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Alleviation of salt-induced oxidative stress in rice seedlings by proline and/or glycinebetaine

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Abstract

The effects of proline and/or glycine betaine (GB) application on growth, photosynthetic pigments, H_2O_2 content, and activities of antioxidant enzymes in rice (*Oryza sativa* L. cv. KDML105) under salt stress were investigated. The H_2O_2 content and the activities of superoxide dismutase (SOD), glutathione reductase (GR), and ascorbate peroxidase (APX) but not catalase (CAT) increased under salinity. Under 160 mM NaCl, the CAT activity was maintained on the pre-stress level in the presence of proline, whereas in the presence of GB, the GR activity increased more than without GB application. A co-application of 30 mM proline and 1 mM GB did not reduce the increase in H_2O_2 caused by the NaCl stress more than applying each of the osmoprotectants and no synergistic effect on the antioxidant enzymes was observed. However, the application of both the osmoprotectants was the most effective in alleviating degradation of photosynthetic pigments.

Additional key words: ascorbate peroxidase, carotenoids, catalase, chlorophyll, glutathione reductase, hydrogen peroxide, NaCl, *Oryza sativa*, osmoprotectants, superoxide dismutase.

Introduction

Several environmental factors (salinity, drought, nutrient imbalances, UV radiation, extreme temperatures, *etc.*) adversely affect growth, development, and final yield of plants. Different abiotic stresses may provoke osmotic stress and oxidative stress which lead to similar cellular adaptive responses, such as accumulation of compatible solutes, induction of stress proteins, and activation of reactive oxygen species (ROS) scavenging systems which comprise non-enzymatic and enzymatic antioxidants (Zhu 2002, Apel and Hirt 2004). Major ROS scavenging enzymes of plants include superoxide dismutase (SOD: EC 1.15.1.1), ascorbate peroxidase (APX: EC 1.11.1.11), catalase (CAT: EC 1.11.1.6), and glutathione reductase (GR: EC 1.8.1.7), and non-enzymatic antioxidants include ascorbic acid and glutathione. A further common stress response in plants is the production of different types of compatible organic solutes (Serraj and Sinclair 2002) and among them, proline and glycinebetaine (GB) play a pivotal role in osmotic adjustment (Flowers *et al*. 1977, Greenway and Munns 1980, Rhodes and Hanson 1993, Hasegawa *et al*. 2000) and also contribute to detoxification of ROS and stabilization of enzymes/ proteins (Yancey *et al*. 1982, Bohnert and Jensen 1996). Effects after application of these solutes have been studied in many crop plants. Under salt stress, GB application maintains or enhances growth and yield in rice (Rahman *et al*. 2002, Demiral and Türkan 2006, Farooq *et al*. 2008, Cha-um *et al*. 2013), wheat (Ma *et al*. 2006, Raza *et al*. 2006), and maize (Anjum *et al*. 2011), whereas proline can counteract growth inhibition induced by NaCl in rape (Makela *et al*. 1999), rice (Deivanai *et al*. 2011), and maize (Ali *et al*. 2007).

Rice, an important crop worldwide, is relatively sensitive to environmental stresses. Beneficial effects of GB or proline application under salt or drought stress were shown (Sobahan *et al*. 2009, 2012, Cha-Um and

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Abbreviations: APX - ascorbate peroxidase (EC 1.11.1.11); CAT - catalase (EC 1.11.1.6); GB - glycinebetaine; GR - glutathione reductase (EC 1.6.4.2); GSSG - oxidized glutathione; H_2O_2 - hydrogen peroxide; ROS - reactive oxygen species; SOD - superoxide dismutase (EC 1.15.1.1).

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Kirdmanee 2010, Deivanai *et al*. 2011, Mohammed and Tarpley 2011), but an effect of co-application of both proline and GB during NaCl treatment has not been reported. Therefore, we studied the role of exogenous proline and/or GB application on salt stress in the salt sensitive (Wanchananan *et al*. 2003, Kong-ngern *et al*. 2012) Thai jasmine rice.

