Use of spermidine reduced the oxidative damage in onion seedlings under salinity by modulating antioxidants

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This research studies the role of spermidine (Spd) in conferring tolerance in onion seedlings under oxidative stress, caused by NaCl. Stress condition was applied on two months old onion seedlings by adding 10 g L⁻¹ of NaCl, where 100 µM of Spd was sprayed twice daily before counting the stress duration. Under salinity stress, seedlings were observed for 7 days, and data were measured on relative leaf water, proline, reactive oxygen species (ROS), lipid peroxidation (as malondialdehyde, MDA), amine oxidases, enzymatic and non-enzymatic antioxidants in leaves. Salinity stress decreased the relative water content (RWC), where Spd application delayed the loss of RWC. Contrariwise, Spd increased the proline content in salinity stressed seedlings up to five days. Salinity increased the contents of superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and MDA continuously and significantly with stress duration. More importantly, application of Spd decreased the ROS and MDA contents in stressed seedlings more effectively, up to three days of stress. Spd maintained higher activities of polyamine oxidase (PAO) and diamine oxidase (DAO) under salinity. Higher activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR) in presence of Spd over salinity during the study period, suggested their ROS scavenging role under salinity stress. Conversely, glutathione peroxidase (GPX) and dehydroascorbate reductase (DHAR) played important role in reducing the oxidative stress for 3 to 5 days. Spd also maintained higher reduced glutathione (GSH), ascorbic acid (ASA) and their redox homeostasis in leaves during the study period. Thus, Spd observably confirms better tolerance in short term salinity.

Key words: Spermidine, oxidative damage, salinity, antioxidants, onion seedlings.

INTRODUCTION

Onion is the most important spice crops in Bangladesh. However, the production of this crop is hampered in coastal soil of southern districts of Bangladesh. Tidal flash of sea water increases soil salinity which is a major
environmental stress affecting plants growth and productivity of the crop. In plants, salinity causes oxidative stress by producing reactive oxygen species (ROS) such as superoxide radicals \( \left( \text{O}_2^- \right) \), singlet oxygen \( \left( \text{O}_2 \right) \), hydroxyl radicals \( \left( \cdot \text{OH} \right) \) and \( \text{H}_2\text{O}_2 \) (Hasegawa et al., 2000; Apel and Hirt, 2004). Higher ROS causes damage to cell organelles like proteins, DNA, lipids, pigments and carbohydrates which ultimately lead to cell death (Apel and Hirt, 2004; Gill and Tujeta, 2010). Conversely, higher methylglyoxal (MG) production under salinity causes potential damage to the cell organelles (Yadav et al., 2005a, b). Hence, higher concentration of ROS and MG under stress is essentially needed to be reduced in cell, to survive and grow.

To survive under such situation, plants hold antioxidant system in cell to reduce the oxidative damage by ROS (Gill and Tujeta, 2010). Plants have both enzymatic and non-enzymatic antioxidants which take part in scavenging of ROS produced during various environmental stresses. Among the enzymatic antioxidant in plants, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione S-transferase (GST), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) are important. On the other hand, non-enzymatic antioxidants like ascorbic acid (ASA) and reduced glutathione (GSH) play important role in maintaining the enzymatic activities (Rohman et al., 2016; Gill and Tujeta, 2010). Alternatively, glyoxalase-I (Gly-I) and glyoxalase-II (Gly-II) detoxify MG in cell (Yadav et al., 2005b). It has been repeatedly reported that, enzymes both the antioxidant and glyoxalase system are important to lessen the toxicity of ROS and MG under stress (Singla-Pareek et al., 2008; Noctor et al., 2012; Saxena et al., 2011).

