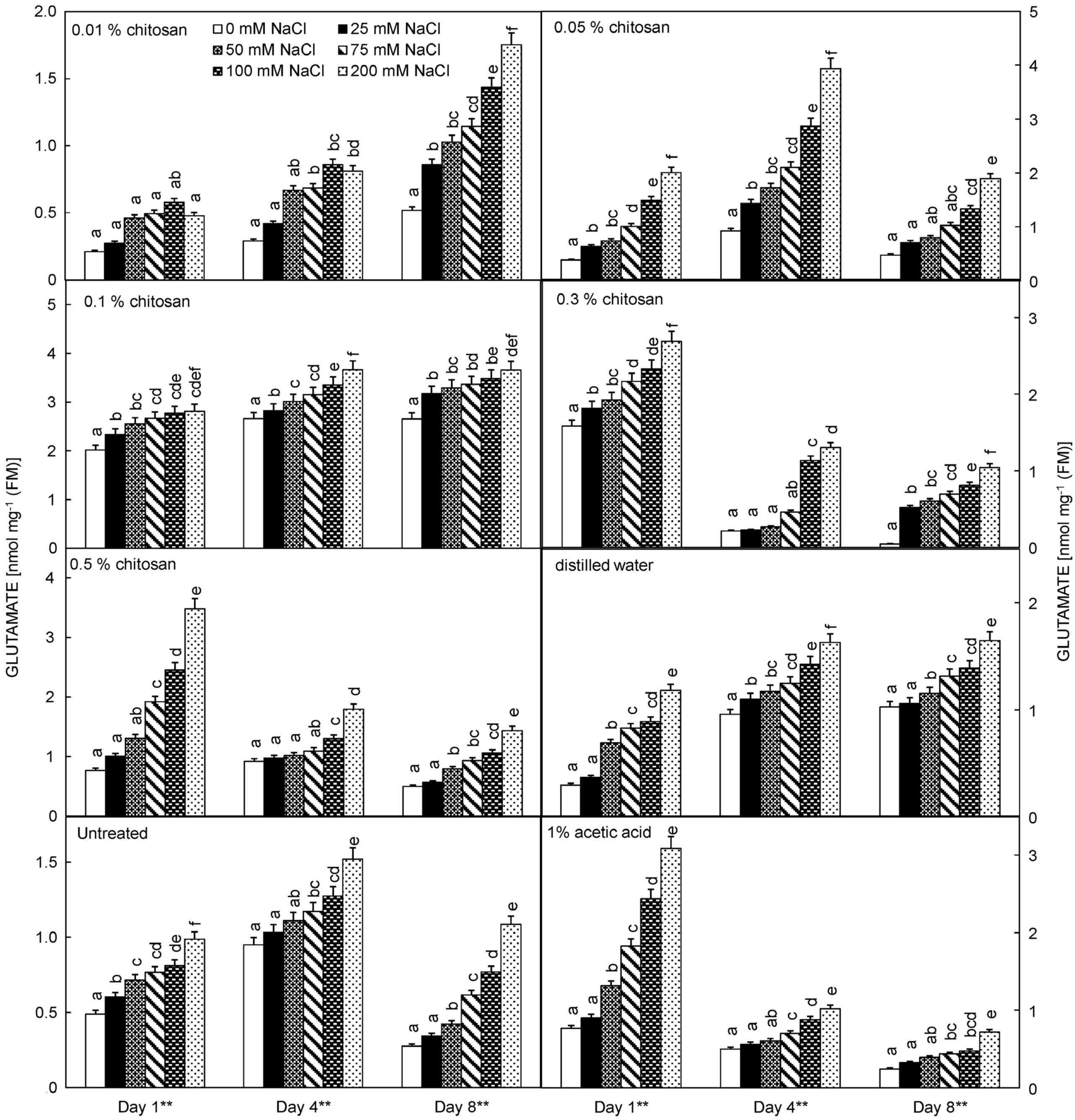

Fig. 5. Content of glutamate in wheat seeds exposed to eight treatments (0.01, 0.05, 0.1, 0.3, and 0.5% chitosan, distilled water, untreated, and 1% acetic acid) and supplemented with 0, 25, 50, 75, 100, and 200 mM of NaCl. For each day under different NaCl treatments, columns with *different letters* show statistically different ($P \leq 0.05$) values according to LSD test. $^{**}P$ value ≤ 0.01.

in rice seedlings treated with chitosan under osmotic stress (Pongprayoon *et al.* 2013). The same results were reported in safflower and sunflower where MDA content was decreased by chitosan treatment under salt stress (Jabeen and Ahmad 2013). Reduced MDA accumulation might be due to the induction of effective ROS scavenging under the 0.01, 0.1, 0.3, and 0.5% chitosan pretreatments. This result showed that the pretreatment of durum wheat seeds with chitosan had a protective effect on salt-induced membrane damage. Consequently, the activation of the ROS scavenging system is an essential component in modulating chitosan reactions for plant growth stimulation under salt stress during the seed germination stage.