Materials and methods

Growth conditions and treatments: Seeds of *Oryza sativa* L. var. *indica* cv. Khao Dawk Ma Li 105 (KDML105) were obtained from the Department of Agriculture, the Ministry of Agriculture and Cooperatives (Bangkok, Thailand). They were dehusked and sterilized with 70 % (v/v) ethanol for 2 min and then with 2 % (m/v) sodium hypochlorite for 20 min. The seeds were rinsed three times with sterile water and germinated in a nutrient broth medium (NB) (Li *et al*. 1993) containing 0.8 % (m/v) agar at a temperature of 25 - 28 °C, a 16-h photoperiod, and an irradiance of 200 μmol m^{-2} s^{-1}. After 7 d, germinated seeds were transferred to a Limpinuntana's nutrient solution (Limpinuntana 1978) and grown under the previously mentioned conditions for 14 d. Three-week-old seedlings were exposed to a nutrient solution supplemented with 0 (control), 60, 120, or 160 mM NaCl with or without various concentrations of GB (1, 5, 15, or 30 mM) or proline (15, 30, or 45 mM) or both 30 mM proline and 1 mM GB for 3 d and then returned to the control medium. Leaves were harvested after 3 d of the salt stress and after 3 d of recovery. The samples were randomly selected and oven-dried at 60 °C for 72 h for determination of dry mass. Relative growth rate (RGR) was determined following the method of Beadle (1993) and it was calculated from the increase in dry mass at the beginning and end of the treatment using an equation: $RGR = (\ln M_f - \ln M_i)/(t_f - t_i)$, where M is the shoot or root dry mass, t is the time, and subscripts denote the initial (day 0) and final (day 6) samplings.

Measurement of pigment content: Chlorophyll (Chl) *a* and Chl *b* and carotenoids were extracted in 2 cm^3 of *N,N*-dimethyl formamide by grinding 0.05 g of rice leaves in a chilled mortar and pestle. The homogenate was centrifuged (15 000 *g*, 4 °C, 10 min,) and the absorbances of the resulting supernatant were taken at 461, 625, and 664 nm using a spectrophotometer (*DU 640*, *Beckman*, Coulter, USA). Chl *a*, Chl *b,* and carotenoid content was calculated according to Arnon (1949).

Enzyme extractions and assay: Enzyme extracts for evaluating SOD, CAT, APX, and GR activities were prepared by freezing leaf samples (0.5 g) in liquid nitrogen to prevent proteolytic activity, followed by grinding with 5 cm^3 of a cold extraction buffer (a 0.1 M phosphate buffer, pH 7.5) containing 0.5 mM ethylene-diaminetetraacetic acid (EDTA), 5 mM dithiothreitol, and a 0.1 % (m/v) protease inhibitor, except that in the case of APX activity, 5 mM ascorbic acid was added to the extraction buffer. Homogenates were filtered and centrifuged (15 000 *g*, 4 °C, 20 min) prior to harvesting the supernatant as the enzyme extract.

The SOD activity was estimated by recording the decrease in absorbance of formazan produced by superoxide and nitroblue tetrazolium (NBT) by the enzyme (Dhindsa *et al*. 1981). A reaction mixture (1.2 cm^3) contained 13.33 mM methionine, 75 μM NBT, 0.10 mM EDTA, a 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate, and the enzyme extract (50 μg of protein). The reaction was started by adding 2 μM riboflavin and placing the tubes under two 15 W fluorescent lamps (at ~30 cm distance) for 15 min. The reaction was stopped by switching off the light and putting the tubes in the dark for 10 min. The complete reaction mixture without the enzyme served as control, whilst the non-irradiated complete reaction mixture served as blank. The absorbance was recorded at 560 nm, and one unit (U) of enzyme activity was taken as the amount of enzyme which reduced the absorbance reading to 50 % in comparison with the control.

The CAT activity was assayed from the rate of H_2O_2 decomposition as measured by the decrease of absorbance at 240 nm at room temperature following the modified procedure of Aebi (1974). A reaction mixture (1.2 cm^3) contained a 50 mM potassium phosphate buffer (pH 7.0) and the enzyme extract (50 μg of protein). The reaction was initiated by adding 16.67 mM H_2O_2.