Spermidine, a triamine of polyamine (PA) group, plays important role in growth and development of plants (Martin-Tanguy, 2001). PAs are also well known for their antioxidant properties as well as their cell membrane stability (Zhao and Yang, 2008). Due to cationic nature, PAs are reported to stabilize protein, DNA and lipids of cell membrane (Bouchereau et al., 1999). In addition, they have been reported to have defensive role under abiotic stresses (Alcázar et al., 2006). Importantly, cellular level of PAs shows positive correlation with plant tolerance towards environmental stresses (Nada et al., 2004; He et al., 2002; Shen et al., 2000; Roy and Ghosh, 1996; Besford et al., 1993; Krishnamurthy and Bhagwat et al., 1989). Yiu et al. (2008) reported Spd mediated higher activities of antioxidant enzymes in welsh onion (Allium fistulosum L.), under submerged condition.

Previously, enhanced activities of glyoxalases and GSTs in onion (Allium cepa L.) by exogenous Spd under salinity were reported, where the activities of the enzymes were upregulated with lower MG content (Islam et al., 2016). Therefore, exogenous Spd might have important role in reducing of ROS and regulating of related physiological activities under salinity. Considering these, we applied exogenous Spd to examine its role in maintaining ROS and related physiological activities, by measuring enzymatic and non-enzymatic antioxidants in onion under saline stress.

MATERIALS AND METHODS

Plant material and stress treatment

Seedlings of two months old (Allium cepa L. var BARI Piaj-3) were used as plant material. They were grown in plastic bucket (30 L), under green house of Bangladesh Agricultural Research Institute (BARI), and the seedlings were imposed to salinity stress by adding NaCl saline solution (10 g L\(^{-1}\)). An EC meter (Hanna 993310) was used to measure salinity level. Spermidine at 100 μM concentration was sprayed twice daily. Saline was added until the level became 16 dS m\(^{-1}\), to attain salinity level of 16 dS m\(^{-1}\).

When the salinity level attained 16 dS m\(^{-1}\), addition of both saline water and Spd was stopped, and salinity duration was counted. Soil surface was sealed with polythene to maintain the soil moisture. This condition was maintained for seven days. A control without salinity and Spd was maintained under same condition. Data were measured at 1, 3, 5 and 7 days of stress, in fully expanded leaves on different parameters.

Measurement of relative water content

Relative water content (RWC) was calculated according to the method of Barrs and Weatherley (1962). Data on fresh weight (FW), turgid weight (TW) and dry weight (DW) of leaves were recorded. The below formula was used to calculate RWC:

\[
\text{RWC} \% = \left( \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \right) \times 100
\]

Measurement of \( \text{O}_2^- \) generation rate and \( \text{H}_2\text{O}_2 \)

Superoxide radical generation rate was measured according to Rohman et al. (2016). Method of Yu et al. (2003) was used in measuring \( \text{H}_2\text{O}_2 \).

Measurement of lipid peroxidation

Heath and Packer (1968) method was monitored to measure the level of lipid peroxidation, which was assayed as melondialdehyde (MDA), a peroxidation product of polyunsaturated fatty acid of the membrane lipid.

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Determination of proline

Ninhydrin was used to produce prolin’s reaction which was used to estimate proline following the method of Bates et al. (1973).

Extraction and measurement of ascorbate and glutathione

Half gram of fresh onion leaves were homogenized in 3 ml extraction buffer containing 5% meta-phosphoric acid and 1 mM EDTA. Homogenates were centrifuged at 11,500×g for 15 min by 4°C, and the supernatant was used in analysis of ascorbate and glutathione. Method of Huang et al. (2005) was used to measure ascorbate while Yu et al. (2003) was used to assay for glutathione pool.

Determination of protein

Content of protein was determined according to Bradford (1976) where, BSA was used as standard.

Enzyme extraction and assays

Half gram of leaf tissue was homogenized in 1 ml of 50 mM K-phosphate buffer (pH 7.0), which contains 100 mM KCl, 1 mM ascorbate, 5 mM β-mercaptoethanol and 10% (w/v) glycerol. The homogenates were centrifuged at 11,500×g for 10 min, and the supernatants were used for determination of enzyme activity. All process was carried out below 4°C.

