EFFECT OF SPERMIDINE ON PHYSIOLOGY AND ANTIOXIDANTS UNDER SALINITY STRESS IN ONION SEEDLINGS

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EFFECT OF SPERMIDINE ON PHYSIOLOGY AND ANTIOXIDANTS UNDER SALINITY STRESS IN ONION SEEDLINGS

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Dedicated to

My

Beloved Parents

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CERTIFICATE

This is to certify that the thesis entitled ***"Effect of Spermidine on Physiology and Antioxidants Under Salinity Stress in Onion Seedlings"*** *submitted to the Department of Horticulture, Sher-e-Bangla Agricultural University, Dhaka in partial fulfillment of the requirements for the degree of* ***MASTER OF SCIENCE*** *in* ***HORTICULTURE****, embodies the result of a piece of bona fied research work carried out by* ***TANJINA ISLAM****, Registration No.* ***08-03165*** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that any help or source of information received during the course of this investigation has been duly acknowledged.

Dated: December, 2014
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ABSTRACT

In this study, protective role of spermidine (Spd) on NaCl induced oxidative damage in onion seedlings was investigated for better understanding the salinity tolerance mechanism. Onion seedlings were subjected to salinity stress of 16 dSm^{-1} and 100 µM of Spd were sprayed twice daily. Data were analyzed on Chlorophyll (Chl), Carotenoid (Car), Leaf water, Proline, Reactive Oxygen Species (ROS), Methylglyoxal (MG), lipid peroxidation as Malondialdehyde (MDA), Polyamines, Glutathione and Ascobate related antioxidant enzymes and Glyoxalase (Gly) systems. Salinity reduced the Chl, Car and Relative Water Content (RWC) of leaves where foliar spray of Spd reduced the loss of these contents. Contrary, Spd increased the proline contents in salinity stressed seedlings. The contents of $O_2^{\bullet-}$, H_2O_2, MDA were decreased in the Spd supplied leaves. However, Spd effectively maintained the ROS and MDA at first 3 day of stress, while Glutathion (GSH), Ascorbic acid (ASA) and their redox homeostasis were maintained higher throughout the study period. Higher increments of Polyamine Oxidase (PAO) and Diamine Oxidase (DAO) activities upto 3 day suggested its protective role via H_2O_2 signal transduction pathway. In presence of Spd, higher activities of superoxide dismutase (SOD), Ascorbate Peroxidase (APX) and Monodehydroascorbate reductase (MDHAR) over salinity during the study period suggested its ROS scavenging role. However, higher activities of Glutathione Peroxidase (GPX) and Dehydroascorbatereductase (DHAR) by Spd indicated maintenance of ROS and ASA, respectively. Similarly, Spd increased activities of Gly-I and Gly-II upto 3 day of stress concurrently with lower MG suggested the improvement of MG detoxification system. Higher activity of Glutathione-*S*-Transferase (GST) by Spd in saline stress seedling might involve in protein stabilization and detoxification role in onion plant. Since GST activity increased strongly in presence or absence of Spd under salinity, attempt was taken to test its accumulation. Therefore, from different plant parts it was found that both root and bulb showed higher GST activity. However, onion bulb was chosen for easy availability. In chromatographic separation of onion bulb, three component GSTs were separated among which one GST eluted at 120 mM KCl was found to be accumulated under salinity in western blotting analysis. Taken together, in short term salinity, foliar spray of Spd might be useful to reduce the oxidative damage through increment of antioxidants and glyoxalase system.

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LIST OF ABBREVIATED TERMS

ABBREVIATION	ELABORATION
$^1O_2$	Singlet oxygen
APX	Ascorbate peroxidase
ASA	Ascorbic acid
ASH	Ascorbic acid
Car	Carotenoid
CAT	Catalase
Chl	Chlorophyll
Chl*a*	Chlorophyll *a*
Chl*b*	Chlorophyll *b*
DAO	Diamine oxidase
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DNA	Deoxyribonucleic acid
Gly-I	Glyoxalase I
Gly-II	Glyoxalase II
GOPX	Guaicol peroxidase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathion
GSSG	Oxidixed glutathione
GST	Glutathione-*S*- transferase
H_2O_2	Hydrogen peroxide
MDA	Malondialdehyde
MDHAR	Monodehydroascorbate reductase
MG	Methylglyoxal
$O_2^{\bullet-}$	Superoxide radical
$OH^{\bullet}$	Hydroxyl radical