The effect of chitosan on glutamate decarboxylase (*GAD*) mRNA transcript abundance in response to salt stress: GAD is the enzyme that catalyzes the decarboxylation of glutamate to form GABA and CO_2

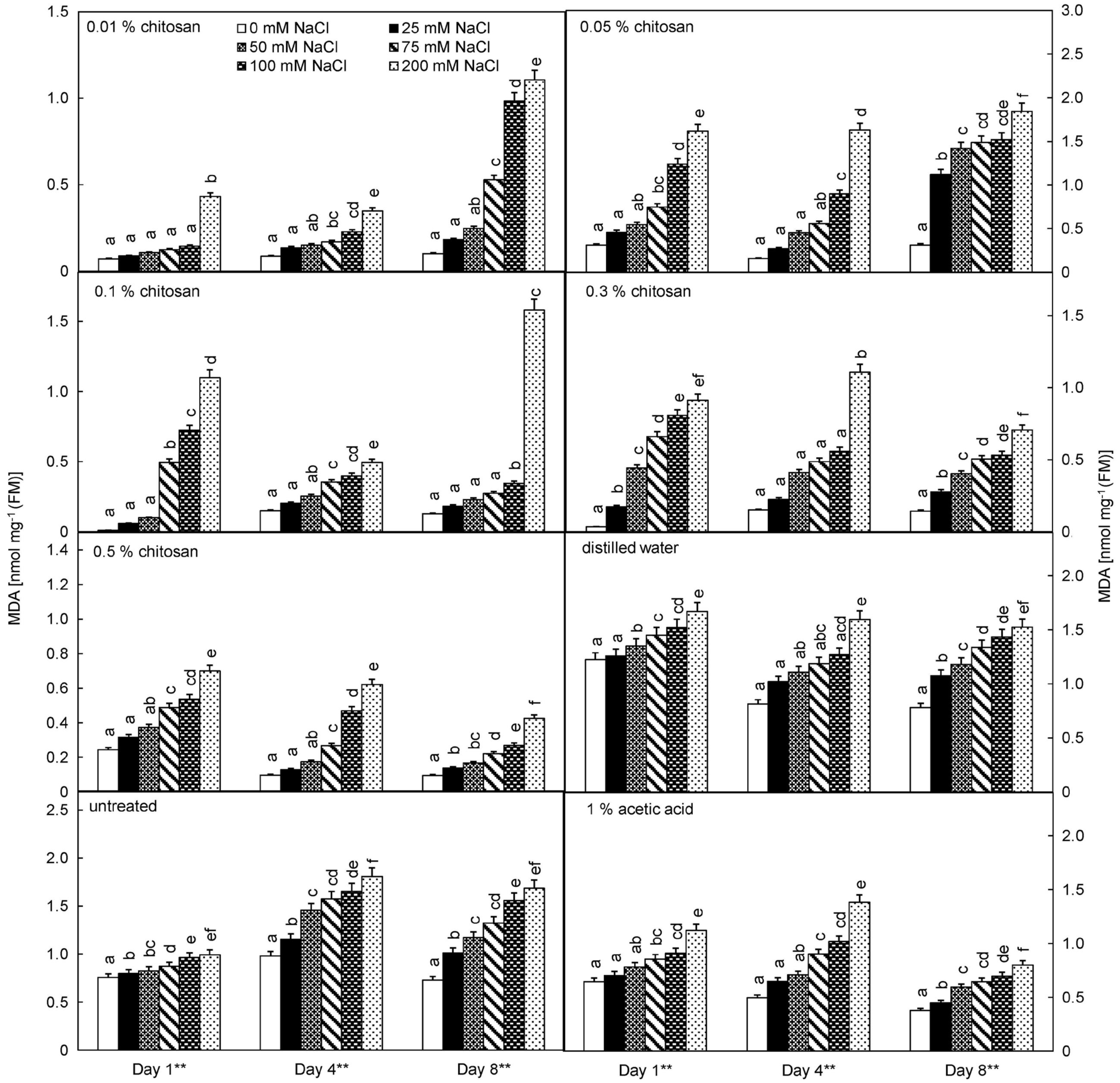

Fig. 6. Content of malondialdehyde (MDA) in wheat seeds exposed to eight treatments (0.01, 0.05, 0.1, 0.3, and 0.5% chitosan, distilled water, untreated, and 1% acetic acid) and supplemented with 0, 25, 50, 75, 100, and 200 mM of NaCl. For each day under different NaCl treatments, columns with *different letters* show statistically different ($P \leq 0.05$) values according to LSD test. $^{**}P$ value ≤ 0.01.

