

# Exogenously applied poly- $\gamma$ -glutamic acid alleviates salt stress in wheat seedlings by modulating ion balance and the antioxidant system

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**Abstract** Salt stress is a main abiotic stress that limits agricultural productivity in many parts of the world. To investigate whether poly- $\gamma$ -glutamic acid ( $\gamma$ -PGA) can alleviate the negative effects of salt stress on wheat, a foliar application of 400 mg/L  $\gamma$ -PGA was applied to wheat seedlings, which were then subjected to 150 mM NaCl. Our results showed that after application of  $\gamma$ -PGA, the plant height, the plant weight, and the antioxidant enzymes including superoxide dismutase, peroxidase, and catalase were significantly increased compared with the treatment of 150 mM NaCl alone. Meanwhile,  $\gamma$ -PGA application also resulted in high accumulation of  $K^+$  and decreased storage of  $Na^+$  in wheat leaves. These results suggest that  $\gamma$ -PGA treatment may improve salt tolerance of wheat by diminishing ionic imbalances and enhancing antioxidant capacity. Our results indicate that exogenous  $\gamma$ -PGA could alleviate the damage caused by salt stress.

**Keywords** Wheat · Salt stress ·  $\gamma$ -PGA · Antioxidant · Ion balance

## Introduction

Wheat is the most important stable crop in the world and provides almost 20% of the total necessary calories for the

world's population (Hajihashemi et al. 2007). Salinity stress has been reported to disturb the integrity of cell membranes in wheat and barley and cause an increase in membrane permeability and decrease in lipid partiality (Mansour et al. 1993). Currently, salinity has become a widespread problem; presumably, by the year 2050, 50% of arable lands will be salinized (Wang et al. 2003). Salt stress is a major factor limiting growth and crop production in the world (Bohnert and Jensen 1996). High salinity induces ion and osmotic pressure imbalance (Xiong et al. 2002). The accumulation of toxic concentrations of salt in old leaves causes ion toxicity and accelerates the senescence of leaves and finally results in leaf death (Katerji et al. 2003). Osmotic stress restricts water availability and leaf growth in plants (Tuna et al. 2008). High salinity also induces several morphological, physiological, and metabolic responses, leading to gradual peroxidation of lipids and antioxidant enzyme inactivation (Garg and Manchanda 2009).

In order to reduce oxidative damage, plants have produced an enzymatic and non-enzymatic defense system to alleviate oxidative stress. The active oxygen-scavenging enzymes include superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) (Li et al. 2011; Ma et al. 2012). SOD is a major scavenger of toxic  $O_2^-$  radicals and is subsequently converted to hydrogen peroxide ( $H_2O_2$ ) by peroxidase (Mutlu et al. 2009; Triantaphylides and Havaux 2009). POD disintegrates  $H_2O_2$  by oxidation of co-substrates such as phenolic compounds or antioxidants, whereas CAT decomposes  $H_2O_2$  and  $O_2$  (Noctor and Foyer 1998). In contrast, non-enzyme antioxidants include glutathione, ascorbate, proline, and flavonoids (Li et al. 2011). It is therefore necessary to find methods for reducing the adverse effects of salt stress and increasing wheat productivity.

Poly- $\gamma$ -glutamic acid ( $\gamma$ -PGA) is a water-soluble, biodegradable polypeptide (Xu et al. 2013); it is a biopolymer composed of D-glutamic acid and L-glutamic acid monomers connected by amide linkages via *Bacillus subtilis* fermentation (Birrer et al.

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1994; Shih and Van 2001).  $\gamma$ -PGA is used in many fields, such as food, medicine, cosmetics, and agriculture (Shih and Van 2001).  $\gamma$ -PGA is able to hold as much water as hydrogels, allowing its use as a soil conditioner for crop fields in dry deserts. It is reported that  $\gamma$ -PGA can greatly raise the dry weight of the roots of both cucumber seedlings (Wang et al. 2008) and Chinese cabbage (Xu et al. 2014).  $\gamma$ -PGA application on wheat resulted in increased spike number, 1000 seed weight, seed number per spike, and dry matter production (Xu et al. 2013).  $\gamma$ -PGA treatment increased the chlorophyll content of tomato leaves during the flowering stage (Jiang et al. 2014).