ABBREVIATION	ELABORATION
PA	Polyamine
PAO	Polyamine oxidase
POD	Peroxide dismutase
ROS	Reactive oxygen species
RWC	Relative water content
SOD	Superoxide dismutase
Spd	Spermidine

LIST OF CHEMICALS

β-mercapttoethanol	Glycine
1-Chloro-2, 4-dinitrobeenzene	HCl
2-Nitro-5-thiobenzoic acid	Horse raddishperoxide
2-Thiobarbituric acid	Hydrogen peroxide
5,50-Dithio-bis(2-nitrobenzoic acid)	Hydroxyapatite
Acetone	KOH
Acrylamide	L-Ascorbic acid
Albumin from Bovine Serum	Methanol
Ammonium per-oxisulfate	Methyl glyoxal
Ammonuim Sulfate	$MgSO_4.7H_2O$
Ascorbate oxidase	Na_2CO_3
Beta mercapto ethanol	Na_2EDTA
Bromophenol Blue	$Na_2H_2PO_4.2H_2O$
Catalase	$Na_2HPO_4.12H_20$
Charcoal	N-acetyl-L-cysteine
Citric acid (Monohydrade)	NaCl
Coomassie Brilliant Blue (CBB)	NADPH
DEAE (DE-52, UK)	NH_3 Solution
Dehydroascorbate	Ninhydrin
Dithiothreitol	Nitrobletetrazolium
Ethanol	Nicotinamide adenosine dinucleotide
Ethylenediamine tetraacitic acid	Phosphate
Formaldehyde	Nitroblue tetrazolium
Glacial acetic acid	Perchloric acid
Glutathione (Oxidize)	Phosphoric Acid
Glutathione (Reduced)	Potassium (Dibasic)
Glutathione Reductase	Potassium (Monobasic)
Glycerol	Potassium chloride

Proline	Spermidine
Putrscine	Sulphosalycylic acid
Riboflavin	TEMED (tetramethyl ethylene diamine)
S-D-Lactoylglutathione	Toluene
Silver nitrate	Trichloroacetic acid
Sodium chloride	Tris (hydroxylmethyl) aminomethane
Sodium dodecyl sulfate	Xanthine
Sodium hydroxide	Xanthine oxidase

CHAPTER I

INTRODUCTION

Soil salinity is a major environmental stress that drastically affects plants growth and productivity of several crops including onion by creating low osmotic potential outside the seeds, thereby inhibiting or slowing down seed germination and also retarding seedling growth because of both osmotic and ion toxicity effects (Khajeh-Hosseini *et al.,* 2003). Salinity also induced secondary stresses such as nutritional imbalance and oxidative stress (Zhu, 2002). Numerous attempts have been made to improve the salinity tolerance of a variety of crops by traditional breeding programs *(*Duan *et al.*, 2008*),* but the progress to develop such salt-resistant plants is very slow and commercial success is limited due to multigenic responces of the adaptive process.

Salinity stress causes oxidative stress in plant and produce reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydroxyl radicals ($OH^{\bullet}$) and concomitantly H_2O_2 (Hasegawa *et al.,* 2000; Apel and Hirt, 2004) and methyl glyoxal (MG) (Yadav *et al.*, 2005a, b). In plant cell, ROS are highly reactive and toxic which can lead to cell death by causing damage to proteins, lipids, DNA and carbohydrates (Apel and Hirt, 2004; Noctor *et al.*, 2002). On the other hand, methylglyoxalase (MG) is potential cytotoxic and can react with and modify other molecules including DNA and proteins (Yadav *et al.,* 2005b) whereas proteins being one of the major targets of ROS. Therefore, ROS and MG are highly toxic and must be detoxified by cellular responses to survive and grow.

