Exogenous ascorbic acid and hydrogen peroxide alleviates salt-induced oxidative stress in rice (Oryza sativa L.) by enhancing antioxidant enzyme activities and proline content

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ABSTRACT
Exogenous protectants such as ascorbic acid (AsA) and signaling molecules such as hydrogen peroxide (H₂O₂) could be a promising approach to mitigate salt-induced oxidative stress in rice. The present study was carried out to examine the effects of exogenous application of hydrogen peroxide (H₂O₂) and ascorbic acid (AsA) on the growth, content of photosynthetic pigments, proline, ascorbic acid and antioxidant enzymes of rice (Oryza sativa L.) cv. BRRI dhan36 seedlings grown in greenhouse hydroponically under 125mM NaCl. The plants exposed to the NaCl stress exhibited a significant reduction in growth, leaf photosynthetic pigments, ascorbate peroxidase (APX) as well as increased in proline, ascorbic acid and catalase (CAT) activity. The AsA and H₂O₂ application not only mitigated the inhibitory effects of salt stress but also induced a stimulatory effect on all the studied growth parameters. The activities of antioxidant enzymes (CAT and APX) as well as proline contents of rice plants were significantly increased after AsA and H₂O₂ application. The present study; therefore, suggests that exogenous AsA and H₂O₂ confer tolerance to salt stress in rice by enhancing proline accumulation and antioxidant defense systems, in addition exogenous H₂O₂ maintain higher endogenous ascorbate content.

KEYWORDS: Catalase, ascorbate peroxidase, proline, rice, salinity stress.

INTRODUCTION
Rice (Oryza sativa L.) is one of the main staple food in Bangladesh. The total production of rice has become static over the years with the gradual decrease of crop-growing areas. The static production of rice is still attributable to the lack of suitable improved cultivars for different environmental conditions. Adverse climatic changes pose a great threat for the agriculture and the future food security. Among the various abiotic stresses salinity is one of the most threatening factors in coastal area in Bangladesh. Coastal areas constitute about 2.5 million ha which amount to about 11% of the total cropland.

Recent report showed that salinity is the second most widespread soil problem next to drought in rice growing countries and is considered as a serious constraint to increased rice production worldwide [1]. The induction of salt tolerance in plants is crucial to maintain their economic yield. This can be achieved either through genetic modifications or chemical treatments [2]. The strategies of plant breeding and genetic
engineering are long-term and complex endeavours to develop salt tolerance that have had limited success [3]. Alternatively, the exogenous application of plant growth regulating compounds is an efficient and technically simpler approach to cope with the deleterious effects of salinity on plants [4, 5]. Exogenous application of protectants, plant growth regulators, fertilizers, and non-enzymatic antioxidants has been successfully used to minimize the adverse effects of salinity on plant growth and yield [6, 7].

Ascorbic acid is a naturally occurring compound with antioxidant activity that plays a pivotal role in plant cell adaptation to salinity stress.[8]. It has been used to counteract the adverse effects of salt stress in many crop plants [9, 10, 11] as well as functions in whole plant metabolism [12]. Furthermore, experimental studies on different plants have shown that exogenous application of AsA may reduce salt-induced adverse effects and results in a significant increment of growth and yield [10, 11, 13]. AsA is absorbed readily after exogenous application [14, 15] and moves within the plant [16]. Therefore, foliar application of AsA improves salt tolerance of crop plants in a number of ways [17, 18, 19].

In plants, hydrogen peroxide is one of the major and the most stable ROS and regulates basic processes, such as acclimation, defense and development [20]. The generation of H$_2$O$_2$ is increased due to a wide variety of stresses, and some authors have suggested that H$_2$O$_2$ is a key factor mediating the phenomena of acclimation and cross-tolerance [21]. Recent investigations have revealed that H$_2$O$_2$ is a central component of the signal transduction cascade involved in plant adaptation to a changing environment [21]. H$_2$O$_2$ plays a dual role in plants: at low concentrations, it acts as an acclimatory signal, triggering tolerance to various stresses [22, 23], and at high concentrations, it organizes programmed cell death [24]. Exogenous application of H$_2$O$_2$ at low concentrations signals the induction of defense responses in plants against oxidative stresses and assists in triggering stress resistance mechanism [25]. In maize, exogenously applied H$_2$O$_2$ increases salt tolerance by increasing the activities of antioxidants [26].