(Shelp *et al.* 2012). *GAD* mRNA transcript abundance of chitosan treated and untreated durum wheat seeds were analyzed on the 8th day post-germination at different NaCl concentrations (Fig. 7). Our data showed a significant ($P \leq 0.05$) increase in the abundance of *GAD* mRNA transcripts with the increase of NaCl concentration under all chitosan treatments. Seeds treated with 0.3% chitosan showed an elevation in *GAD* mRNA transcription at 50, 75, 100, and 200 mM NaCl treatments compared to untreated seeds. Whereas seeds treated with the other chitosan treatments in addition to distilled water and 1% acetic acid treatment showed a decrease in *GAD* mRNA transcription compared to untreated seeds. The elevated *GAD* mRNA transcription in 0.3% chitosan-treated seeds suggests the vital need for GABA shunt metabolism that is mediated by the high production of GAD protein and its enzymatic activity to convert glutamate into GABA under salt stress. Seeds treated with 0.3% chitosan showed reduced content of GABA and GABA metabolites despite the increase of *GAD* mRNA expression in wheat seeds. This outcome is explained by increased GABA shunt pathway metabolites consumption through high production and activation of GAD enzyme leading to the influx of more GABA into the mitochondrial matrix. GABA is then consumed by GABA

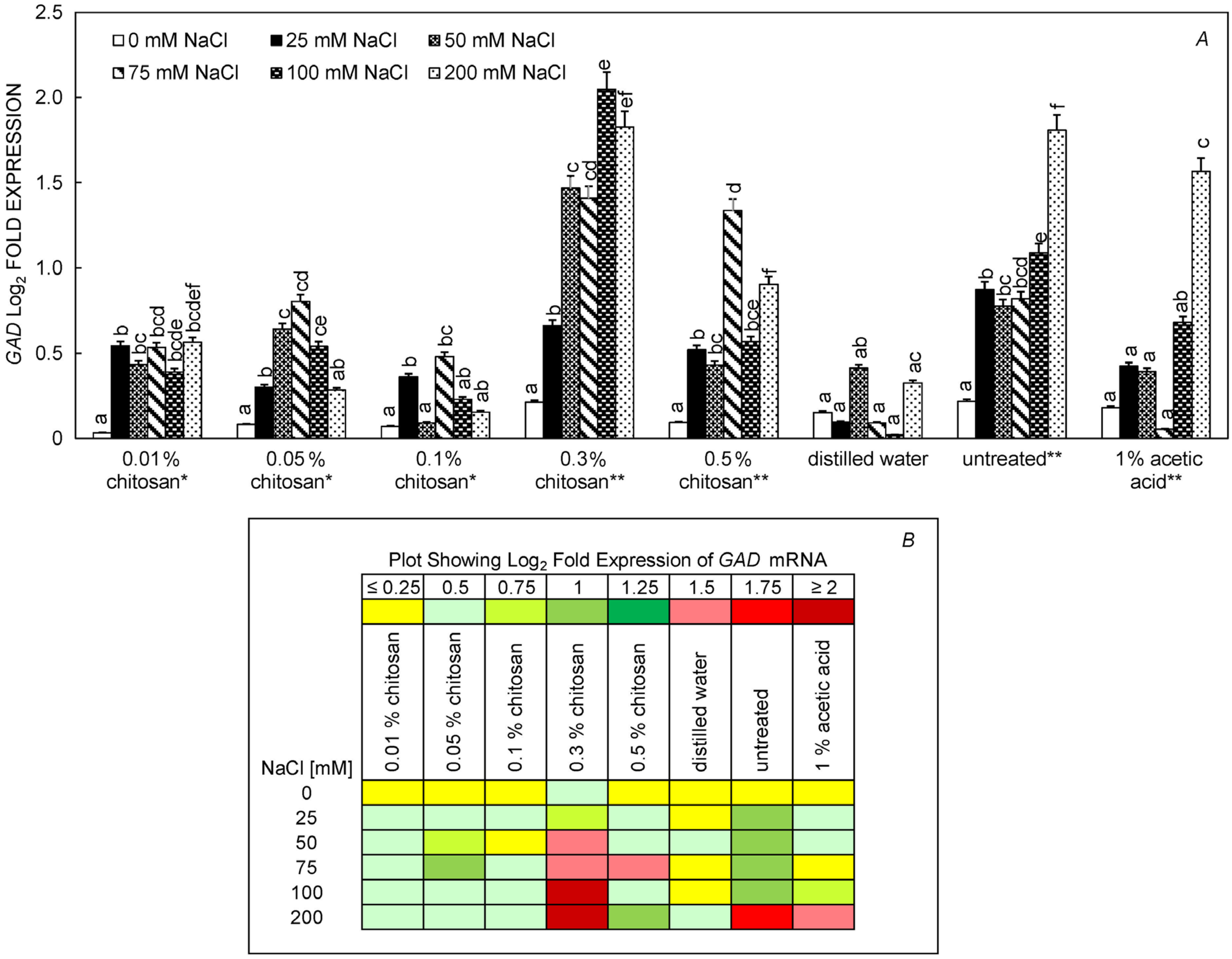

Fig. 7. Log$_2$ fold expression of *GAD* (*A*) and color plot of *GAD* expression (*B*) in wheat seeds exposed to eight treatments (0.01, 0.05, 0.1, 0.3, and 0.5% chitosan, distilled water, untreated, and 1% acetic acid) and supplemented with 0, 25, 50, 75, 100, and 200 mM of NaCl at the 8th day post-germination. For each seed treatment under different NaCl treatments, columns with *different letters* show statistically different ($P \leq 0.05$) values according to LSD test. **P* value ≤ 0.05, ***P* value ≤ 0.01.