To protect cells from ROS-induced cellular injury, plants have evolved a complex antioxidant system that plays a significant role in ROS signaling in plants (Apel & Hirt, 2004; Noctor *et al.,* 2002). Efficient scavenging of ROS produced during various environmental stresses including salinity requires the action of several non-enzymatic as well as enzymatic antioxidants present in the tissue. The antioxidant defenses of plants include 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), along with non-enzymatic components such as ascorbic acid (ASA) and glutathione (GSH) (Rohman *et al.*, 2015). On the other hand, MG is detoxified and GSH homeostasis is maintained via glyoxalase system (Yadav *et al.,* 2005b), which consists of two enzymes: glyoxalase-I (Gly-I) and glyoxalase-II (Gly-II). It was reported that the coordinated inductions or regulations of both the antioxidant and glyoxalase pathway enzymes are necessary to obtain substantial tolerance in plants against oxidative stress (Singla-Pareek *et al.,* 2008; Saxena *et al.,* 2011).

Polyamines (PAs), including the diamine putrescine (Put), triamine spermidine (Spd) and tetramine spermine (Spm) are ubiquitous low-molecular-weight aliphatic amines that are involved in regulation of plant growth and development (Martin-Tanguy, 2001) and are well known for their anti-senescence and anti-stress effects due to their acid neutralizing and antioxidant properties, as well as for their membrane and cell wall stabilizing abilities (Zhao and Yang, 2008). Because of their cationic nature at physiological pH, PAs are able to interact with proteins, nucleic acids, membrane phospholipids and cell wall constituents, thereby stabilizing these molecules (Bouchereau *et al.,* 1999). Apart from their implication in growth and development, PAs have been reported to be involved in defense response to biotic and abiotic stresses (Alcázar *et al.,* 2006). It has been shown that a high cellular level of PAs correlates with plant tolerance to a wide array of environmental stresses such as salinity (Krishnamurthy and Bhagwat *et al.,* 1998), oxidative stress (Langebartels *et al.,* 1991; Kurepa *et al.,* 1998), low and high temperatures (Roy and Ghosh, 1996; Shen *et al.,* 2000; He *et al.,* 2002), hyperosmosis (Besford *et al.,* 1993), and hypoxia (Nada *et al.,* 2004). Treatment with a polyamine biosynthetic enzyme inhibitor reduces stress tolerance, but the concomitant treatment with exogenous polyamines restores it (Lee *et al.,* 1997; He *et al.,* 2002), supporting the view that polyamines play essential roles in environmental stress tolerance of plants. It has been shown that stress-tolerant plants had significantly higher endogenous PAs levels as

compared to sensitive ones under stress. (Lee *et al.*, 1997). Several biochemical and physiological effects were elicited by exogenously applied PAs including Spd under environmental stress. It was reported that exogenous spermidine was effective in enhancing the activity of peroxidase under salinity stress and the salt-induced increase in reducing sugar and free proline level was further promoted by spermidine in indica rice (Roychoudhury *et al.*, 2011). Moreover, it has been demonstrated that over expression of spermidine synthase gene in transgenic *Arabidopsis thaliana* maintained higher levels of spermidine content and enhanced tolerance to salinity, chilling, hyperosmosis and drought relative to the wild-type plants, which suggests that spermidine plays an important role in stress signaling pathway as a signaling regulator, leading to build a stress tolerance mechanisms for plants (Kasukabe *et al.*, 2004). Previous studies have indicated that spermidine was involved in a number of environmental stress, not only exogenous application but also transgene (such as spermidine synthase gene) could alleviate damage from these stresses (Gill and Tujeta, 2010). However, increase in stress tolerance mechanism under salinity is still not clear. Onion has been reported to be model crop for stress mitigating antioxidants (Hossain *et al.*, 2010; Rohman *et al*., 2010). Spermidine has been reported to maintain antioxidant enzyme activities of welsh onion (*Allium fistulosum* L.) under water logging condition where it is able to moderate radical scavenging system to lessen oxidative stress in this way (Yiu *et al.*, 2008). Therefore, onion may be important crop to understand the underlying salinity tolerance mechanism in presence of Spd exogenously. Considering this fact the study was undertaken with the following objectives:

1. Protective roles of antioxidants, amine oxidases and glyoxalases from oxidative damage in onion seedlings under salinity stress.
2. Role of exogenous Spd in regulation of salinity induced physiology, antioxidants, amine oxidases, glyoxalases as well as pigment.