Diamine oxidase (DAO, EC: 1.4.3.6) and polyamine oxidases (PAO, EC: 1.5.3.11) activities were measured by the method of Gao et al. (2005), with few modifications. Fresh samples of onion leaves were homogenized in 100 mM phosphate buffer (pH 6.5) and the homogenate was centrifuged for 20 min at 4°C by 10,000×g. The supernatant was used for enzyme assay. The reaction mixture contained 2.5 ml of phosphate buffer (100 mM, pH 6.5), 0.2 ml of 4-aminoantipyrine/N,N-dimethylbenzidine reaction solution, 0.1 ml of horseradish peroxidase (250 U/ml), and 0.2 ml of the enzyme extract. The reaction was initiated by addition of 0.1 ml Putrescine (final concentration of 20 mM) for DAO determination and 0.1 ml Spd (final concentration of 20 mM) for PAO determination. The change of absorbance at 550 nm per minute by 0.001 was considered as, one unit enzyme activity.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed according to Spitz and Oberley (1989) based on the competition between SOD and an indicator molecule NBT for superoxide production from xanthine and xanthine oxidase. Activity of one unit was defined as, the amount of protein required to inhibit NBT reduction by 50%. The Catalase (CAT, EC: 1.11.1.6) activity was assayed by Otsizár et al. (2011) while extinction coefficient of 39.4 M⁻¹cm⁻¹ was used to compute the activity.

Ascorbate peroxidase (APX, EC: 1.11.1.11) activity was computed by Nakano and Asada (1981) while Glutathione peroxidase (GPX, EC: 1.11.1.9) activity was calculated by Elia et al. (2003). The activities of glutathione (GR, EC: 1.6.4.2) and monodehydroascorbate reductase (MDHAR, EC: 1.6.5.4) were assayed according to the methods of Hossain and Fujita (2010). In case of assay activity of dehydroascorbate reductase (DHAR, EC: 1.6.5.1), method of Nakano and Asada (1981) was monitored.

Statistical analysis

Data were analyzed by statistical software SAS (9.1 version) and the means were separated by Tukey’s tests following randomized complete block design (RCBD). Value presented in table and figures are, mean of three independent experiments (each experiment consists of three replications). Probability level at P≤0.05 was considered as significant.

RESULTS

Changes in relative water content (RWC) and proline

The salinity reduced the leaf RWC gradually with stress duration, and at 5 and 7 days, RWC was significantly lower in the stressed seedlings than control (Figure 1A). Foliar application of Spd reduced the loss of water and restored the RWC in salinity stress seedlings by 7, 15, 14 and 14 % at 1, 3, 5 and 7 days, respectively. On the other hand, salinity stress significantly increased the proline content in onion seedlings (Figure 1B).

In salinity treated seedlings, the content was 0.55, 2.6, 2.4 and 1.9 fold higher over control seedlings at 1, 3, 5 and 7 days, respectively. Exogenous foliar spray of Spd further increased the content up to 5 days of stress and decreased subsequently.

Effect of Spd on ROS and lipid peroxidation

The formation rate of O₂⁻ increased continuously and
Changes in polyamine related enzymes

Under saline stress, the activity of polyamine oxidase (PAO) increased slightly up to 3 days and decreased afterward (Figure 3A). On the other hand, the activity of diamine oxidase (DAO) increased by saline stress where maximum (47% over control), increment was observed at 3 days of stress (Figure 3B). Spd increased both activities over salinity. However, both of the activities were the highest at 3 days of stress.

Changes in antioxidant enzymes

The activity of superoxide dismutase (SOD) increased under saline stress (Figure 4A). However, after 5 day of stress, it decreased. The increments of the activity under salinity over control were 20, 22, 47 and 25% at 1, 3, 5 and 7 days, respectively. Application of Spd in saline stressed seedlings further increased the activity over salinity by 10, 21, 20 and 25% at 1, 3, 5 and 7 days, respectively. However, CAT activity was almost similar in the seedlings both under salinity with or without Spd (Figure 4B).

