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BRIEF COMMUNICATION

Pretreatment with NaCl induces tolerance of rice seedlings to subsequent Cd or Cd + NaCl stresses

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Abstract

Rice (*Oryza sativa* L.) seedlings were grown hydroponically in Hoagland's nutrient solution under controlled conditions to investigate the effects of NaCl pretreatment on their response to subsequent application of cadmium (Cd) alone and Cd + NaCl combination. The Cd stress caused growth retardation in all plants, significantly reduced pigment content, stomatal conductance (g_s), and net photosynthetic rate (P_N). Cd stress significantly increased malondialdehyde and proline content. Compared to Cd treatment alone, combination stress had more detrimental effects on the above parameters. However, the NaCl pretreatment was beneficial in improving the plant growth and plant tolerance to Cd alone or combination stress.

Additional key words: antioxidative enzymes, malondialdehyde, *Oryza sativa*, photosynthesis, proline, stomatal conductance.

Cadmium is one of the most toxic trace pollutants which enter soil-plant system due to discharge of industrial waste water and the excessive use of phosphate fertilizers (Waisberg *et al.* 2003). Salinity is also one of the significant abiotic stresses limiting plant growth and development especially in arid and semi-arid regions. Up to 20 % of the irrigated land is already affected by salt (Abogadallah 2010). Salinity and Cd may occur at the same place which lead to plants suffering from multiple abiotic stresses and plant injury could be enhanced (Shafi *et al.* 2009). Some authors found that Cd content was increased in plants after application of phosphate fertilizers containing Cd or NaCl (Smolders *et al.* 1998, Weggler-Beaton *et al.* 2000). Therefore, it is very important to study the relationships between NaCl and Cd stress.

Salinity and heavy-metal Cd stresses not only disrupt cell membrane function and elicit lipid peroxidation, but also disturb redox homeostasis in plant cells which induce a burst of reactive oxygen species (ROS; Mühling and Läuchli 2003). The generation of ROS is one of the main causes of injuries in plants exposed to NaCl and/or Cd stresses (Shah *et al.* 2001, Leyva *et al.* 2011). Plants can scavenge ROS by antioxidant systems consisting of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), *etc.* (Shao *et al.* 2008). In addition to antioxidant systems, osmoprotector proline also plays an important role in resisting abiotic stresses (Sharma and Dietz 2006).

It has also been reported that cross-adaptation is an important strategy employed by higher plants to resist combined abiotic stresses (Boussiba *et al.* 1975). For example, NaCl pretreatment alleviated following salt stress in mungbean (Saha *et al.* 2010), soybean (Umezawa *et al.* 2000), and *Bruguiera cylindrica* (Atreya *et al.* 2009). NaCl pretreatment also enhanced the antioxidative response in the halophyte *Hordeum*

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Abbreviations: CAT - catalase ; g_s - stomatal conductance; MDA - malondialdehyde; P_N - net photosynthetic rate; POD - peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase.

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maritimum under potassium deficiency (Hafsi *et al.* 2010). However, very little is known about NaCl and Cd interactions. The objective of the present study was to investigate the effects of NaCl pretreatment on plant response to Cd and Cd+NaCl stresses.

The experiments were carried out with rice (*Oryza sativa* L. cv. Liaoxing No. 1). Seeds, surface sterilized in 2 % (m/v) sodium hypochlorite solution for 10 min, were subsequently triple-rinsed with sterile distilled water. Seeds were then soaked in sterile deionized water at 28 °C for 6 h and then transferred between two pieces of filter paper moistened with sterile deionized water. The seeds were germinated at 28 °C in the dark for 48 h. After germination, the seedlings were grown hydroponically in Hoagland's nutrient solution in a growth chamber at a 16-h photoperiod, photosynthetic photon flux density (PPFD) of 800 μmol m^{-2} s^{-1}, day/night temperature of 27/20 °C, and air humidity of 80 %. The nutrient solution was renewed every 2 d. When rice seedlings were 10-d-old, the NaCl was gradually added to the nutrient solution of pre-treated plants (the first day NaCl concentration was 25 mM, the 2nd - 5th day 50 mM). Then, 5 μM $CdCl_2$ or 5 μM $CdCl_2$ + 50 mM NaCl were added to the nutrient solution of pretreated or non-pretreated plants. Each parameter was determined at 10th day after pretreatment. All treatments were conducted in triplicate.