Many studies have reported that  $\gamma$ -PGA plays an important role in plant growth and regulation. However, there is meager information regarding the effects of  $\gamma$ -PGA on seedling growth under salt stress. It was reported that  $\gamma$ -PGA could enhance the tolerance of *Brassica napus* L. by promoting proline accumulation under salt stress (Lei et al. 2016). In this study, wheat seedlings were used to assess the effect of foliar application of  $\gamma$ -PGA on the growth of seedlings subjected to salt stress. This study shows that foliar application of  $\gamma$ -PGA alleviated the negative effects of salt stress on wheat by modulating ion balance and the antioxidant system. These results provide an insight into the role of  $\gamma$ -PGA in the alleviation of salt stress in wheat seedlings and information on the potential use of  $\gamma$ -PGA to regulate plant growth in salinity soil.

## Materials and methods

### Plant material, growth, and treatment conditions

Wheat seeds were disinfected with 70% ethanol for 1 min and then rinsed with distilled water for several times. The seeds were swamped in water for 12 h and moved to wet filter paper to germinate in the darkness in a temperature-controlled chamber at 30 °C for 24 h. The germinated seeds were transferred to a vermiculite culture with standard Hoagland solution. The solution was changed every 3 days. The seedlings were kept under a 16/8-h day/night cycle with day/night temperatures of 24/20 °C. After growing 7 days, wheat seedlings with two leaves were randomly divided into four treatment groups (Shalata and Neumann 2001; Wang 2017; Xie et al. 2015): (1) seedlings sprayed with distilled water alone, (2) seedlings sprayed with 400 mg/L  $\gamma$ -PGA, (3) seedlings sprayed with  $\gamma$ -PGA for 12 h before addition of 150 mM NaCl, and (4) seedlings treated with 150 mM NaCl. Each pot contained 12 seedlings, and three pots were analyzed for each treatment at each time point. Seedlings were sprayed until leaves were completely wetted (about 30 mL for each pot). All the experiments were repeated three times. Based on our pre-experiments, the concentrations of 150 mM NaCl and 400 mg/L  $\gamma$ -PGA were selected for our experiments (Figs. S1 and S2).

### Biomass assay

After 7 days of NaCl treatment, the seedlings had three leaves, and the shoot of wheat seedlings were collected for determination of fresh and dry weight. Dry weight was weighted after drying the seedlings at 105 °C for 5 min and then at 80 °C for 48 h.

### Measurement of relative conductivity, chlorophyll content, and lipid peroxidation (MDA)

The method described by Barcia et al. (2014) was used to analyze relative conductivity. Chlorophyll was extracted using 80% acetone under the darkness for 24 h, and the chlorophyll concentration was calculated according to Arnon (1949). The MDA content was measured using the method of Madhava Rao and Sresty (2000).

### Antioxidant enzyme extraction and analysis

Fresh leaves (0.2 g) were finely grounded in 2 mL extraction buffer (50 mM phosphate buffer (pH = 7.8)) containing 1 mM EDTA and 2% (w/v) PVP at chilled condition. After centrifugation at 10,000×g for 20 min at 4 °C, the crude extraction was transferred to another tube and used for enzyme determination. The SOD activity was determined following the method of Giannopolitis and Ries (1977), and POD and CAT were analyzed basing on the produce described by Cakmak and Marschner (1992) and Bergmeyer (1970), respectively. The activity of antioxidant enzymes was determined on fresh weight basis.

### Assay of H<sub>2</sub>O<sub>2</sub> content

H<sub>2</sub>O<sub>2</sub> content was analyzed according to Chang and Kao (1998) with some modifications. In our experiment, we changed the concentration of sulfuric titanium from 2% (w/v) to 20% (w/v).

### Histochemical staining using diaminobenzidine tetrahydrochloride and nitroblue tetrazolium

Diaminobenzidine tetrahydrochloride (DAB) staining was performed using wheat leaves placed in 1 mg/mL DAB (pH = 3.8) solution for 8 h in the darkness. Chlorophyll was removed by boiling in 95% ethanol for 15 min. Observations were made under a light microscope (Thordal-Christensen et al. 1997).