transaminase (GABA-TA) and succinic semialdehyde dehydrogenase (SSADH) to form succinate that feeds the TCA cycle and critically participates in saccharide and protein metabolism during seed germination and supports mitochondrial respiration under salt stress in wheat (Che-Othman *et al.* 2020).

Previous studies reported that *GAD* expression increased with several environmental conditions such as heat shock in cowpea (Mayer *et al.* 1990), low pH in tobacco (Bown *et al.* 2006), salt stress in wheat and barley (Al-Quraan *et al.* 2013, 2019) and chlorsulfuron herbicide treatment in lentil (Al-Quraan *et al.* 2015) which led to an increase in GABA content. Other studies reported that *GAD* expression was increased under cadmium stress in different tomato species (Chaffei *et al.* 2004, Hédiji *et al.* 2010) which increased GABA production. Our study suggested that chitosan treatment could significantly enhance *GAD* expression, GAD activity, and GABA abundance under salt stress. Similarly, Geng *et al.* (2020) showed that chitosan treatment induced sucrose, GABA, and polyamines accumulation to enhance salt tolerance in *Agrostis stolonifera*. Chitosan as a polycationic polymer could elicit cellular changes including the transport of Ca^{2+} ions (Yin *et al.* 2010). During GABA shunt activation in plants, the GAD enzyme is activated by binding to the Ca^{2+}/CaM complex that resulted in GABA accumulation under abiotic stress (Shelp *et al.* 1999, Bouché and Fromm 2004, Ranty *et al.* 2006). Furthermore, chitosan could activate several genes and increase the production of proteins and phenolic compounds through the phenylpropanoid pathway which increased the plant tolerance against pathogen infection and stressful conditions (Hadwiger *et al.* 1986). Hernández-Hernández *et al.* (2018) showed that application of chitosan-polyvinyl alcohol hydrogels (Cs-PVA) and copper nanoparticles (Cu-NPs) promoted the expression of jasmonic acid and the superoxide dismutase genes in tomato under salt stress. Our study demonstrated that chitosan significantly enhanced the accumulation of GABA and amino acids metabolism to maintain the C:N balance and improve salt tolerance in durum wheat seeds during germination.

In conclusion, pre-treatment of durum wheat seeds with chitosan under salt stress improved seed germination by enhanced germination percentage, seedling length, and seedling fresh and dry masses. Data also showed that seeds priming with low concentrations of chitosan could significantly improve the salt tolerance of wheat under all NaCl concentrations. In addition to hydro-priming with distilled water, seeds treated with chitosan increased seeds' moisture content and promoted seed germination through elevation of water uptake and adjusting the seed osmotic potential. The improvement by chitosan treatment was associated with the enhancement of GABA, glutamate, and alanine accumulation under salt stress. Under all chitosan treatments used in this study, the GABA, alanine, and glutamate content was negatively correlated with germination percentage, seedling length, and seedling fresh and dry masses in response to all salt treatments (Table 3 Suppl.). Chitosan priming enhanced wheat seed tolerance to salt stress by activating the GABA shunt pathway and increasing the capability of seeds to germinate under salt stress by maintaining cell metabolic stability. A significant increase in MDA content in response to increasing NaCl concentration was recorded. Chitosan priming of wheat seeds had a protective effect on salt-induced membrane damage through enhanced ROS scavenging abilities. Chitosan treatment significantly enhanced *GAD* expression and GABA abundance under salt stress. *GAD* expression in seeds treated with 0.3% chitosan was high which suggests the need for increased activity of GAD-mediated conversion of glutamate to GABA during seed germination under salt stress.

References

Akçay N., Bor M., Karabudak T. *et al*.: Contribution of gamma amino butyric acid (GABA) to salt stress responses of *Nicotiana sylvestris CMSII* mutant and wild type plants. - J. Plant Physiol. **169**: 452-458, 2012.

ALKahtani M.D.F., Attia K.A., Hafez Y.M. *et al*.: Chlorophyll fluorescence parameters and antioxidant defense system can display salt tolerance of salt acclimated sweet pepper plants treated with chitosan and plant growth promoting rhizobacteria. - Agronomy **10**: 1180, 2020.