Under salinity, the APX activity decreased after 3 day of stress (Figure 5A). Application of Spd increased the activity in the seedlings over salinity, where the increments were 23, 21, 19 and 39% at 1, 3, 5 and 7 days, respectively. Alternatively, saline stress increased the GPX activity (3, 32, 55 and 29% over control at 1, 3, 5 and 7 days, respectively) where the activity decreased after 5 days of stress (Figure 5B). Application of Spd increased the activity over salinity by 14 and 15% at 1 and 3 days, respectively; however, this activity decreased gradually.

The important enzymes, MDHAR, DHAR and GR of ASA-GSH cycle were also measured which maintain ASA and GSH. In this study, saline stress decreased MDHAR activity with duration, though, significant variation was not found between the activities under control and salinity (Figure 6A). Notably, application of Spd increased the activity over salinity, where increase was higher by 6, 19, 22 and 21% at 1, 3, 5 and 7 days, respectively. In contrast, salinity increased the DHAR activity over control. In application of Spd, the activity was found to increase stressed seedlings up to 3 days of stress (Figure 6B).

Saline stress also increased the activity of GR with stress duration (Figure 6C). As compared to control, the activity was higher by 5, 28, 40 and 38% at 1, 3, 5 and 7 days, respectively. Notably, application of Spd further increased the activity in stressed seedlings by 15, 23, 10 and 20% at 1, 3, 5 and 7 days, respectively.

Changes in ascorbate and glutathione

A continual decrease was observed in ascorbic acid (ASA) content under salinity stress (Figure 7A). As
Figure 3. Changes in activities of PAO (A) and DAO (B) in leaves of onion seedlings by Spd under salinity stress. Values present in the bars are mean ± SE. Similar letters between the bars are not significant at P≤5%.

Figure 4. Changes in activities of SOD (A) and CAT (B) in leaves of onion seedlings by Spd under salinity stress. Values present in the bars are mean ± SE. Similar letters between the bars are not significant at P≤5%.
Figure 5. Changes in activities of APX (A) and GPX (B) in leaves of onion seedlings by Spd under salinity stress. Values present in the bars are mean ± SE. Similar letters between the bars are not significant at P≤5%.

compare to control, salinity reduced the ASA content by 15, 36, 43 and 60% at 1, 3, 5 and 7 days, respectively, while application of Spd maintained the ASA content higher over salinity by 7, 16, 16 and 17% in stressed seedlings correspondingly.

Contrary, the DHA contents were observed to increase continuously with duration of saline stress, and as compared to control, 32, 34, 33 and 52% higher DHA was found at 1, 3, 5 and 7 days, respectively (Figure 7B). The application of Spd in saline stressed seedlings also reduced the oxidation of ASA, resulting in decrease of DHA content (15, 17, 16 and 23% at 1, 3, 5 and 7 days, respectively) as well. Importantly, Spd maintained the ascorbate redox in saline stressed seedlings by 11, 18, 20 and 31% at 1, 3, 5 and 7 days, respectively (Figure 7C).

Saline stress also caused continual and significant decrease in GSH content in onion seedlings, while 7, 24, 51 and 62% reduction was observed at 1, 3, 5 and 7 days, respectively (Figure 8A). In presence of Spd, saline treated seedlings showed 8, 14, 40 and 57% higher GSH at 1, 3, 5 and 7 days, respectively. Conversely, salinity increased GSSG content significantly and continuously (Figure 8B). As compared to control, the content was 1.8, 2.4, 5.6 and 6.4 folds higher at 1, 3, 5 and 7 days, respectively. Application of Spd decreased GSSG in saline stress seedlings, maintaining higher glutathione redox (Figure 8C).

DISCUSSION

Leaf water relationship is a very important factor to maintain physiological and biochemical processes in plants. In this study, salinity caused loss of leaf water in onion seedlings (Figure 1A). Osmotic adjustment in plants is very essential to maintain structure and function of cell components (Lambers et al., 2006; Hasegawa et al., 2000) while exogenous Spd increased the leaf water content (Figure 1A).