Nitroblue tetrazolium (NBT) staining was used to determine superoxide anion content. Wheat leaves were submerged in 10 mM PBS buffer (pH = 7.8) containing 1 mg/mL NBT; the mixture was placed under a vacuum for 20 min, and chlorophyll was removed by boiling in 95% ethanol

(Garrett et al. 1990). The leaves were then preserved in 50% glycerin for observation.

### Measurement of Na<sup>+</sup> and K<sup>+</sup> concentration

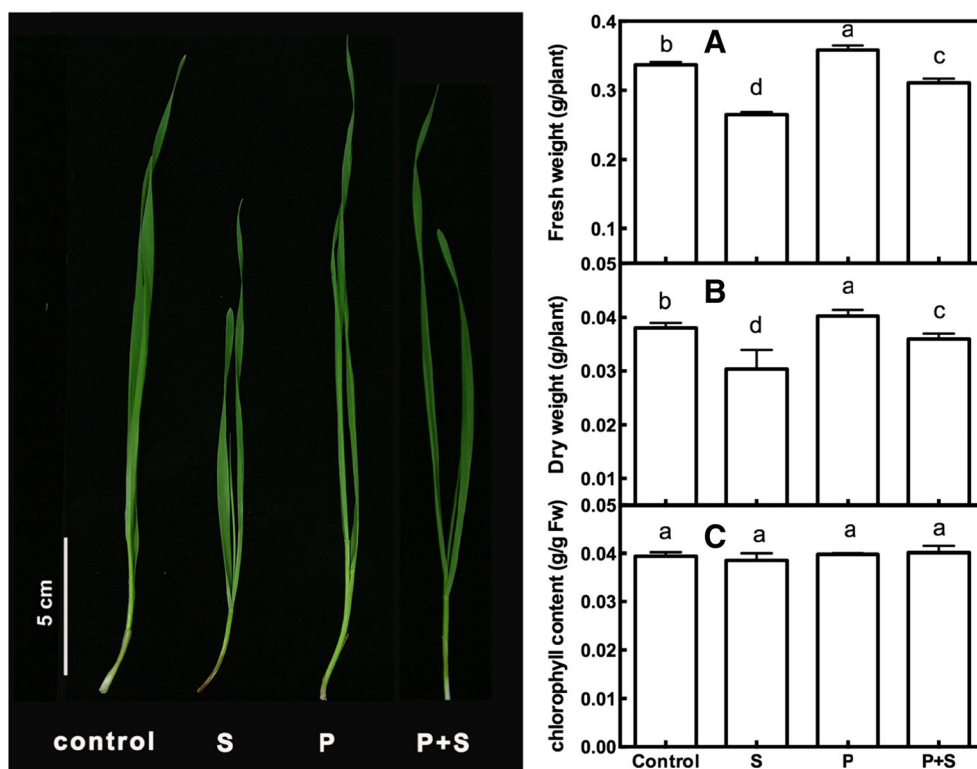
Elemental analysis was according to the method described by Zarcinas et al. (1987).

## Results

### Effect of foliar application of $\gamma$ -PGA on growth parameters and chlorophyll content in wheat seedlings under salt stress

To investigate the effects of exogenous  $\gamma$ -PGA in wheat, the biomass of wheat seedlings under salt stress was analyzed. The data suggested that salt decreased the fresh and dry weights by 21 and 20%, respectively, compared with the control. However, the fresh and dry weights only decreased by 7.7 and 5.4%, respectively, under  $\gamma$ -PGA treatment with salt stress (Fig. 1a,b). Salt stress also significantly reduced wheat height, and exogenous application of  $\gamma$ -PGA alleviated this effect. However, the chlorophyll content in wheat seedlings subjected to salt stress was not significantly different from that of the control (Fig. 1c).