The APX activity was assayed according to the method of Nagano and Asada (1981). The activity was determined by following the decrease of absorbance at 290 nm (the coefficient of absorbance of 2.8 mM^{-1} cm^{-1}). A reaction mixture contained a 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.2 mM H_2O_2, and the enzyme extract (50 μg of protein).

The GR activity was assayed according to the method of Smith *et al.* (1988). A reaction mixture contained a 66.67 mM potassium phosphate buffer (pH 7.5), 0.333 mM EDTA, 0.5 mM 5,5-dithiobis-2-nitrobenzoic acid in a 0.01 M potassium phosphate buffer (pH 7.5), 0.0667 mM NADPH, the enzyme extract (50 μg of protein), and distilled water to make up a final volume of 1.2 cm^3. The reaction was initiated by adding 0.667 mM glutathione disulphide (GSSG; oxidized glutathione). The increase in absorbance at 412 nm was recorded at 25 °C over a period of 5 min.

Protein content in the enzyme extract was measured according to the method of Bradford (1976) using bovine serum albumin as standard.

H_2O_2 content was measured by the modified method of Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing 100 mg of leaf tissue with liquid nitrogen

followed by grinding with 3 cm^3 of a 50 mM phosphate buffer (pH 7.0). The homogenate was filtered and centrifuged (6 000 *g*, 4 °C, 25 min), and 0.9 cm^3 of the supernatant was mixed with 0.3 cm^3 of 1 % (v/v) $TiCl_4$ in conc. HCl and centrifuged (6 000 *g*, 4 °C, 15 min). The absorbance of the yellow supernatant was measured at 410 nm. H_2O_2 content was then calculated by comparison the absorbance with absorbances based on a standard curve drawn using different known concentrations of H_2O_2.

Statistical analysis: All analyses were completely randomized with ten replicates per treatment. Statistical analysis was performed using one-way *ANOVA* and differences between the mean values were compared using Duncan's test at $P < 0.05$.

Results

The H_2O_2 content in leaves increased significantly by 12 h after the initiation of the salt stress and reached a maximum after 3 d (data not shown). Based on these results, we selected the three-day NaCl exposure for further experiments. The H_2O_2 content and SOD, CAT, GR, and APX activities in seedlings grown under 0 - 160 mM NaCl for 3 d in the presence or absence of GB and/or proline were determined. The results reveal a significant increase in H_2O_2 content in NaCl dose-dependent manner with the maximum H_2O_2 content at 160 mM NaCl (Table 1). Simultaneously, increased SOD, GR, and APX activities were observed under the salt stress (Table 1). For GR, all three concentrations of NaCl (60, 120, and 160 mM) induced a broadly similar (~1.4- to 1.45-fold) and significant increase in the activity, whereas increases in SOD and APX activities were statistically significant at 160 mM NaCl. In contrast, all the NaCl concentrations led to a significant reduction (1.39-fold at 60 mM NaCl and ~1.6-fold at 120 and 160 mM NaCl) in CAT activity.

To determine whether exogenous proline alleviate salt-induced oxidative stress, we applied various proline concentrations (0, 15, 30, or 45 mM) along with 160 mM NaCl and evaluated leaf H_2O_2 content and enzyme activities after 3 d. Only the addition of 30 mM proline was effective in reducing the NaCl-mediated increase in H_2O_2 back to the control level (Table 1). With respect to SOD activity, the addition of 15 or 30 mM proline did not cause any significant effect, but 45 mM proline significantly reduced the SOD activity. The proline application at all the three concentrations counteracted the NaCl-mediated decrease in CAT activity as well as the NaCl-mediated increase in GR activity. The application of 15 and 45 (but not 30) mM proline abolished the NaCl-mediated increase in APX activity (Table 1).

The addition of 1 mM GB to the 160 mM NaCl salt-stressed rice plants significantly reduced (~1.4-fold) the increment of H_2O_2 content when compared with the salt-stressed rice plants without GB application, but did not reduce the values to that of the unstressed control plants (Table 1). In contrast, 5 and 15 mM GB had essentially no effect on the H_2O_2 content, whereas 30 mM GB significantly increased it.