The osmotic adjustment in presence of Spd might be due to proline synthesis. Proline accumulation in onion seedlings was correlated with RWC (Figure 1B). Previously, exogenous Spd was also reported to increase with proline content and RWC in other plants under salinity stress, which demonstrated that osmolyte level, was modulated by PAs (Roychoudhury et al., 2011; Duan et al., 2008). Proline functions as an osmoprotectant for osmotic adjustment as well as scavenger of ROS (Yancey et al., 1982), and as compatible solute (Ashraf
Figure 6. Changes in activities of MDHAR (A), DHAR (B) and GR (C) in leaves of onion seedlings by Spd under salinity stress. Values present in the bars are mean ± SE. Similar letters between the bars are not significant at P≤5%.

and Foolad, 2007; Kocsy et al., 2005). Proline biosynthesis in higher plants is preceded through polyamine cycle where Spd is very important to regulate the proline content (Szabados and Savoure, 2009; Sanchez et al., 2001). Previously, under drought stress, Li et al. (2014) reported that exogenous Spd promoted polyamine cycle. In this study, higher proline content by exogenous Spd could play important role in osmotic adjustment under salinity stress, which might be involved in membrane stability. However, decreased proline content after 5 days of stress might be due to insufficient Spd as its application was stopped before counting the stress duration.

Production of ROS like superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) is a common phenomenon in crop under abiotic stress including salinity (Huang et al., 2005; Noctor et al., 2002; Hernández et al., 2000), but at higher concentration, they are the major cellular components to cell death (Foyer and Noctor, 2005; Foyer et al., 1994). To protect the cell organells from the toxicity of ROS, plant deploy antioxidant activity (Gill and Tijet, 2010). In this study, we observed profound increases in O$_2^-$ and H$_2$O$_2$ contents under salinity in onion seedlings (Figure 2A, B). ROS-scavenging enzymes as well as antioxidant molecules in plants protect cell organells by lessening the damage from O$_2^-$ and H$_2$O$_2$, where O$_2^-$ is first dismutated into H$_2$O$_2$ by the interference of SOD in different cell organells (Bowler et al., 1992).

In this study, exogenous Spd improved SOD activity in onion which was associated with lower O$_2^-$ generation. Therefore, SOD activity played an important role in dismutation of O$_2^-$ in onion seedlings under salinity stress. Melondialdehyde, a product of lipid peroxidation by ROS under environmental stresses including salinity,
causes damage to plasmalemma and organelle membranes (Garg and Manchanda, 2009). In the experiment, both MDA and \( \text{H}_2\text{O}_2 \) were increased significantly (Figure 2B, C), which can cause membrane damage in onion. Higher MDA content under salinity in plants was also reported previously (Saleethong et al., 2011; Moschou et al., 2008; Rohman et al., 2016). Reduction of MDA in Spd treated seedlings might be resulting from comparatively lower concentration of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \).

Superoxide dismutase deploys the primary protection against \( \text{O}_2^- \) to reduced the oxidative damage. Exogenous Spd increased SOD activity which correlated negatively with \( \text{O}_2^- \) generation (Figure 4A). Therefore, the increased SOD activity by the addition of Spd, dismutated the NaCl-stress which mediate higher \( \text{O}_2^- \) in onion seedlings. However, the increased SOD activity by Spd addition up to 5 days of stress, suggested its better role under short-term salinity stress.

Excessive accumulation of \( \text{H}_2\text{O}_2 \) is one of the most important indicators of oxidative stress (Apel and Hirt, 2004). \( \text{H}_2\text{O}_2 \), produced by intervention of SOD, is highly cytotoxic (Gill and Tujeta, 2010). On the other hand, CAT is considered as the strongest decomposer of \( \text{H}_2\text{O}_2 \) (Scandalios, 2005). However CAT activity did not increase in the onion seedlings under saline stress (Figure 4B). Foliar application of Spd also failed to increase the activity in saline stressed seedlings. Unlike other \( \text{H}_2\text{O}_2 \) scavenging enzymes, enzymatic reaction of CAT is independent of other cellular substrates for instituting its activity (Scandalios, 2005). However, under salinity CAT activity almost unchanged in the presence or absence of Spd suggesting that, the enzyme did not play important role in decomposition of \( \text{H}_2\text{O}_2 \) under saline

**Figure 7.** Changes in ASA (A), DHA (B) and Ascorbate redox [ASA/(ASA+DHA)] (C) in leaves of onion seedlings by Spd under salinity stress. Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level.
stress in onion seedlings.