**Fig. 1** Effect of exogenous  $\gamma$ -PGA (400 mg/L) on the regulation of fresh weight (a), dry weight (b), and chlorophyll content (c) in wheat seedlings under NaCl (150 mM) treatment for 7 days. The different treatments included distilled water (Control), 150 mM salt stress (S), 400 mg/L  $\gamma$ -PGA (P), and 150 mM salt stress + 400 mg/L  $\gamma$ -PGA (P + S). Bars are means  $\pm$  standard error of three replicates. Different letters indicate significant differences among treatments at the 0.05 level based on Duncan's test



### Effect of exogenous $\gamma$ -PGA on lipid peroxidation and relative conductivity in wheat seedlings under salt stress

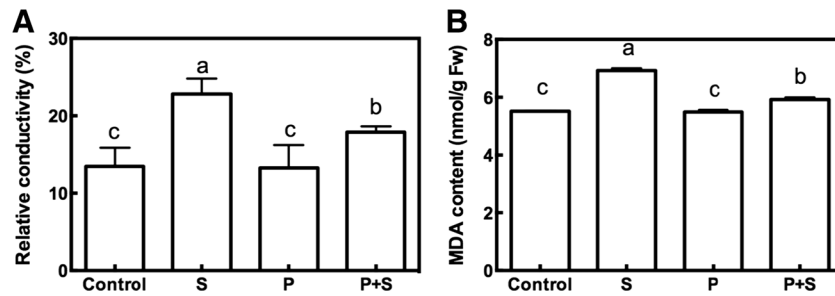
The extent of lipid peroxidation is indicated by the MDA content. Salt stress induced MDA content, and this induction was significantly mitigated by treatment with 400 mg/L  $\gamma$ -PGA, although  $\gamma$ -PGA treatment alone did not have a significant effect compared with the control (Fig. 2b). The relative conductivity increased quickly under salt stress, and this effect was diminished by exogenous application of 400 mg/L  $\gamma$ -PGA (Fig. 2a).

### Effect of $\gamma$ -PGA on antioxidant enzyme activity in wheat seedlings under salt stress

The effect of exogenous  $\gamma$ -PGA on the activities of SOD, POD, and CAT in leaves is shown in Fig. 3. Salt stress significantly decreased SOD (Fig. 3a), POD (Fig. 3b), and CAT (Fig. 3c) activities in leaves compared with those of the control. In contrast, exogenous application of  $\gamma$ -PGA increased these activities under salt stress. As seen in Fig. 3, treatment with  $\gamma$ -PGA alone notably enhanced the activities of SOD, POD, and CAT compared with those of the control.

### H<sub>2</sub>O<sub>2</sub> content and DAB and NBT staining

H<sub>2</sub>O<sub>2</sub> content was significantly increased, rising sharply at 12 h, under salt stress; however, H<sub>2</sub>O<sub>2</sub> content was decreased



**Fig. 2** Effect of exogenous  $\gamma$ -PGA (400 mg/L) on the regulation of relative conductivity (a) and MDA content (b) in wheat seedling under NaCl (150 mM) treatment for 7 days. The different treatments included distilled water (Control), 150 mM salt stress (S), 400 mg/L  $\gamma$ -PGA (P),

and 150 mM salt stress + 400 mg/L  $\gamma$ -PGA (P + S). Bars are means  $\pm$  standard error of three replicates. Different letters indicate significant differences among treatments at the 0.05 level based on Duncan’s test

after application of  $\gamma$ -PGA. There was no significant difference between  $\gamma$ -PGA alone treatment and the control (Fig. 4). DAB and NBT staining showed the same results. The red staining represents DAB polymerization products, indicating  $H_2O_2$  formation. The blue staining indicates NBT polymerization, revealing  $O_2^-$  as the product. Little staining was observed in the control and  $\gamma$ -PGA-treated leaves, and stronger reddish brown and blue staining appeared in wheat leaves under salt stress, with more reddish and blue coloration seen in those treated with 150 mM NaCl alone.

$K^+/Na^+$  ratio, which was enhanced by exogenous  $\gamma$ -PGA application in wheat seedlings (Fig. 5c).