The present study was carried out to evaluate the potential of exogenously applied AsA and H$_2$O$_2$ in alleviation of salt stress in rice seedlings and study proline accumulation, antioxidant enzyme activities, endogenous ascorbate content and growth under NaCl stress in the presence of AsA and H$_2$O$_2$ treatments.

**MATERIALS AND METHODS**

**Experimental conditions:**

The experiment was conducted at the laboratory of the Department of Biochemistry and Molecular Biology, Bangladesh Agricultural University, Mymensingh, during January to March 2016. Plants were grown in hydroponic solution using Peter 20:20:20 and FeSO$_4$.5H$_2$O solution for 21 days.

**Plant materials and Treatments:**

BRRI dhan56 (High yielding rice variety) was used in this study. Six treatment combinations viz. T$_0$=control (no NaCl or no AsA or no H$_2$O$_2$), T$_1$=125mM NaCl, T$_2$= 150 mg/L AsA, T$_3$= 125 mM NaCl+150 mg/L AsA, T$_4$=1mM H$_2$O$_2$, T$_5$=125 mM NaCl+1mM H$_2$O$_2$ were used. NaCl was used to develop salinity. The experiment was laid out in a completely randomised block design with three replications. Initially, plants of T$_2$ and T$_3$ treatment groups treated by exogenous 150 mg/L ASA and T$_4$ and T$_5$ treatment group were treated by 1mM H$_2$O$_2$ for 7 days with nutrient solution. After 7 days, salinity was imposed in T$_1$, T$_3$ and T$_5$ treatment groups.

**Growth parameter:**

Shoot length and root length were recorded after 21 days of treatments. Ten randomly selected plants of each treatment were used for data recording. Root length was measured from the base to the top of root whereas shoot length was measured from the base to the tip of the longest leaf of the shoot.

**Biochemical and Enzymatic determination:**

After 21 days, leaf sample was collected and further biochemical analyses were performed. Chlorophyll and proline content were measured as previously described [27, 28]. Enzymes assays for catalase (EC: 1.11.1.6) and ascorbate peroxidase (EC 1.11.1.11) were determined by the method as described previously [29]. AsA content was determined by the titrimetric method using dye ( ) solution.

**Statistical analysis:**

Data for all parameters were statistically analyzed using the MSTAT-C statistical package. Statistically different groups were compared using an LSD test (P<0.01).

**Results:**

**Protective effect of ascorbic acid and H$_2$O$_2$ on growth of rice against salt stress:**
Salt stress caused a significant decrease in growth (root length and shoot length) of rice when they were exposed to 125 mM NaCl (Table 1). Exogenous application of AsA and H$_2$O$_2$ significantly increased root and shoot growth while seedlings were under salinity stress.

**Effect of exogenous AsA and H$_2$O$_2$ on chlorophyll, proline and AsA contents:**

Our present data shows that a significant (P≤ 0.01) reduction in chlorophyll-a, chlorophyll-b as well as proline contents under salt stress. Intriguingly, there was no change in case of AsA contents. Interestingly, exogenous application of AsA and H$_2$O$_2$ significantly (P≤0.01) increased Chlorophyll-a, chlorophyll-b, proline and AsA contents (Table 1). But these exogenous protectants could not increase chlorophyll contents up to the control both in non-saline and saline condition. On the other hand exogenous H$_2$O$_2$ highly increased proline content and significantly increase AsA content compared to saline and non-saline condition.

**Activity of antioxidant enzymes induced by exogenous AsA and H$_2$O$_2$ under salt stress:**

To investigate whether AsA and H$_2$O$_2$ enhance antioxidant defense system of rice under salt stress, activities of major ROS-scavenging antioxidants enzyme viz. CAT and APX were measured. Salt stress caused significant increase in CAT and decrease in APX activities in rice. Exogenous application of AsA and H$_2$O$_2$ significantly increase CAT and APX activities compared to salt stress condition and non-stress condition (Table 1).