Seedling height and root length were determined immediately after harvesting. For determination of shoot and root dry masses, samples were oven-dried at 105 °C for 30 min and then kept at 80 °C for 24 h to obtain a constant mass.

The content of chlorophylls (Chl) and carotenoids (Car) was determined spectrophotometrically (Shanghai analytical instrument factory, Shanghai, China) according to Lichtenthaler (1987). Net photosynthetic rate (P_N) and stomatal conductance (g_s) were measured with a portable photosynthesis system (*LI-6400*, *Li-Cor*, Lincoln, NE, USA) at PPFD of 800 μmol m^{-2} s^{-1} and CO_2 concentration of 400 μmol(CO_2) mol^{-1}(air).

Enzymes were extracted at 4 °C from about 0.5 g of tissue from the second leaves in a mortar and a pestle with 5 cm^3 of extraction buffer, containing 0.1 M phosphate buffer (pH 7.8), 0.1 mM EDTA, and 1 % (m/v) polyvinylpyrrolidone (PVP). Homogenates were centrifuged at 10 000 *g* for 15 min and the supernatants were used as the crude extracts for superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) determinations. SOD activity was assayed as the inhibition of the photochemical reduction of β-nitroblue-tretrazolium chloride (NBT). One unit of SOD was defined as the amount of enzyme, which induced a 50 % inhibition of NBT reduction measured as absorbance decrease at 560 nm (Costa *et al.* 2002). CAT activity was determined as the decomposition of H_2O_2 causing the decline in absorbance at 240 nm (Cakmak and Horst 1991). POD activity was measured with guaiacol as the substrate. Increase in the absorbance due to oxidation of guaiacol was measured at 470 nm (Kochba *et al.* 1977). Protein content was estimated by the method of Lowry (1951) using bovine serum albumin as a standard. The estimation of malondialdehyde (MDA) content was done as described by Heath and Packer (1968). Proline content was determined by the method of Bates *et al.* (1973).

Pronounced damaging effects occurred in the rice plants exposed to the Cd or Cd+NaCl stresses. Root length, and shoot and root dry masses were prominently reduced in comparison with the control plants (Table 1). The NaCl pretreated plants were adapted to stresses because exposure to the 50 mM NaCl or Cd+NaCl stresses induced lower decrease of growth parameters in the pretreated plants than in the non-pretreated ones.

The growth reduction could be at least partially due to the effect of this heavy metal on P_N (Metwally *et al.* 2003). In this study, P_N exhibited a significant ($P < 0.05$) decrease under the Cd or Cd+NaCl stresses in comparison with the controls. However, NaCl pretreatment had a significant ($P < 0.05$) positive effect on the P_N of plants under the Cd or Cd+NaCl stresses (Table 1). The decrease in P_N might be due not only to the reduced g_s but also to reduced non-stomatal components and other factors (Djanaguiraman *et al.* 2006). The results of this study show that g_s decreased significantly under the Cd or Cd+NaCl stresses, however, less in the pretreated plants than the non-pretreated plants (Table 1). Also the total Chl content as well as Car content significantly decreased under the Cd or Cd+NaCl stresses compared to the controls, and again the reduction was less in the pretreated plants than in the non-pretreated ones (Table 1).

MDA content (a characteristics of lipid peroxidation) shows a significant ($P < 0.05$) increase under the Cd or Cd+NaCl stresses indicating the existence of oxidative stress. The present study also recorded that the MDA content in the NaCl pretreated plants was less increased than in the non-pretreated ones. The enhanced content of MDA could be attributed to the increased ROS production or by inhibition of antioxidative defense systems. However, activities of antioxidative enzymes (SOD, POD, and CAT) were increased in the Cd treated plants which is consistent with the observations of Tao *et al.* (2012). In contrast, Shafi *et al.* (2009) observed significant decline in CAT activity when wheat plants were exposed to Cd stress. Martin *et al.* (2012) reported that SOD activity was unaffected in *Thlaspi arvense* plants exposed to Cd stress. These different results may be partly due to variation among species, developmental phase, Cd concentration, and exposure time. In our experiments, the combined Cd+NaCl stress caused a more marked increase of the SOD and POD activities in the rice plants in comparison with Cd alone but not of the CAT activity (Table 1). Maybe, the Cd and NaCl stresses show synergistic effects on oxidative stress. According

Table 1. Effects of NaCl pretreatment on growth characteristics, net photosynthetic rate, stomatal conductance, content of chlorophylls and carotenoids, antioxidative enzyme activities, and MDA and proline content in rice seedlings under Cd alone (NaCl + Cd) and Cd+NaCl combination stresses (NaCl +. Each value represents mean ± SD ($n = 3$). The different letters indicate significant differences at $P < 0.05$.