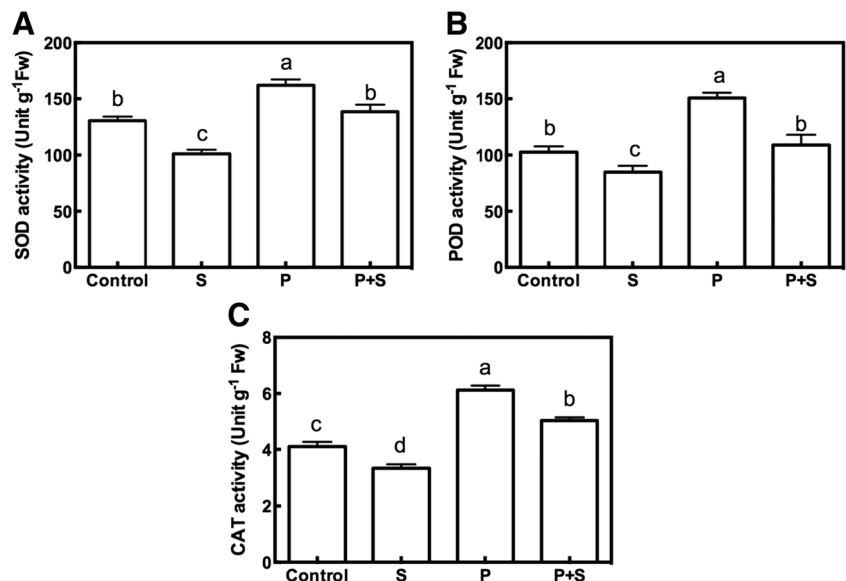
**Effect of  $\gamma$ -PGA on  $Na^+$  and  $K^+$  contents in wheat leaves under salt stress**

Salt stress promoted absorption of  $Na^+$  and decreased absorption of  $K^+$  in the leaves (Fig. 5a, b). Foliar application of  $\gamma$ -PGA inhibited the absorption of  $Na^+$  in the leaves of wheat seedlings. However,  $\gamma$ -PGA had no effect on the accumulation of  $K^+$ . Salt stress resulted in an obvious decrease in the

