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BRIEF COMMUNICATION

Enhancement of antioxidant enzyme activities in rice callus by ascorbic acid under salinity stress

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Abstract

Ascorbic acid (AsA) is naturally occurring compound with antioxidant activity and plays a pivotal role in plant cell adaptation to salinity stress. The objective of this work was to assess the influence of exogenous AsA on the embryogenic callus of indica rice (*Oryza sativa* L.) cv. MRQ74 cultivated under saline conditions. NaCl (200 mM) decreased callus fresh and dry masses, relative growth rate, and K^+ and Ca^{+2} content, and increased Na^+ content and Na^+/K^+ ratio. Application of AsA (0.5 or 1 mM) alleviated these effects of salinity. Activities of peroxidase, catalase, superoxide dismutase, as well as content of proline increased due to the NaCl treatment, and these parameters were mostly further increased by 0.5 mM AsA. Thus, AsA can increase callus tolerance to NaCl stress.

Additional key words: catalase, embryogenic callus, peroxidase, proline, salt tolerance, superoxide dismutase.

The most harmful aspects of salinity stress are usually associated with a nutritional imbalance and low osmotic potential of the soil solution (Alhasnawi *et al.* 2014a). Ascorbic acid (AsA) is important component in phytohormone-regulated signalling networks that regulate the response of plant cells to different stresses including salinity (Khan *et al.* 2011, Alhasnawi *et al.* 2015). An antioxidant protection system, which include non-enzymatic antioxidants, such as glutathione and ascorbic acid; and enzymatic antioxidants, such as ascorbate peroxidase (APX), catalase (CAT), peroxidase (POX), and superoxide dismutase (SOD), regulates a potential harmful effect of reactive oxygen species (ROS) (Alhasnawi *et al.* 2014b) accumulated under stress conditions. Nevertheless, when plants are exposed to salt stress, oxidative damage can occur due to imbalance between ROS accumulation and detoxification (Gomez *et al.* 1999).

In vitro cultures are important in many aspects of molecular breeding and plant biotechnology (Kadhimi *et al.* 2014). They are also used for evaluation or even induction of stress resistance. Thus, in this investigation, we aimed to investigate the effect of AsA on rice embryogenic callus growth and activities of antioxidative enzymes.

Indica rice *(Oryza sativa* L.) cv. MRQ74 was used in our experiments. Selected mature seeds were dehusked and surface sterilized following the method described by Zinnah *et al.* (2013). The sterilized seeds were cultured in petri dishes in a callus induction medium containing a semi-solid Murashige and Skoog (1962; MS) medium supplemented with 3 mg dm^{-3} 2,4-dichlorophenoxy-acetic acid (2,4-D), 0.1 mg dm^{-3} 6-benzylamino-purine (BAP), and 30 g dm^{-3} sucrose. The pH of the media was 5.5 - 5.8,

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Abbreviations: ABA - abscisic acid; APX - ascorbate peroxidase; AsA - ascorbic acid; BAP - 6-benzylaminopurine; CAT - catalase; 2,4-D - 2,4-dichlorophenoxyacetic acid; ET - ethylene; GA_3 - gibberellic acid; JA - jasmonic acid; MS - Murashige and Skoog; POD - peroxidase; PVP - polyvinylpyrrolidone; RGR - relative growth rate; ROS - reactive oxygen species; SA - salicylic acid; SOD - superoxide dismutase.
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and 3 g dm^{-3} of *Gelrite* was added prior to autoclaving. The cultures were incubated at a temperature of 25 ± 2 °C in the dark, and the calli was subcultured to a fresh medium after two weeks. Then, high-quality embryogenic calli (100 mg) were selected and transferred into test tubes (one per tube) containing the above mentioned medium supplemented with NaCl (0 or 200 mM) and AsA (0, 0.5, and 1 mM). The calli were subcultured every two weeks for three months and fresh mass (f.m.), dry mass (d.m.), and relative growth rate [RGR = (ln final f.m. - ln initial f.m.) / cultivation time] were recorded.