Al-Quraan N.A., Al-Ajlouni Z.I., Obedat D.I.: The GABA shunt pathway in germinating seeds of wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) under salt stress. - Seed Sci. Res. **29**: 250-260, 2019.

Al-Quraan N.A., Ghunaim A.I., Alkhatib R.Q.: The influence of chlorsulfuron herbicide on GABA metabolism and oxidative damage in lentil (*Lens culinaris* Medik) and wheat (*Triticum aestivum* L.) seedlings. - Acta Physiol. Plant. **37**: 227, 2015.

Al-Quraan N.A., Locy R.D., Singh N.K.: Expression of calmodulin genes in wild type and calmodulin mutants of *Arabidopsis thaliana* under heat stress. - Plant Physiol. Biochem. **48**: 697-702, 2010.

Al-Quraan N.A., Locy R.D., Singh N.K.: Implications of paraquat and hydrogen peroxide-induced oxidative stress treatments on the GABA shunt pathway in *Arabidopsis thaliana* calmodulin mutants. - Plant Biotechnol. Rep. **5**: 225-234, 2011.

Al-Quraan N.A., Sartawe F.A., Qaryouti M.M.: Characterization of γ-aminobutyric acid metabolism and oxidative damage in wheat (*Triticum aestivum* L.) seedlings under salt and osmotic stress. - J. Plant Physiol. **170**: 1003-1009, 2013.

Al-Tawaha A.M., Seguin P., Smith D.L., Beaulieu C.: Foliar application of elicitors alters isoflavone concentrations and other seed characteristics of field-grown soybean. - Can. J. Plant Sci. **86**: 677-684, 2006.

Al-Tawaha A.R., Turk M.A., Al-Tawaha A.R.M. *et al.*: Using chitosan to improve growth of maize cultivars under salinity conditions. - Bulg. J. Agric. Sci. **24**: 437-442, 2018.

Al-Tawaha A.R.M., Al-Ghzawi A.L.A.: Effect of chitosan coating on seed germination and salt tolerance of lentil (*Lens culinaris* L.). - Res. Crop. **14**: 489-491, 2013.

Altman A.: From plant tissue culture to biotechnology: scientific revolutions, abiotic stress tolerance, and forestry. - In Vitro Cell. Dev.-Pl. **39**: 75-84, 2003.

Bano A., Fatima M.: Salt tolerance in *Zea mays* (L). following inoculation with *Rhizobium* and *Pseudomonas*. - Biol. Fert. Soils **45**: 405-413, 2009.

Batushansky A., Kirma M., Grillich N. *et al.*: Combined transcriptomics and metabolomics of *Arabidopsis thaliana* seedlings exposed to exogenous GABA suggest its role in plants is predominantly metabolic. - Mol. Plant **7**: 1065-1068, 2014.

Bergmeyer H.U.: Methods of Enzymatic Analysis. 3rd Edition. Vol. I. Pp. 574. Verlag Chemie, Weinheim 1983.

Bistgani Z.E., Siadat S.A., Bakhshandeh A. *et al.*: Interactive effects of drought stress and chitosan application on physiological characteristics and essential oil yield of *Thymus daenensis* Celak. - Crop J. **5**: 407-415, 2017.

Bouché N., Fromm H.: GABA in plants: just a metabolite? - Trends Plant Sci. **9**: 110-115, 2004.

Bown A.W., MacGregor K.B., Shelp B.J.: Gamma-aminobutyrate: defense against invertebrate pests? - Trends Plant Sci. **11**: 424-427, 2006.

Chaffei C., Pageau K., Suzuki A. *et al.*: Cadmium toxicity induced changes in nitrogen management in *Lycopersicon esculentum* leading to a metabolic safeguard through an amino acid storage strategy. - Plant Cell Physiol. **45**: 1681-1693, 2004.

Che-Othman M.H., Jacoby R.P., Millar A.H., Taylor N.L.: Wheat mitochondrial respiration shifts from the tricarboxylic acid cycle to the GABA shunt under salt stress. - New Phytol. **225**: 1166-1180, 2020.

Chookhongkha N., Sopondilok T., Photchanachai S.: Effect of chitosan and chitosan nanoparticles on fungal growth and chilli seed quality. - Acta Hortic. **973**: 231-237, 2012.

Cramer G.R., Ergül A., Grimplet J. *et al.*: Water and salinity stress in grapevines: early and late changes in transcript and metabolite profiles. - Funct. Integr. Genomic. **7**: 111-134, 2007.

El Hadrami A., Adam L.R., El Hadrami I., Daayf F.: Chitosan in plant protection. - Mar. Drugs **8**: 968-987, 2010.

Fait A., Fromm H., Walter D. *et al.*: Highway or byway: the metabolic role of the GABA shunt in plants. - Trends Plant Sci. **13**: 14-19, 2008.

Farouk S., Mosa A.A., Taha A.A. *et al.*: Protective effect of humic acid and chitosan on radish (*Raphanus sativus* L. var. *sativus*) plants subjected to cadmium stress. - J. Stress Physiol. Biochem. **7**: 99-116, 2011.