The activity of APX increased in salinity stressed seedlings (Figure 5A). To reduce H$_2$O$_2$ to water, APX needs ASA to generate monodehydroascorbate which disproportionates to ASA and DHA (Apel and Hirt, 2004). Salinity stress might inactivate the APX activity by reducing ASA content (Figure 7A). Exogenous Spd enhanced the activity by increasing ASA content which indicates the H$_2$O$_2$ scavenging role of Spd. GPX activity which increased remarkably, suggests the role of H$_2$O$_2$ metabolism in onion seedlings. Application of Spd treatment increased the activities of GPX up to 5 days of stress, suggesting the role of Spd in converting H$_2$O$_2$ into H$_2$O more efficiently in early days of salinity stress (Figure 5A, B).

Ascorbic acid plays an important role by maintaining enzymatic activity of ascorbate-glutathione cycle and thus improves plant tolerance to adverse environmental conditions including salinity stress by effectively reducing ROS, produced under stress conditions (Apel and Hirt, 2004; Shalata and Neumann, 2001; Nakano and Asada, 1981). In this study, the content of ASA decreased gradually and significantly with duration of salt stress resulting in more oxidation to generate GSSG (Figure 8A, C). In ascorbate-glutathione cycle, ASA is maintained by MDHAR and DHAR enzymes with ASA the key reductant in plant cells for H$_2$O$_2$ metabolism (Mehlhorn et al., 1996; Nakano and Asada, 1987). The increased contents of DHA in this study resulted in the oxidation of ASA (Figure 7B). Conversely, higher activity of DHAR is use in maintaining ASA contents under salinity. Spd-induced MDHAR and DHAR activities suggested higher maintenance of ASA and its redox in onion seedlings (Figure 6A, 6B, 7C).

On the other hand, GSH participates in scavenging of ROS either directly or indirectly in ascorbate-glutathione and thus it is a key non-enzymatic antioxidant (Noctor et al., 2002). The essential role of GSH is due to its capability to restore ASA through reduction of DHA, passing through the ascorbate-glutathione cycle (Apel
and Hirt, 2004). GSH is also used in glyoxalase to detoxify cytotoxic MG by acting as a substrate. Furthermore, in plant cells, GR is the key enzyme for maintaining GSH, which is also necessary in speeding up scavenging H$_2$O$_2$ (Saha et al., 2015).

Salinity stress caused a significant decrease in GSH levels at 3, 5 and 7 days saline stress (Figure 8A), and at the same time, GSSG levels also increased significantly at 1, 3, 5 and 7 days saline stress (Figure 8B). Spd treatment significantly decreased the level of GSSG by GR mediated recycling which ultimately maintained higher GSH content (Figure 8A, B). Hence, the results suggested the contribution of Spd in maintaining glutathione reductase during the stress period. This result was collaborated with other recent findings, where exogenous PAs including Spd upregulated the GR activity under salt stress (Erat et al., 2008).

**Conclusion**

Considering the above results, saline stress caused over production of ROS and MDA in onion seedlings. Application of foliar Spd reduced ROS and MDA through up-regulating activities of SOD, APX, GPX, MDHAR, DHAR and GR as well as maintaining ASA and GSH. Importantly, many of the enzymatic antioxidants showed higher activities at 3 to 5 days of stress.

However, the CAT activity remained almost unchanged under salinity stress with or without Spd application. In this preliminary study, we used only one dose of Spd. Multiple dose can be examined for further study.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

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