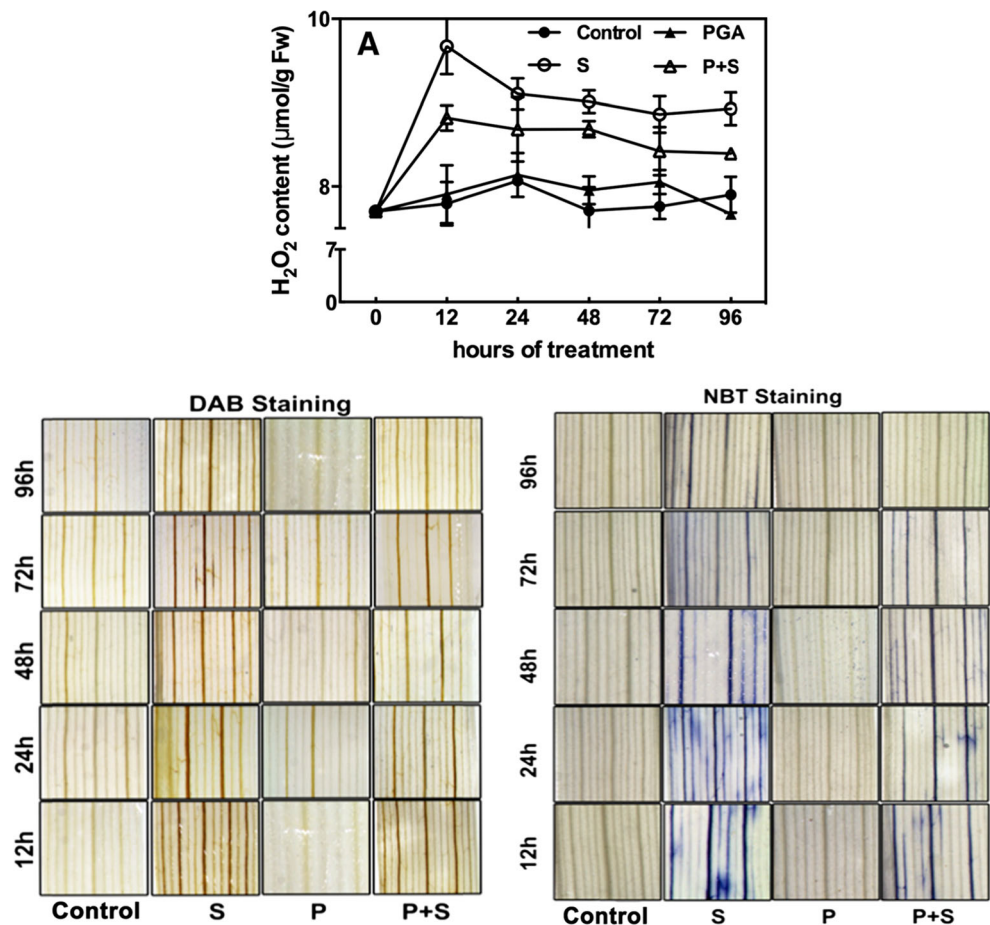
**Discussion**

Salt stress decreases the metabolic activity of plant cells, indicated by inhibition of their growth. In this study, we found that the growth of wheat seedlings was significantly inhibited by 150 mM salt. However, exogenous application of  $\gamma$ -PGA decreased the side effects of salt stress on the growth of wheat seedlings. It has been reported that treatment with 500 mg/L  $\gamma$ -PGA protected rice seedlings from the adverse effects of Cd by enhancing shoot length (Kotabin et al. 2015). Chlorophyll content is widely used as an index of abiotic tolerance in plants. While our results showed that 7 days of salt stress did not affect chlorophyll content, Chen et al. (2014) reported that under salt treatment, the chlorophyll content in wheat seedlings was decreased sharply after 21 days. This

**Fig. 3** Effect of exogenous  $\gamma$ -PGA (400 mg/L) on the regulation of SOD activity (a), POD activity (b), and CAT activity (c) in wheat seedling under NaCl (150 mM) treatment for 7 days. The different treatments included distilled water (Control), 150 mM salt stress (S), 400 mg/L  $\gamma$ -PGA (P), and 150 mM salt stress + 400 mg/L  $\gamma$ -PGA (P + S). Bars are means  $\pm$  standard error of three replicates. Different letters indicate significant differences among treatments at the 0.05 level based on Duncan’s test



**Fig. 4** Effect of exogenous  $\gamma$ -PGA (400 mg/L) on the regulation of  $H_2O_2$  content (a) and DAB staining in different hours after treatment (left) and NBT staining in different hours after treatment (right) in wheat seedling under NaCl (150 mM) treatment for 7 days. The different treatments included distilled water (Control), 150 mM salt stress (S), 400 mg/L  $\gamma$ -PGA (P), and 150 mM salt stress + 400 mg/L  $\gamma$ -PGA (P + S). Bars are means  $\pm$  standard error of three replicates

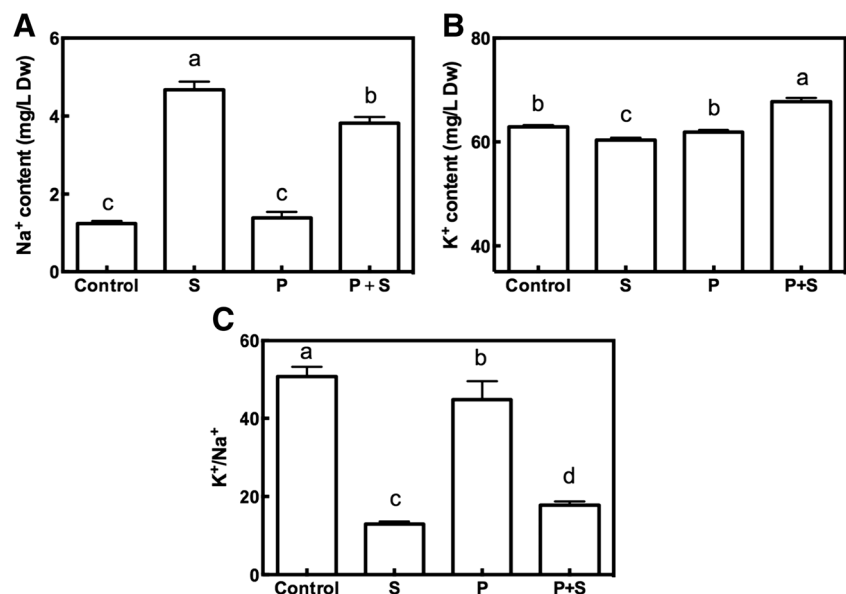


discordance may be attributed to the different application durations of the salt treatments used in each study.