Fraire-Velázquez S., Balderas-Hernández V.E.: Abiotic stress in plants and metabolic responses. - In: Vahdati K., Leslie C. (ed.): Abiotic Stress - Plant Responses and Applications in

Agriculture. Pp. 25-48. InTech Open, New York 2013.
Franco F., Iriti M.: Callose synthesis as a tool to screen chitosan efficacy in inducing plant resistance to pathogens. - Caryologia **60**: 121-124, 2007.
Furbank R.T., White R., Palta J.A., Turner N.C.: Internal recycling of respiratory CO_2 in pods of chickpea (*Cicer arietinum* L.): the role of pod wall, seed coat, and embryo. - J. Exp. Bot. **55**: 1687-1696, 2004.
Garg N., Singla R.: Variability in the response of chickpea cultivars to short-term salinity, in terms of water retention capacity, membrane permeability, and osmo-protection. - Turk. J. Agric. For. **33**: 57-63, 2009.
Geng W., Li Z., Hassan M.J., Peng Y.: Chitosan regulates metabolic balance, polyamine accumulation, and Na^+ transport contributing to salt tolerance in creeping bentgrass. - BMC Plant Biol. **20**: 506, 2020.
Guan Y.J., Hu J., Wang X.J., Shao C.X.: Seed priming with chitosan improves maize germination and seedling growth in relation to physiological changes under low temperature stress. - J. Zhejiang Univ. Sci. B **10**: 427-433, 2009.
Hadwiger L., Kendra D., Fristensky B., Wagoner W.: Chitosan both activates genes in plants and inhibits RNA synthesis in fungi. - In: Muzzarelli R., Jeuniaux C., Gooday G.W. (ed.): Chitin in Nature and Technology. Pp. 209-214. Springer, Boston 1986.
Hahm M.S., Son J.S., Hwang Y.J. *et al.*: Alleviation of salt stress in pepper (*Capsicum annum* L.) plants by plant growth-promoting rhizobacteria. - J. Microbiol. Biotechnol. **27**: 1790-1797, 2017.
Hampson C.R., Simpson G.M.: Effects of temperature, salt, and osmotic potential on early growth of wheat (*Triticum aestivum*). I. Germination. - Can. J. Bot. **68**: 524-528, 1990.
Hédiji H., Djebali W., Cabasson C. *et al.*: Effects of long-term cadmium exposure on growth and metabolomic profile of tomato plants. - Ecotox. Environ. Safe. **73**: 1965-1974, 2010.
Hernández-Hernández H., Juárez-Maldonado A., Benavides-Mendoza A. *et al.*: Chitosan-PVA and copper nanoparticles improve growth and overexpress the SOD and JA genes in tomato plants under salt stress. - Agronomy **8**: 175, 2018.
Hijaz F., Nehela Y., Killiny N.: Application of gamma-aminobutyric acid increased the level of phytohormones in *Citrus sinensis*. - Planta **248**: 909-918, 2018.
Ibrahim E.A.: Seed priming to alleviate salinity stress in germinating seeds. - J. Plant Physiol. **192**: 38-46, 2016.
Jabeen N., Ahmad R.: The activity of antioxidant enzymes in response to salt stress in safflower (*Carthamus tinctorius* L.) and sunflower (*Helianthus annuus* L.) seedlings raised from seed treated with chitosan. - J. Sci. Food Agr. **93**: 1699-1705, 2013.
Jaleel C.A., Gopi R., Sankar B. *et al.*: Studies on germination, seedling vigour, lipid peroxidation and proline metabolism in *Catharanthus roseus* seedlings under salt stress. - S. Afr. J. Bot. **73**: 190-195, 2007.
Kaplan F., Kopka J., Haskell D.W. *et al.*: Exploring the temperature-stress metabolome of *Arabidopsis*. - Plant Physiol. **136**: 4159-4168, 2004.
Kinnersley A.M., Turano F.J.: Gamma aminobutyric acid (GABA) and plant responses to stress. - Crit. Rev. Plant Sci. **19**: 479-509, 2000.
Kishor P.B.K., Sangam S., Amrutha R. *et al.*: Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. - Curr. Sci. **88**: 424-438, 2005.
Li R., He J., Xie H. *et al.*: Effects of chitosan nanoparticles on seed germination and seedling growth of wheat (*Triticum aestivum* L.). - Int. J. Biol. Macromol. **126**: 91-100, 2019.