Content of Na^+, Ca^{+2}, and K^+ in the calli were measured using an atomic absorption spectrometer, model *AAS 3110* (*U.S. Instrument Division*, Norwalk, CT, USA).

The calli (2 g) from the various treatments were homogenized using an ice-chilled pestle and mortar with 4 cm^3 of a 0.1 M phosphate buffer (7.2 pH), to which 0.1 g of polyvinylpyrrolidone was added. After centrifugation at 5 000 *g* and 4 °C for 10 min, the supernatant was utilized for assessment of POD, CAT, and SOD activities. Activity of POD was estimated by the method of Racusen and Foote (1965) with some modifications according to Munir (2009). In this procedure, 1 % (v/v) guaiacol ($C_7H_8O_2$) was used as substrate. The activity of the enzyme was determined by recoding absorbance at 470 nm using a UV-visible spectrophotometer (model *U-1900*, *Hitachi*, Kyoto, Japan). One unit of POD activity was defined as the change in absorbance by 0.1 units per minute under the specified conditions. Activity of CAT was carried out following the method of Beers and Sizer (1952) with a slight modification according to Sajid (2010). The disappearance of H_2O_2 was determined in the supernatant by recording absorbance at 240 nm. One unit of CAT activity was defined as the amount of the enzyme decomposing 1 μmol of H_2O_2 per min at pH 7.0. Activity of SOD was estimated following the procedure of Maral *et al.* (1977) with some modifications according to Munir (2009). This method is based on the capability of the enzyme to inhibit photochemical decomposition of nitroblue tetrazolium. Absorbance was measured at 560 nm and one unit of SOD activity inhibited the maximum reduction of nitroblue tetrazolium by 50 % under the conditions of assay. Protein content was estimated by the biuret method (Racusen and Johnstone 1961). Proline content was estimated by the ninhydrin method according to Bates *et al.* (1973).

Statistical analyses of data were performed by *ANOVA* at the α = 0.05 level. The experiments were organized in a completely randomized design with 10 replicates. Significant differences between means of all recorded parameters were analyzed using Duncan's multiple range test. The statistical analyzes were conducted using the *SAS* system *v. 6.1.7600* for *Windows*.

The rice calli were analyzed upon exposure to NaCl (0 or 200 mM) and AsA (0, 0.5, and 1 mM) for three months. The salt stress significantly decreased fresh and dry masses and RGR but less when NaCl was combined with AsA (Table 1). The inhibition of growth of the calli might be result of reduced water availability in the culture medium similarly as was observed by Priya *et al.* (2011). Salt stress can also induce ion imbalance, which may result in ion toxicity (Reddy and Vaidyanath 1986). The present results agree with those found in Basmati rice calli, which exhibit a decrease in both fresh mass and dry mass under NaCl stress (Reddy and Vaidyanath 1986, Priya *et al.* 2011). Ahmad *et al.* (2007) examined six genotypes of rice and RGR of calli decreases under NaCl stress.

The applied AsA (0.5 or 1 mM) improved growth of the calli under the NaCl stress compared with NaCl alone (Table 1). The present results are consistent with the beneficial impacts of AsA application; *e.g.*, AsA partially mitigates the adverse impact of salinity on cell enlargement and division (Beltagi 2008). Ascorbic acid is also involved in regulation of cell cycle progression (Conklin 2001). Khan *et al.* (2011) mentioned that AsA currently holds an important position in plant physiology, mainly due to its possession of cellular reductant, its antioxidant properties, and its diverse functions in plant growth, development, and responses to abiotic stresses. The role of AsA in division and cell elongation by modifying the cell cycle has shown Saeidi-Sar *et al.* (2013).