Treatment	control	Cd	NaCl + Cd	Cd+NaCl	NaCl + Cd+NaCl
Seedling height [cm]	24.19 ±2.36a	22.03 ±1.31a	21.26 ±3.12a	22.43 ±2.65a	21.89 ±3.34a
Root length [cm]	15.35 ±1.63a	12.51 ±1.49c	11.45 ±1.57c	14.76 ±1.74ab	13.33 ±1.86bc
Shoot dry mass [g plant^{-1}]	0.114±0.012a	0.076±0.006b	0.053±0.008c	0.093±0.01ab	0.087±0.008b
Root dry mass [g plant^{-1}]	0.051±0.005a	0.035±0.003bc	0.026±0.007c	0.042±0.004a	0.033±0.006ab
P_N [μmol m^{-2}s^{-1}]	13.87 ±0.54a	9.21 ±0.32c	7.96 ±0.16c	12.45 ±0.37ab	11.56 ±0.45b
g_s [mmol m^{-2}s^{-1}]	104.00 ±1.76a	72.00 ±2.23c	65.00 ±1.45c	95.00 ±2.39b	98.00 ±1.03b
Chl content [mg g^{-1}(FM)]	2.87 ±0.17a	2.12 ±0.19c	1.98 ±0.21c	2.73 ±0.20b	2.45 ±0.11b
Car content [mg g^{-1}(FM)]	0.51 ±0.01a	0.38 ±0.03c	0.35 ±0.03c	0.43 ±0.02b	0.45 ±0.01b
MDA content [nmol g^{-1}(FM)]	14.51 ±1.56c	23.82 ±2.01a	25.06 ±1.39a	17.36 ±2.37b	19.27 ±2.19b
SOD activity [U mg^{-1}(protein)](	25.75 ±2.54d	36.32 ±5.69c	48.19 ±5.01b	46.04 ±6.10b	56.55 ±3.49a
POD activity [U mg^{-1}(protein)]	13.59 ±2.43d	20.46 ±2.38c	25.62 ±3.08b	23.90 ±2.53b	30.37 ±1.32a
CAT activity [U mg^{-1}(protein)]	37.45 ±0.83a	42.32 ±1.38a	39.39 ±2.32a	32.34 ±1.29a	35.48 ±1.75a
Proline content [μg g^{-1}(FM)]	21.26 ±1.07c	36.87 ±3.69b	34.51 ±2.41b	49.23 ±2.44a	43.35 ±2.61a

to Guo *et al*. (2012), combined Cd and elevated O_3 had a significantly synergic effect on oxidative stress in wheat shoots. However, Welfare *et al*. (2002) reported that NaCl and ozone in combination had an antagonistic effect on two chickpea cultivars. Therefore, further studies are required to understand the mechanisms of plant responses to multiple stresses. In the NaCl pretreated plants, the increase in the SOD and POD activities were even more pronounced when compared with the plants directly exposed to Cd or Cd+NaCl (Table 1). The increase of antioxidative enzyme activities could be due to induced adaptation in NaCl pretreated plants which afford better survival of plants exposed to subsequent ROS production induced by the Cd or Cd+NaCl stresses.

Proline content increased significantly when the plants were exposed to the Cd or Cd+NaCl stresses. The plants pretreated with NaCl had significantly higher proline content than the non-pretreated plants. It is possible to suppose that the elevated content of proline protected the rice seedlings from harmful effect of the subsequent Cd or Cd+NaCl stresses.

In conclusion, the Cd stress inhibited rice seedlings growth and decreased pigment content, P_N, and g_s whereas MDA and proline content was increased. The combination of the Cd stress and the NaCl stress show a synergistic effect on all the estimated parameters. NaCl pretreatment had the positive effects by improving the plant growth and tolerance to Cd alone or Cd+NaCl.

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