Li Z., Yu J., Peng Y. *et al.*: Metabolic pathways regulated by γ-aminobutyric acid (GABA) contributing to heat tolerance in creeping bentgrass (*Agrostis stolonifera*). - Sci. Rep.-UK **6**: 30338, 2016.
Li Z., Zhang Y., Zhang X. *et al.*: Metabolic pathways regulated by chitosan contributing to drought resistance in white clover. - J. Proteome Res. **16**: 3039-3052, 2017.
Lin W., Hu X., Zhang W. *et al.*: Hydrogen peroxide mediates defence responses induced by chitosans of different molecular weights in rice. - J. Plant Physiol. **162**: 937-944, 2005.
Lindsey III B.E., Rivero L., Calhoun C.S. *et al.*: Standardized method for high-throughput sterilization of *Arabidopsis* seeds. - J. Vis. Exp. **128**: e56587, 2017.
Ma L., Li Y., Yu C. *et al.*: Alleviation of exogenous oligochitosan on wheat seedlings growth under salt stress. - Protoplasma **249**: 393-399, 2012.
Mahdavi B.: Seed germination and growth responses of isabgol (*Plantago ovata* Forsk) to chitosan and salinity. - Int. J. Agric. Crop Sci. **5**: 1084-1088, 2013.
Mahdavi B., Rahimi A.: Seed priming with chitosan improves the germination and growth performance of ajowan (*Carum copticum*) under salt stress. - Eurasia J. Biosci. **7**: 69-76, 2013.
Maron J.L., Crone E.: Herbivory: effects on plant abundance, distribution and population growth. - Philos. T. Roy. Soc. B **273**: 2575-2584, 2006.
Mayer R.R., Cherry J.H., Rhodes D.: Effects of heat shock on amino acid metabolism of cowpea cells. - Plant Physiol. **94**: 796-810, 1990.
Mazzucotelli E., Tartari A., Cattivelli L., Forlani G.: Metabolism of γ-aminobutyric acid during cold acclimation and freezing and its relationship to frost tolerance in barley and wheat. - J. Exp. Bot. **57**: 3755-3766, 2006.
Miransari M., Smith D.L.: Plant hormones and seed germination. - Environ. Exp. Bot. **99**: 110-121, 2014.
Mordecai E.A.: Pathogen impacts on plant communities: unifying theory, concepts, and empirical work. - Ecol. Monogr. **81**: 429-441, 2011.
Muzzarelli R.A.A., Aiba S., Fujiwara Y. *et al.*: Filmogenic properties of chitin/chitosan. - In: Muzzarelli R., Jeuniaux C., Gooday G.W. (ed.): Chitin in Nature and Technology. Pp. 389-402. Springer, Boston 1986.
Orlita A., Sidwa-Gorycka M., Paszkiewicz M. *et al.*: Application of chitin and chitosan as elicitors of coumarins and furoquinolone alkaloids in *Ruta graveolens* L. (common rue). - Biotechnol. Appl. Biochem. **51**: 91-96, 2008.
Parvin K., Hasanuzzaman M., Bhuyan M.H.M.B. *et al.*: Comparative physiological and biochemical changes in tomato (*Solanum lycopersicum* L.) under salt stress and recovery: role of antioxidant defense and glyoxalase systems. - Antioxidants **8**: 350, 2019.
Pongprayoon W., Roytrakul S., Pichayangkura R., Chadchawan S.: The role of hydrogen peroxide in chitosan-induced resistance to osmotic stress in rice (*Oryza sativa* L.). - Plant Growth Regul. **70**: 159-173, 2013.
Rajaram S., Van Ginkel M.: Mexico, 50 years of international wheat breeding. - In: Bonjean A.P., Angus W.J. (ed.): The World Wheat Book: A History of Wheat Breeding. Pp. 579-604. Lavoisier Publishing, Paris 2001.
Ranty B., Aldon D., Galaud J.-P.: Plant calmodulins and calmodulin-related proteins: multifaceted relays to decode calcium signals. - Plant Signal Behav. **1**: 96-104, 2006.
Renault H., Roussel V., El Amrani A. *et al.*: The *Arabidopsis pop2-1* mutant reveals the involvement of GABA transaminase in salt stress tolerance. - BMC Plant Biol. **10**: 20, 2010.
Roberts M.R.: Does GABA act as a signal in plants? Hints from molecular studies. - Plant Signal Behav. **2**: 408-409, 2007.