**Discussion:**

Salt stress caused a remarkable reduction in all growth parameters measured in rice plants exposed to 125 mM saline stress. Exogenous application of AsA reduced the adverse effect of salt stress on different growth parameters of rice seedlings (Table 1). Excess salt induced ionic and osmotic stresses that disturbed metabolism and led to reduction of plant development [30]. AsA induced increase in growth of rice plants under saline conditions might be the role of AsA in increasing cell division and/or cell enlargement [17]. Previous study revealed that AsA plays a multiple roles in plant growth, functioning in cell division, cell wall expansion, and other development processes [31]. It has also been observed that exogenous application of AsA can also counteract salt-induced growth inhibition in plants, e.g., brassica [11], durum wheat [32], barley [33], and sunflower [34]. [17] observed that AsA applied at 100 mg/L improved growth of a salt tolerant cultivar of wheat under saline conditions and this AsA-induced enhancement of growth was found to be associated with enhanced photosynthetic efficiency. Similar growth promoting effect of AsA was also observed in chickpea [9]. In our study, H$_2$O$_2$ application significantly increased root length under salinity stress. Similar result was found by [35] that H$_2$O$_2$ significantly improved the growth of salt stressed plants.

Chlorophyll is one of the most important pigment components, providing photosynthetic ability of a plant. Chlorophyll-a and chlorophyll-b contents decreased in rice cultivars due to salt stress (Table 1). In our study, exogenous AsA and H$_2$O$_2$ alleviated the negative effect of salt stress and improved chlorophyll content (Table 1). These results are totally different to those of [36] in which a significant decrease in chlorophyll content has been observed in salt stressed wheat plants due to exogenously applied ascorbic acid. However, in contrast, in many reports an improvement in photosynthetic pigments has been reported due to exogenous application of ascorbic acid, e.g., in Brassica campestris [11] and Chickpea [9]. On the other hand, exogenous H$_2$O$_2$ application could be associated with the H$_2$O$_2$-mediated increase in AsA and glutathione (GSH) concentrations, which act as an antioxidants and photosynthetic machinery protector from salt-induced ROS. It has been shown that pre-treatment of seeds with H$_2$O$_2$ increase the net photosynthetic rate in wheat seedlings [37, 38].

The activities of antioxidant enzymes (CAT and APX) in the rice plants were increased significantly in exogenously applied AsA condition under the NaCl stress as well as only after the AsA application (Table 1). These higher CAT and APX activities help plants in defense to possible oxidative damage. [39] suggested that improved antioxidative defense system due to foliar application of ascorbic acid alleviates the detrimental effects of salinity and increased resistance to salinity in the maize plants. Similarly to our results, [17, 13] reported an increase in antioxidant enzyme activities in wheat plants after an AsA application.

As well as, salt stress increased CAT activities but decreased APX activity in plants and in exogenously H$_2$O$_2$ treated plants under salt stress produced significantly increased CAT and APX antioxidant enzymes (Table 1). [40] found that exogenous H$_2$O$_2$ is able to moderate activities of antioxidant enzymes reducing stress intensity at low light. Recently, [41] found that Panax ginseng seedlings treated with 100 µM H$_2$O$_2$ for 2 days showed enhanced salinity tolerance and increased activities of CAT and APX.

Proline accumulation is often used as stress indicator in plants [42, 43]. Its content significantly increased in the rice seedlings exposed to the salt stress (Table 1) and might serve as a compatible solute [44]. It has been found that a salt stress up-regulated the enzymes involved in proline biosynthesis [45]. The proline content of the rice leaves also shows an increasing trend after the AsA application under the NaCl stress (Table 1). Similar results have been reported in maize [46], barley [33], where applications of different levels of AsA increase the proline content of plants.
Also, proline content increased with the H₂O₂ application under non saline and salt stress compared to control. Maximum increase of about 3 times in proline content was noted with the 1mM H₂O₂ under salt stressed plants compared to control. Proline can protect plants from stress through different mechanisms, including osmotic adjustment, detoxification of ROS, protection of membrane integrity, and stabilization of proteins/enzymes [47]. Exogenous H₂O₂ may increase the proline content in *Nitraria tangutorum*, which could be alleviated by H₂O₂ scavengers [48]. In the present study, accumulation of proline content under salt stress was increased by the application of H₂O₂ and reversed the deleterious effects of salt stress. The reversal of adverse effects of salt stress on photosynthesis by H₂O₂ may be attributed to the accumulation of proline which maintained the cellular osmotic adjustments by increasing osmotic potential and water potential and protected photosynthetic machinery from salt stress by acting as an oxygen radical scavenger. [49] suggested that H₂O₂ might be involved in signal transduction events, leading to proline accumulation in maize seedlings, and that the H₂O₂-induced proline accumulation.