CHAPTER II

REVIEW OF LITERATURE

Salinity impairs in the growth and yield and causes oxidative stress .Though a number of physiological and biochemical process has been associated with the adaptive responses, the underlying tress tolerant mechanism is still not clear. A reasonable number of literature exists has been suggesting that Spd is important PAs able to interact with proteins, nucleic acids, membrane phospholipids and cell wall constituents, thereby stabilizing these molecules,. It is also suggested that Spd plays an important role in stress signaling pathway as a signaling regulator, leading to build a stress tolerance mechanisms for plants. However, information on role of exotic spermidice in plant under salinity is limited. Therefore, I discussed the following reviews to make a foundation of my study.

Singh and Sharma (2013) studied the response of antioxidant enzyme activities in seedlings of different sorghum cultivars under mannitol stress. Seven-day old seedlings were subjected to 100-500 mM mannitol stress which resulted in the decreases in shoot/root length and relative water content thus indicating the primary response to these tissues at phenotypic level. The level of lipid peroxidation as well as the specific activity of antioxidant enzymes such as peroxidase, catalase and superoxide dismutase increased at higher conc. except at 200 mM conditions. The level of catalase and peroxidase decreased at 500 mM concentration in the two different cultivars whereas the activity of superoxide dismutase consistently increased in response to the mannitol stress. They demonstrated that drought responsiveness tolerance in sorghum cultivars during germination is associated with enhanced activity of antioxidant enzymes.

Kolarovic *et al.* (2009) found significant activation of antioxidative defence mechanisms correlated with drought induced oxidative stress tolerance in maize, and this phenomenon was shown to be characteristic of the drought-tolerant cv. Nova. Activities of some ROS-scavenging enzymes, superoxide dismutase (SOD), guaiacol peroxidase (POX), catalase (CAT) and ascorbate peroxidase (APX) were enhanced

significantly 4h after the start of drought exposure in the drought-tolerant cv. Nova. Furthermore, a significant increase in the ascorbate pool was observed in this cultivar. On the other hand, in the drought-sensitive cv. Ankora only SOD and POD activities and the thiol pool were increased. No changes in APX activity or the level of ascorbate were recorded in cv. Ankora.

Naji and Debaraj (2011) reported the effects of high temperature and salt stress on *Macrotyloma uniflorum* (Lam.) Verdc. (Horse gram), on antioxidants and antioxidant enzymes, where both treatments caused typical stress responses. Oxidative stress indicators such as H_2O_2, TBARS, and proline were significantly elevated. Similarly, the antioxidant enzymes superoxide dismutase (SOD), guaiacol peroxidase (POX) and acid phosphates (AP) were significantly elevated while catalase (CAT) was reduced. These treatments had contrasting effects on glutathione reductase (GR) and b-amylase (EC 3.2.1.1). While temperature stress caused increase in GR and decrease in b-amylase, salt stress caused a counter effect. Contrast was also observed in ascorbate and glutathione which increased in temperature stress and reduced in salt stress. SDS-PAGE analysis indicated entirely different protein profiles in temperature and salt stressed seedlings

DaCosta and Huang (2007) exhibited that resistant of some grass species are correlated with higher antioxidant system. They variation in drought resistance for colonial bentgrass (*A. capillaris* L.), creeping bentgrass (*A. stolonifera* L.), and velvet bentgrass was associated with differences in antioxidant enzyme levels in response to drought. Plants of 'Tiger II' colonial bentgrass, 'L-93' creeping bentgrass, and 'Greenwich' velvet bentgrass were maintained in a growth chamber under two watering treatments: 1) well-watered control and 2) irrigation completely withheld for 28 d (drought stress). Prolonged drought stress caused oxidative damage in all three bentgrass species as exhibited by a general decline in antioxidant enzyme activities and an increase in lipid peroxidation. Compared among the three species, velvet bentgrass maintained antioxidant enzyme activities for a greater duration of drought treatment compared with both colonial bentgrass and creeping bentgrass. Higher antioxidant enzyme capacity for velvet bentgrass was associated with less

lipid peroxidation and higher turf quality, leaf relative water content, and photochemical efficiency for a greater duration of stress compared with colonial bentgrass and creeping bentgrass. Finally they concluded that bentgrass resistance to drought stress was associated with higher oxidative scavenging ability.