The application of GB reduced the SOD activity in a dose-dependent manner. The NaCl-mediated increase in SOD activity decreased back to the control level at 5 mM GB and to values about 60 % of the control at 15 and 30 mM GB. In contrast, the NaCl-mediated decrease

Table 1. Effects of 60, 120, and 160 mM NaCl-induced stress and the application of proline and/or GB on H_2O_2 content [μmol g^{-1}(f.m.)] and SOD [U mg^{-1}(protein)], CAT [μmol(H_2O_2) mg^{-1}(protein) min^{-1}], GR [μmol(GSSG) mg^{-1}(protein) min^{-1}], and APX [μmol(ascorbate) mg^{-1}(protein) min^{-1}] activities in leaves of rice cv. KDML105 after a 3-d exposure. Means ± SD, $n = 10$. Means with different letters are significantly different ($P < 0.05$).

Treatments	H_2O_2 content	SOD	CAT	GR	APX
0 mM NaCl	90.73 ± 9.78a	31.76 ± 2.73a	56.93 ± 1.55b	26.08 ± 2.21a	309.38 ± 37.79a
60 mM NaCl	105.50 ± 3.15b	32.26 ± 1.10a	41.00 ± 2.43a	35.87 ± 2.92b	307.85 ± 64.43a
120 mM NaCl	134.24 ± 15.51c	36.92 ± 1.14a	35.38 ± 2.74a	37.34 ± 2.13b	342.76 ± 31.76a
160 mM NaCl	180.32 ± 12.47d	43.34 ± 5.72b	36.21 ± 4.13a	36.22 ± 1.93b	442.10 ± 23.21b
160 mM NaCl + 15 mM proline	173.46 ± 36.21b	39.73 ± 2.77bc	55.74 ± 7.00b	21.84 ± 3.88ab	381.77 ± 37.51b
160 mM NaCl + 30 mM proline	94.26 ± 6.19a	43.05 ± 2.67c	58.57 ± 1.45b	22.76 ± 2.04b	462.94 ± 20.65c
160 mM NaCl + 45 mM proline	236.19 ± 4.52c	34.67 ± 1.16ab	59.22 ± 2.79b	19.25 ± 3.56a	323.52 ± 43.78a
160 mM NaCl + 1 mM GB	127.56 ± 11.69b	33.80 ± 3.54bc	26.98 ± 0.73a	40.94 ± 3.68c	358.67 ± 50.03ab
160 mM NaCl + 5 mM GB	188.21 ± 11.58c	28.45 ± 1.05ab	34.79 ± 3.97b	56.16 ± 2.48d	332.82 ± 20.84ab
160 mM NaCl + 15 mM GB	186.96 ± 13.01c	21.29 ± 1.67a	22.64 ± 0.45a	62.86 ±1.94e	302.43 ± 40.75a
160 mM NaCl + 30 mM GB	215.06 ± 9.34d	21.47 ± 0.46a	27.45 ± 1.41a	60.51 ± 3.11de	273.02 ± 60.49a
160 mM NaCl + 30 mM proline + 1 mM GB	124.13 ± 11.51b	27.82 ± 3.68a	35.18 ± 2.06b	36.45 ± 1.83b,c	343.52 ± 45.66a

Table 2. Effects of exogenous proline (30 mM) and/or GB (1 mM) on relative growth rate (RGR) of shoots and roots [g g^{-1} d^{-1}] and photosynthetic pigments [mg g^{-1} (f.m.)] of rice cv. KDML105 after a salt stress (160 mM NaCl) for 3 d and recovery for 3 d. Means ± SD, n = 10. Means with different letters are significantly different (P < 0.05).