Plants under salt stress use the antioxidant system to resist the adverse effects of salt stress on their growth. MDA is one of the final products of lipid peroxidation in plant cell

membranes and a marker of oxidative damage in plants exposed to stress (Meloni et al. 2003). Under cold stress,  $\gamma$ -PGA protected rape seedlings by reducing MDA content (Lei et al. 2015). In our study, a significant increase in lipid peroxidation was observed under salt stress. Nevertheless, exogenous

**Fig. 5** Effect of exogenous  $\gamma$ -PGA (400 mg/L) on the regulation of  $Na^+$  content (a),  $K^+$  content (b), and  $K^+/Na^+$  (c) in wheat seedling leaves under NaCl (150 mM) treatment for 7 days. The different treatments included distilled water (Control), 150 mM salt stress (S), 400 mg/L  $\gamma$ -PGA (P), and 150 mM salt stress + 400 mg/L  $\gamma$ -PGA (P + S). Bars are means  $\pm$  standard error of three replicates. Different letters indicate significant differences among treatments at the 0.05 level based on Duncan's test



application of  $\gamma$ -PGA prevented lipid peroxidation and protected cells from salt stress. Salt-induced inhibition on plant growth could also be attributed to accumulation of ROS, which can damage macromolecules and cellular structures. It was reported that elevated expression of antioxidant system components has been linked to the decreased oxidative damage under different stresses, because high antioxidase activity can eliminate ROS quickly and protect plants from stress damage (Wang et al. 2010). Our results suggested that under salt stress, SOD, POD, and CAT activities were decreased significantly, while  $\gamma$ -PGA alleviated the deleterious effects of the salt stress. Moriyama et al. (2015) revealed that cross-linked catechol-PGA nanoparticles have  $H_2O_2$  scavenging activity.  $\gamma$ -PGA can also serve as a capping agent to scavenge intracellular ROS formed (Stevanović et al. 2011; Stevanović et al. 2012). It has also been reported that  $\gamma$ -PGA has potential for use in the food, cosmetic, and biomedical industries in the development of novel products with radical scavenging activity (Lee et al. 2014). This study revealed that  $H_2O_2$  content was decreased in wheat seedlings when  $\gamma$ -PGA was applied under salt stress. Histochemical staining supported this result. After DAB and NBT staining, we found that  $H_2O_2$  and  $O_2^-$  were diminished after application of  $\gamma$ -PGA. We therefore presumed that  $\gamma$ -PGA acts as a  $H_2O_2$  scavenging agent to alleviate salt stress by modulation of the antioxidant system.

Salt-induced inhibition of plant growth could also be attributed to ion toxicity. Plant cells under salt stress show increased toxic level of cellular  $Na^+$  and restricted absorption of macroelement  $K^+$ . This causes a rapid reduction in the cytosolic  $K^+/Na^+$  ratio and to salt sensitivity. Reducing the  $Na^+$  accumulate in leaves is thought as an important mechanism of salt stress (Tester and Davenport 2003). Our results showed that the  $Na^+$  concentration in wheat seedlings was increased markedly under 150 mM NaCl and that the  $K^+$  concentration was decreased. After foliar application of  $\gamma$ -PGA, the accumulation of  $Na^+$  was inhibited and  $K^+$  uptake stimulated in the leaves of salt-stressed wheat seedlings. This is consistent with reports that  $\gamma$ -PGA can slow down the decreasing rate of  $K^+$  uptake under stress and promote  $K^+$  accumulation.  $\gamma$ -PGA was also reported to be a sorbent for increasing Ca and N absorption (Tanimoto et al. 2001; Xu et al. 2013). These results suggest that  $\gamma$ -PGA alleviates ion-specific toxicity by decreasing cellular accumulation of  $Na^+$ .

## Conclusion

In conclusion, salt stress reduced the growth of wheat seedlings, and 400 mg/L  $\gamma$ -PGA alleviated salinity damage by increasing SOD, POD, and CAT activities and decreasing ion toxicity in wheat seedlings. These results indicate that

exogenously applied  $\gamma$ -PGA can protect wheat seedlings from the adverse effects of salt stress.

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