AA is a small soluble antioxidant molecule fulfills essential metabolic functions in plant cells [50]. According to [51], the ascorbic acid could react directly with hydroxyl radicals, superoxide, and singlet oxygen and thus provides protection by scavenging free radicals. Although salinity had increased the leaf AsA contents, it did not promote any significant alteration. Exogenous AsA and H₂O₂ increased endogenous AsA content. Similar result was shown by [17] where exogenous application of AsA increased endogenous level of AsA of wheat. Also, [35] have been reported that, H₂O₂ pre-treatment in maize plants increased AsA content.

Overall mechanism of AsA and H₂O₂ to bring salt tolerance in rice seedlings is shown in Figure 1.

![Fig. 1: Overall mechanism of AsA and H₂O₂ to bring salt tolerance in rice seedlings.](Image)

Table 1: Effect of exogenous AsA and H₂O₂ on different morphological and biochemical parameters. Values represent the mean of three replications. Same letter in a column represents insignificant difference at p<0.01.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Morphological parameters</th>
<th>Biochemical parameters</th>
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<tbody>
<tr>
<td></td>
<td>Shoot length (cm)</td>
<td>Root length (cm)</td>
</tr>
<tr>
<td>T₀  (Control)</td>
<td>58.39±1.97a</td>
<td>17.82±0.42b</td>
</tr>
<tr>
<td>T₁ (125mM NaCl)</td>
<td>50.44±1.25a</td>
<td>12.97±0.62c</td>
</tr>
<tr>
<td>T₂ (150mg/L AsA)</td>
<td>53.11±0.78a</td>
<td>15.82±0.72bc</td>
</tr>
<tr>
<td>T₃ (125mM NaCl+ 150mg/L AsA)</td>
<td>53.84±0.85a</td>
<td>14.98±0.98d</td>
</tr>
<tr>
<td>T₄ (1mM H₂O₂)</td>
<td>44.27±2.26a</td>
<td>15.35±0.21d</td>
</tr>
<tr>
<td>T₅ (125mM NaCl+ 1mM H₂O₂)</td>
<td>44.34±1.26a</td>
<td>13.49±0.61b</td>
</tr>
<tr>
<td>CV (%)</td>
<td>2.95</td>
<td>4.23</td>
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</table>

1. Salinity accelerates ROS production.
2. ROS impaired growth and chlorophyll content.
3. Exogenous AsA enhanced proline and ROS scavenging enzymes.
4. Exogenous H₂O₂ increased endogenous ascorbate, proline and ROS scavenging enzymes.
5. These parameters mitigate adverse effect of ROS on growth and photosynthetic efficiency.
Conclusion:
The present study and the available literature reviewed in the discussion suggest that exogenous AsA and H$_2$O$_2$ are effective in improving salt stress tolerance in rice seedlings. Enhancement of enzymatic antioxidants and proline might be responsible for regulation of ROS during NaCl stress. The elevated activity of antioxidant system, at least in part, protected the photosynthetic machinery and increased the tolerance of the rice plants to the NaCl stress. However, still very little is known about the role of AsA and H$_2$O$_2$ in plants. Although some researchers have successfully used AsA and H$_2$O$_2$ in different plants for mitigating adverse effect of different abiotic stresses; its roles in tolerances to diversified of stresses in relation to plant architecture, growth and metabolism still demand huge researches.

REFERENCES

388-395.