Treatments	RGR of shoots	RGR of roots	Chl *a* content	Chl *b* content	Carotenoid content
0 mM NaCl	0.11 ± 0.02c	0.17 ± 0.03b	0.33 ± 0.05b	0.12 ± 0.03a	3.70 ± 0.74c
160 mM NaCl	0.04 ± 0.01a	0.06 ± 0.02a	0.22 ± 0.02a	0.10 ±0.02a	2.22 ± 0.08a
160 mM NaCl + 30 mM proline	0.12 ± 0.02c	0.09 ± 0.02a	0.26 ± 0.07ab	0.14 ± 0.05a	2.42 ± 0.32ab
160 mM NaCl + 1 mM GB	0.08 ± 0.01b	0.09 ± 0.02a	0.27 ± 0.07ab	0.14 ± 0.05a	2.59 ± 0.71ab
160 mM NaCl + 30 mM proline + 1 mM GB	0.10 ± 0.01bc	0.09 ± 0.01a	0.36 ± 0.03b	0.25 ± 0.03b	3.33 ± 0.38bc

in CAT activity was not counteracted by any concentration of GB, but instead either no effect (at 5 mM GB) or a further reduction (at 1, 15, and 30 mM GB) in CAT activity was observed (Table 1). The application of GB increased the GR activity above that induced by 160 mM NaCl alone in an initially dose-dependent manner reaching a maximum at 15 mM GB (a ~2.4-fold higher GR activity than in the control and 1.8-fold higher than under NaCl alone). Furthermore, the exogenous GB application under the salt stress also reduced the APX activity in a dose-dependent manner similarly to what we observed for the SOD activity.

When compared to the 160 mM NaCl-stressed plants, the co-application of 30 mM proline and 1 mM GB caused a significant decrease in the H_2O_2 content, however, it was still significantly higher than that in the unstressed control plants and was not significantly different to that induced by 1 mM GB alone. Only the addition of 30 mM proline was effective in reducing the NaCl-mediated increase in H_2O_2 back to the control level. For CAT and GR activities, the co-addition of GB and proline was ineffective. In contrast, the co-addition of proline and GB decreased the SOD and APX activities induced by 160 mM NaCl back to the control levels.

The treatment with 160 mM NaCl for 3 d followed by recovery without NaCl for 3 d brought about a significant decrease in RGR of shoots (~2.7-fold) and roots (~3.1-fold), and also in the Chl *a* (~1.5-fold) and carotenoid (~1.7-fold) content in leaves (Table 2). The salt-stressed (160 mM NaCl) plants with added proline (30 mM) or GB (1 mM) alone or in the co-application alleviated the salt stress-induced reduction of RGR of shoots but not of roots. Proline was more effective than GB alone or the co-application of proline + GB on shoot RGR in the salt stressed plants bringing it back to the control level. In addition, the 160 mM NaCl-mediated reduction in Chl *a* and carotenoid content was alleviated by GB (1 mM) or proline (30 mM), and the co-application of both osmoprotectants increased the photosynthetic pigments to the level near or over that of the control (Table 2).

Discussion

Regarding the effect of the salt stress on leaves of Thai jasmine rice cv. KDML105, the results show that the H_2O_2 content and SOD, GR, and APX activities increased, whereas the CAT activity decreased. These results suggest that the oxidative stress induced by NaCl and characterized by a significant elevation of the H_2O_2 content led to the induction or reduction of antioxidant enzymes. The average SOD activity correlated with the change in the H_2O_2 content. It is possible that H_2O_2 increased probably as result of increased SOD activity (which produces H_2O_2) combined with insufficiently increased APX activity and decreased CAT activity (which removes it). The increased SOD, GR, and APX activities are consistent with previous reports in pea leaves (Hernandez and Almansa 2002) and in a moderately salinity tolerant wheat genotype (Sairam *et al*. 2005), but not in a tobacco By-2 suspension culture, where salt stress has no effect on SOD activity (Hoque *et al*. 2007).

It has been reported that an increase in H_2O_2 content caused by various abiotic stresses results in an increase in CAT activity to protect cells, *e.g.*, in salt-tolerant *Lycopersicon pennellii* (Mittova *et al*. 2003) and *Plantago maritima* (Sekmen *et al*. 2007). However, there are other reports showing, in agreement with our results, a decreased CAT activity in response to various stresses, such as in rice cv. Dongjin under drought stress (Lee *et al*. 2001), and mung bean (Hossain *et al*. 2011), tobacco suspension (Hoque *et al*. 2006), and rapeseed (Hasanuzzaman *et al*. 2011) under salt stress, and mustard seedlings under heat stress (Dat *et al*. 1998). Our report also shows that the GR and APX activities increased significantly under the salt stress, which is consistent with previous reports concerning *Plantago maritima* (Sekmen *et al*. 2007), wheat (Sairam *et al*. 2005), and pea leaves (Hernandez and Almansa 2002). Thus, the increased H_2O_2 generation in leaves of the salt-stressed rice plants might function in signaling oxidative stress which led to the induction of some antioxidative enzymes. The application of proline and/or GB to the