Csiszár *et al.*, (2007) investigated the enzymatic antioxidative defence mechanisms of some regional subspecies of *Allium* (*A. cepa* L., *A. ascalonicum* auct. hort., *A. sativum* L.) cultivated mainly in the western regions of Romania, and two modern Hungarian climate resistant F_1 hybrids were reprted by Csiszár *et al.*, (2007). The variability in the activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione reductase (GR) and glutathione S-transferase (GST) and their changes under soil moisture stress were investigated. 1-week-long water stress revealed that among three *Allium* species, relative water content decreased only in *A. ascalonicum* leaves (up to 16%). Unlike root enzymes, the activities of the shoot enzymes, especially POD, GR and GST showed a stronger correlation with the water content of the leaves after one week of water withdrawal; regression coefficients (R^2) were 0.359, 0.518 and 0.279, respectively. The ancient populations with elevated (or highly inducible) antioxidant enzyme activities were suggested for further research and for breeding of new *Allium* varieties.

Abedi and Pakniyat (2010) identified the responses of antioxidant enzyme activities and their isozyme patterns in seedlings of 10 oilseed rape (*Brassica napus* L.) cultivars under drought stress conditions. Plants were grown under three irrigation regimes (FC; field capacity, 60% FC and 30% FC) in a greenhouse. Drought stress preferentially enhanced the activities of superoxide dismutase (SOD) and guaiacol peroxidase (POD) whereas it decreased catalase (CAT) activity. Licord with the highest level of enzyme activity under both optimum and limited irrigation regimes is reported as the most tolerant cultivar. Whereas Hyola 308 and Okapy, having the lowest enzymes activities, are mentioned as cultivars sensitive to drought stress. The native polyacrylamide gel electrophoresis (PAGE) analysis detected eight SOD isozymes. Oilseed rape leaves contained three isoforms of Mn-SOD and five isoforms of Cu/Zn-SOD. The expression of Mn-SOD was preferentially enhanced by

drought stress. Five POD isoforms were detected in oilseed rape leaves. The intensities of POD-4 and -5 were enhanced under drought stress. They suggested that the appearance of new isozyme bands under drought stress conditions may be used as a biochemical marker to differentiate drought tolerant cultivars under drought stress.

Marok *et al.,* (2013) investigated the level of oxidative stress and the participation of antioxidant systems in two barley genotypes: Express, a variety known to be sensitive to drought, and Saïda, an Algerian landrace selected for its tolerance to water deficit. They observed that upon water stress Express exhibits compared to Saïda accelerated wilting and a higher level of oxidative stress evaluated by HPLC measurements of lipid peroxidation and by imaging techniques. In parallel, Express plants also displayed lower levels of catalase and superoxide dismutase activity. No great difference was observed regarding peroxiredoxins and methionine sulfoxide reductases, enzymes detoxifying peroxides and repairing oxidized proteins, respectively. In contrast, upon water stress and recovery, much higher contents and oxidation ratios of glutathione and ascorbate were measured in Express compared to Saïda. Express also shows during water deficit greater increases in the pools of lipophilic antioxidants like xantophyll carotenoids and α-tocopherol. Altogether, these data show that the differential behavior of the two genotypes involves distinct responses regarding antioxidant mechanisms. They concluded that Saïda is associated with oxidative damage and a lower enzymatic ROS-scavenging capacity, but in parallel with a much stronger enhancement of most mechanisms involving low-molecular weight antioxidant compounds.