The calli exposure to 200 mM NaCl caused a decrease of K^+ and Ca^{2+} content in the calli. Under the salt stress, the application of AsA (0.5 or 1 mM) to the culture media produced a significant increase in the amount of K^+ and Ca^{+2} compared with the salinity alone. A similar result was previously reported by Ahmad *et al.* (2007). The effect of accumulation of Cl^- as consequence of a high concentration of NaCl is associated with an increased accumulation of Na^+. However, the total charges must be balanced in cells; thus, the accumulation of Na^+ decreases the content of Ca^{2+} and K^+. Ahmad *et al.* (2007) reported that salinity affects the plasma membrane by displacing Ca^{2+} by Na^+, which lead to leakage of K^+ from cells due to the alteration in plasma membrane permeability. In addition, increased water stress is associated with an increased quantity of ions that allow plant cells to withstand a damage by water stress *via* osmotic adjustment. In our experiments, AsA (especially 0.5 mM) caused accumulation of K^+ and Ca^{+2} in the calli (Table 1). Sodium chloride in the callus media significantly influenced Na^+ content and Na^+/K^+ ratio. Moreover, the applied AsA decreased Na^+ content and Na^+/K^+ ratio of the salt-treated calli. Similar results have been reported by Sathish *et al.* (1997), Bassuony *et al.* (2008), Khafagy *et al.* (2009), and Alhasnawi *et al.* (2015). In addition, AsA can decrease a stress-induced increase in electrolyte leakage due to a decreased damage of cell membranes (Khan *et al.* 2011). Saeidi-Sar *et al.* (2013) reported the protection of common bean seedlings against NaCl by applied AsA as consequence of its effect on K^+ uptake. Hussein *et al.* (2011) also found that the application of AsA significantly increases K^+ and Ca^{+2} in a wheat flag leaf.

Table 1. The effect of 200 mM NaCl alone or together with 0.5 or 1.0 mM ascorbic acid (AsA) on fresh mass, dry mass, relative growth rate (RGR), content of K^+, Ca^{+2}, and Na^+, Na^+/K^+ ratio, specific activities of peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD), and proline content of rice calli. Means ± SDs, n = 10. Values within rows with different letters are significantly different at $P \leq 0.05$ (according to Duncan's multiple range test).

Parameter	Control	200 mM NaCl	200 mM NaCl + 0.5 mM AsA	200 mM NaCl + 1.0 mM AsA
Fresh mass [mg callus^{-1}]	932.91 ± 22.33^a	500.86 ± 25.14^d	627.10 ± 20.60^b	565.41 ± 24.48^c
Dry mass [mg callus^{-1}]	187.19 ± 10.19^a	98.29 ± 9.04^d	129.68 ± 10.70^b	117.64 ± 7.80^c
RGR [week^{-1}]	64.78 ± 1.74^a	31.18 ± 1.96^d	41.00 ± 1.60^b	36.20 ± 1.90^c
K^+ content [mmol g^{-1}(d.m.)]	0.224 ± 0.016^a	0.088 ± 0.009^c	0.153 ± 0.012^b	0.098 ± 0.014^c
Ca^{2+} content [mmol g^{-1}(d.m.)]	0.035 ± 0.002^c	0.008 ± 0.003^d	0.046 ± 0.004^b	0.072 ± 0.003^a
Na^+ content [mmol g^{-1}(d.m.)]	0.024 ± 0.007^d	0.333 ± 0.017^a	0.255 ± 0.014^c	0.269 ± 0.007^b
Na^+/K^+ ratio	0.11 ± 0.03^d	3.83 ± 0.44^a	1.68 ± 0.20^c	2.80 ± 0.39^b
POD [U mg^{-1}(protein)]	0.010 ± 0.004^d	0.048 ± 0.003^b	0.090 ± 0.005^a	0.032 ± 0.003^c
CAT [U mg^{-1}(protein)]	6.07 ± 0.99^c	9.84 ± 0.90^b	11.22 ± 0.98^a	6.75 ± 0.84^c
SOD [U mg^{-1}(protein)]	8.05 ± 1.42^d	18.92 ± 1.26^c	32.53 ± 1.23^a	25.78 ± 1.75^b
Proline [µmol g^{-1}(f.m.)]	6.11 ± 0.52^d	15.70 ± 0.95^c	27.87 ± 0.94^a	25.54 ± 1.03^b