Sairam R.K., Rao K.V., Srivastava G.C.: Differential response of wheat genotypes to long term salinity stress in relation to oxidative stress, antioxidant activity and osmolyte concentration. - Plant Sci. **163**: 1037-1046, 2002.
Samarah N.H., Al-Quraan N.A., Massad R.S., Welbaum G.E.: Treatment of bell pepper (*Capsicum annuum* L.) seeds with chitosan increases chitinase and glucanase activities and enhances emergence in a standard cold test. - Sci. Hortic.-Amsterdam **269**: 109393, 2020.
Samarah N.H., Wang H., Welbaum G.E.: Pepper (*Capsicum annuum*) seed germination and vigour following nanochitin, chitosan or hydropriming treatments. - Seed Sci. Technol. **44**: 609-623, 2016.
Sathiyabama M., Manikandan A.: Chitosan nanoparticle induced defense responses in fingermillet plants against blast disease caused by *Pyricularia grisea* (Cke.) Sacc. - Carbohydr. Polym. **154**: 241-246, 2016.
Scholz S.S., Malabarba J., Reichelt M. *et al.*: Evidence for GABA-induced systemic GABA accumulation in *Arabidopsis* upon wounding. - Front. Plant Sci. **8**: 388, 2017.
Shao C.-X., Hu J.-J., Song W.-J., Hu W.-M.: [Effects of seed priming with chitosan solutions of different acidity on seed germination and physiological characteristics of maize seedling.] - J. Zhejiang Univ. **31**: 705-708, 2005. [In Chinese]
Shelp B.J., Bown A.W., McLean M.D.: Metabolism and functions of gamma-aminobutyric acid. - Trends Plant Sci. **4**: 446-452, 1999.
Shelp B.J., Bozzo G.G., Trobacher C.P. *et al.*: Strategies and tools for studying the metabolism and function of γ-aminobutyrate in plants. I. Pathway structure. - Botany **90**: 651-668, 2012.
Shewry P.R.: Wheat. - J. Exp. Bot. **60**: 1537-1553, 2009.
Sigler W.V., Turco R.F.: The impact of chlorothalonil application on soil bacterial and fungal populations as assessed by denaturing gradient gel electrophoresis. - Appl. Soil Ecol. **21**: 107-118, 2002.
Strauss S.Y., Zangerl A.R.: Plant-insect interactions in terrestrial ecosystems. - In: Herrera C.M., Pellmyr O. (ed.): Plant-Animal Interactions: An Evolutionary Approach. Pp. 77-106. Wiley-Blackwell Publishing, Oxford 2002.
Tourian N., Sinaki J., Hasani N., Madani H.: Change in photosynthetic pigment concentration of wheat grass (*Agropyron repens*) cultivars response to drought stress and foliar application with chitosan. - Int. J. Agron. Plant Prod. **4**: 1084-1091, 2013.
Tunio S.D., Korejo M.N., Jarwar A.D., Waggan M.R.: Studies on indigenous and exotic weed competition in wheat. - Pak. J. Agric. Agric. Eng. Vet. Sci. **22**: 1-8, 2006.
van Ittersum M.K., Cassman K.G., Grassini P. *et al.*: Yield gap analysis with local to global relevance - a review. - Field Crop. Res. **143**: 4-17, 2013.
Van S.N., Minh H.D., Anh D.N.: Study on chitosan nanoparticles on biophysical characteristics and growth of Robusta coffee in green house. - Biocatal. Agric. Biotechnol. **2**: 289-294, 2013.
Wang M., Chen Y., Zhang R. *et al.*: Effects of chitosan oligosaccharides on the yield components and production quality of different wheat cultivars (*Triticum aestivum* L.) in Northwest China. - Field Crop. Res. **172**: 11-20, 2015.
Wang W., Vinocur B., Altman A.: Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. - Planta **218**: 1-14, 2003.
Yang F., Hu J., Li J. *et al.*: Chitosan enhances leaf membrane stability and antioxidant enzyme activities in apple seedlings under drought stress. - Plant Growth Regul. **58**: 131-136, 2009.
Yang Y., Guo Y.: Elucidating the molecular mechanisms mediating plant salt-stress responses. - New Phytol. **217**: 523-539, 2018.
Yin H., Zhao X., Du Y.: Oligochitosan: a plant diseases vaccine - a review. - Carbohydr. Polym. **82**: 1-8, 2010.
Zhang G., Bown A.W.: The rapid determination of γ-aminobutyric acid. - Phytochemistry **44**: 1007-1009, 1997.
Zhang H., Irving L.J., McGill C. *et al.*: The effects of salinity and osmotic stress on barley germination rate: sodium as an osmotic regulator. - Ann. Bot.-London **106**: 1027-1035, 2010.
Zhao J., Davis L.C., Verpoorte R.: Elicitor signal transduction leading to production of plant secondary metabolites. - Biotechnol. Adv. **23**: 283-333, 2005.
Zhu J.-K.: Plant salt stress. - Trends Plant Sci. **6**: 66-71, 2001.
Zhu J.-K.: Salt and drought stress signal transduction in plants. - Annu. Rev. Plant Biol. **53**: 247-273, 2002.
Zhu X., Liao J., Xia X. *et al.*: Physiological and iTRAQ-based proteomic analyses reveal the function of exogenous γ-aminobutyric acid (GABA) in improving tea plant (*Camellia sinensis* L.) tolerance at cold temperature. - BMC Plant Biol. **19**: 43, 2019.
Zong H., Liu S., Xing R. *et al.*: Protective effect of chitosan on photosynthesis and antioxidative defense system in edible rape (*Brassica rapa* L.) in the presence of cadmium. - Ecotox. Environ. Safe. **138**: 271-278, 2017.