Behera *et al.,* (2009) examined the role of protein and antioxidative enzymes defense system during high salt shock, two *Bruguiera* species *B. parviflora* and *B. gymnorrhiza* of the family Rhizophoraceae. These species were exposed to high salt shock (500 mM NaCl) for a short period of 6days. Total protein content in both the species decreased upon salt shock but the rate of degradation was more rapid in *B. parviflora* than in *B. gymnorrhiza*. SDS-PAGE protein profiling revealed that the

protein having apparent molecular mass 90kDa, 49kDa, 33kDa, 23kDa, 10kDa reduced very first after 4days of salt treatment of *B. parviflora* than in *B. gymnorrhiza*. The specific activities of catalase increased 2.4 times and 2.1 times in *B. parviflora* and *B. gymnorrhiza* upon exposure to high salt. Out of the four isoforms of catalase, CAT-2 activities enhanced 1.5 times and 1.2 times upon initial salt treatment for 4 days and 6 days. APX activity increased 1.3 times and 2.3 times in *B. parviflora* and *B. gymnorrhiza* under salt shock. A single isoform of APX enhanced 1.3 times and 1.7 times in *B. parviflora* and *B. gymnorrhiza*. The GPX activity increased 1.5 times and 1.3 times in leaves of salt treated seedlings of *B. parviflora* and *B. gymnorrhiza*.

Gill and Tuteja (2010) reported that various abiotic stresses lead to the overproduction of reactive oxygen species (ROS) in plants which are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates and DNA which ultimately results in oxidative stress. The ROS comprises both free radical ($O_2^{\bullet-}$, superoxide radicals; OH, hydroxyl radical; HO_2^-, perhydroxy radical and RO, alkoxy radicals) and non-radical (molecular) forms (H_2O_2, hydrogen peroxide and $1O_2$, singlet oxygen). In chloroplasts, photosystem I and II (PSI and PSII) are the major sites for the production of $1O_2$ and $O_2^{\bullet-}$. In mitochondria, complex I, ubiquinone and complex III of electron transport chain (ETC) are the major sites for the generation of $O_2^{\bullet-}$. The antioxidant defense machinery protects plants against oxidative stress damages. Plants possess very efficient enzymatic and non-enzymatic antioxidant defense systems which work in concert to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging of ROS. They concluded that ROS influence the expression of a number of genes and therefore control many processes like growth, cell cycle, programmed cell death (PCD), abiotic stress responses, pathogen defense, systemic signaling and development.

Apel and Hirt (2004) reported that several reactive oxygen species (ROS) are continuously produced in plants as byproducts of aerobic metabolism. Depending on the nature of the ROS species, some are highly toxic and rapidly detoxified by

various cellular enzymatic and nonenzymatic mechanisms. Whereas plants are surfeited with mechanisms to combat increased ROS levels during abiotic stress conditions, in other circumstances plants appear to purposefully generate ROS as signaling molecules to control various processes including pathogen defense, programmed cell death, and stomatal behavior. They describe the mechanisms of ROS generation and removal in plants during development and under biotic and abiotic stress conditions. New insights into the complexity and roles that ROS play in plants have come from genetic analyses of ROS detoxifying and signaling mutants. Considering recent ROS-induced genomewide expression analyses, the possible functions and mechanisms for ROS sensing and signaling in plants are compared with those in animals and yeast.

Saha *et al.,* (2015) studied that the balance between accumulation of stress-induced polyamines and reactive oxygen species (ROS) is arguably a critical factor in plant tolerance to salt stress. Polyamines are compounds, which accumulate in plants under salt stress and help maintain cellular ROS homeostasis. They outline the role of polyamines in mediating salt stress responses through their modulation of redox homeostasis. The two proposed roles of polyamines in regulating ROS—as antioxidative molecules and source of ROS synthesis. Second, the proposed function of polyamines as modulators of ion transport is discussed in the context of plant salt stress. Finally, they highlighted the apparent connection between polyamine accumulation and programmed cell death induction during stress. Thus, they concluded that polyamines have a complex functional role in regulating cellular signaling and metabolism during stress. By focusing future efforts on how polyamine accumulation and turnover is regulated, research in this area may provide novel targets for developing stress tolerance.