Furthermore, high NaCl concentrations lead to production of ROS, and plant salt tolerance is correlated with regulatory mechanisms. Reactive oxygen species can cause oxidative damage to vital molecules, such as proteins, DNA, and lipids, and this damage must be repaired efficiently and continuously (Hajiboland 2013, Hörandl and Hadacek 2013). The induction of ROS-scavenging enzymes, *e.g.*, CAT, POD, and SOD occurs as stress response (Alhasnawi *et al.* 2014b). In this study, we found that the activities of antioxidative enzymes POD, CAT, and SOD increased as result of the NaCl stress relative to the control (Table 1). With the 0.5 mM AsA application to the culture media, POD, CAT, and SOD further increased as compared to the control or 200 mM NaCl alone, however, 1.0 mM AsA decreased these activities (Table 1). From the data reported here, the antioxidative enzyme system was activated by NaCl. Similar results were observed in the calli of other rice cultivar (Swapna 2003), and in the calli of sugarcane (Munir *et al.* 2013). Several studies have shown that AsA plays an important role in improving callus tolerance to abiotic stresses (Amiri *et al.* 2013). Plants with a high antioxidant content show a considerable resistance to oxidative damage (Garratt *et al.* 2002). The importance of SOD, POD, and CAT activities depends on plant species. An increase in ability to scavenge the superoxide radical by SOD should be accompanied by higher H_2O_2 detoxifying enzyme activities. Salt-tolerant plants may have a better protection against ROS *via* accumulation of antioxidant enzymes under salinity (Rahnama and Ebrahimzadeh 2005). The present results agree with those of Shao *et al.* (2008), who found that the application of AsA protects metabolic processes against H_2O_2 and stabilizes membranes. Application of AsA also modifies non-enzymatic antioxidants, such as carotenoids, glutathione, and tocopherols, and stimulates signalling by various plant hormones (Mazid *et al.* 2011). In agreement with this, AsA as antioxidant can alleviate from the influence of NaCl stress on metabolism, development, and growth (Younis *et al.* 2010, Agami 2014).

Proline accumulation under salinity stress is probably one of the most evident metabolic changes that are often observed under stress conditions. Alhasnawi *et al.* (2014c) have reported that proline accumulation in many folds under salinity stress may be caused by a stimulation of its synthesis from glutamate, a lower rate of degradation, and a lower incorporation into proteins. Demiral and Turkan (2005) found that accumulation of the Na^+ ion in the apoplastic solution results in accumulation of proline in cytoplasm, particularly in tolerant rice cultivars. Accumulation and production of free amino acids, particularly proline, under salinity stress are adaptive responses (El Sayed 2013). Ahmad *et al.* (2007) found that a proline increase is not only a signal of cellular damage but also a marker of stress tolerance performing a definite osmoregulatory function. Vanekamp *et al.* (1989) found that a higher content of proline reduces acidity of cytoplasm.

In our experiments, the application of 200 mM NaCl significantly increased proline content of the calli compared with the control. However, the maximum content of proline was observed in the calli that were grown with 200 mM NaCl and 0.5 mM AsA (Table 1). Abd El-Azizi *et al.* (2006) detected an increase in content of proline under NaCl stress when seeds were treated with AsA. The effect of AsA on content of proline suggests that AsA may affect osmotic adjustment in addition to its antioxidant action (Azzedine *et al.* 2011).

In conclusion, AsA improved callus growth under the NaCl stress and increased callus tolerance to the NaCl stress due to increased POD, CAT, and SOD activities and proline content.

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