salt-stressed rice cv. KDML105 improved plant growth. However, the application of 30 mM proline, but not 15 or 45 mM, alleviated the increase in H_2O_2 content caused by the salt stress. These results suggest that the effective concentration may vary with plant species or cultivars, and an over-application may be toxic to some plants resulting in growth inhibition. Thus, the optimal concentration must be carefully determined before use. For example, the addition of 20 - 33 mM proline to mung bean cell cultures mitigates the effects of NaCl stress, whereas concentrations 50 mM or higher inhibit the growth of both salt-stressed and non-stressed cultures (Kumar and Sharma 1989). In alfalfa callus cultures, 10 mM exogenous proline is very effective in alleviating the effects of salt stress, whereas higher concentrations are not beneficial (Ehsanpour and Fatahian 2003). In rice cv. Ratna, the application of 30 mM proline counteracted the adverse effects of salinity on early seedling growth, although higher concentrations of proline resulted in a reduced growth (Roy *et al*. 1993). In addition, the application of 1 mM GB to the NaCl-stressed plants alleviated the salt-stress mediated increase in the H_2O_2 content to a level below that of the salt-stressed plants. Thus, when added separately, the optimal concentrations for exogenous proline and GB were 30 mM and 1 mM, respectively. The higher efficiency of proline than GB in lowering H_2O_2 content could be due to proline ability to directly scavenge ROS during stress (Smirnoff and Cumbes 1989, Chen and Murata 2008, Miller *et al*. 2010).

The application of 30 mM proline together with 1 mM GB (or 15 mM proline together with 0.5 mM GB, data not shown) did not alleviate the NaCl-mediated increase in H_2O_2 content more than the application of each osmoprotectant separately. This difference in the degree of alleviation of the effects of the salt stress between proline and GB might be due to the differences in their effects on CAT, GR, and APX activities and probably also other enzyme activities not investigated in the present experiments. Under the 160 mM NaCl stress, the GR activity decreased to the control level only in the presence of 30 mM proline, but not 1 mM GB alone or both 30 mM proline and 1 mM GB, whereas the application of only 1 mM GB or both 30 mM proline and 1 mM GB led to a decrease in APX activity to the control level. These results disagree with that reported in tobacco suspension where the application of proline led to an increase in APX activity under salt stress (Hoque *et al*. 2007). The CAT activity decreased under the 160 mM NaCl stress in rice and this decrease was not affected by the presence of GB alone or both proline and GB but increased to the control level with the addition of 30 mM proline.

The reductions in the content of Chl *a* and carotenoids in rice leaves and RGR of shoots after the 3-d recovery following the 3-d salt stress (160 mM NaCl), were alleviated by the application of 30 mM proline, 1 mM GB or the co-application of 30 mM proline and 1 mM GB. The effect of salinity on chlorophyll content may be a result of increasing activity of a chlorophyll-degrading enzyme, chlorophyllase (Reddy and Vora 1986). Degradation of β-carotene and formation of zeaxanthin may be involved in protection against photoinhibition (Sharma and Hall 1991). In this report, the increased photosynthetic pigment content under the salt stress due to proline and GB application and co-application of both osmoprotectants might be most effective in alleviating degradation of photosynthetic pigments probably because the osmolytes function in protecting macromolecules and stabilizing protein structures (Hayat *et al*. 2012, Wani *et al*. 2013).

In this study, the positive effect of 30 mM proline + 1 mM GB on the pigment content was observed but no synergistic effect of proline + GB on the antioxidant enzyme activities. However, it remains to be evaluated if the synergy will be observed under lower (suboptimal) proline and GB concentrations. There is a great deal of variation in different plant species in response to the application of proline and GB. Therefore, before the use of osmoprotectants as stress alleviators, the most optimal concentrations, appropriate plant developmental stages, and growth conditions must be carefully determined.

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