Hossain and Fujita (2010) observed that in mung bean seedlings, salt stress (300 mM NaCl) caused a significant increase in reduced glutathione (GSH) content within 24 h of treatment as compared to control whereas a slight increase was observed after 48 h of treatment. Highest oxidized glutathione (GSSG) content was observed after 48 h to treatment with a concomitant decrease in glutathione redox state. Glutathione

peroxidase, glutathione *S*-transferase, and glyoxalase II enzyme activities were significantly elevated up to 48 h, whereas glutathione reductase and glyoxalase I activities were increased only up to 24 h and then gradually decreased. Application of 15 mM proline or 15 mM glycinebetaine resulted in an increase in GSH content, maintenance of a high glutathione redox state and higher activities of glutathione peroxidase, glutathione *S*-transferase, glutathione reductase, glyoxalase I and glyoxalase II enzymes involved in the ROS and methylglyoxal (MG) detoxification system for up to 48 h, compared to those of the control and mostly also salt stressed plants, with a simultaneous decrease in GSSG content, H_2O_2 and lipid peroxidation level. They suggested that both proline and glycinebetaine provide a protective action against salt induced oxidative damage by reducing H_2O_2 and lipid peroxidation level and by enhancing antioxidant defense and MG detoxification systems.

Roxas *et al.* (2000) reported the overexpression of a tobacco glutathione S-transferase with glutathione peroxidase activity (GST/GPX) in transgenic tobacco (*Nicotiana tabacum* L.) enhanced seedling growth under a variety of stressful conditions. In addition to increased GST and GPX activity, transgenic GST/GPXexpressing (GST+) seedlings had elevated levels of monodehydroascorbate reductase activity. GST+ seedlings also contained higher levels of glutathione and ascorbate than wildtype seedlings and the glutathione pools were more oxidized. Thermal or salt-stress treatments that inhibited the growth of wild-type seedlings also caused increased levels of lipid peroxidation. These treatments had less effect on the growth of GST+ seedling growth and did not lead to increased lipid peroxidation. Stress-induced damage resulted in reduced metabolic activity in wild-type seedlings while GST+ seedlings maintained metabolic activity levels comparable to seedlings grown under control conditions. These results indicate that overexpression of GST/GPX in transgenic tobacco seedlings provides increased glutathione-dependent peroxide scavenging and alterations in glutathione and ascorbate metabolism that lead to reduced oxidative damage. It was concluded

that this protective effect is primarily responsible for the ability of GST+ seedlings to maintain growth under stressful conditions.

Csiszár *et al.* (2011) investigated three weeks old *Solanum lycopersicum* L. cvar. Rio Fuego plants, which grew in hydroponic culture, pre-treated with 10-7 M or 10-4 M salicylic acid (SA) and 100 mM NaCl was added to the nutrient solution from the 6th week. The activity of glutathione transferase (GST), glutathione peroxidase (GPOX) and dehydroascorbate reductase (DHAR) were analyzed spectrophotometrically after one week salt stress. All of these activities are connected to GST enzyme family, but the changes were different at the end of the pre-treatment or after the NaCl stress. SA enhanced the GPOX activity in the highest extent by the end of the three-week period, while in glutathione transferase function there was no significant changes. The salt treatment mostly enhanced these enzyme activities but in the SA-pre-treated plants the GST and GPOX activities were elevated in a higher extent. In contrast to the lower SA concentration, pre-treatment with 10-4 M SA maintained the DHAR activities at the control level even in roots. They indicate that the increased antioxidant enzyme activities may be the part of the hardening effect of SA. Finally, they suggested that GSTs can participate in the maintenance of the redox state of cells and improving the salt stress tolerance of tomato plants.

Takesawa *et al.* (2002) studied to develop a rice cultivar that would be suitable for direct-seeding cultivation in cooler temperate regions, we generated transgenic rice plants in which a rice encoding a ζ - class glutathione *S*-transferase (GST) under the control of a maize ubiquitin promoter. GSTs have been suggested to be responsible for tolerance to various stresses such as cold, salt and drought by detoxification of xenobiotic compounds and reactive oxygen species. A total of 87 R0 transgenic rice plants harboring a chimeric *GST* gene were generated using *Agrobacterium* mediated transformation. Three R_2 lines homozygous for the transgene were assayed for GST activity and had higher GST and glutathione peroxidase activities than non-transformants. Seedlings of the transgenic lines demonstrated greatly enhanced germination and growth rates at low temperature grown under submergence. They

suggested that the GST transgenic lines should be useful for breeding rice cultivars suitable for direct-seeding cultivation in cooler temperate regions.

Mohsenzadeh *et al.* (2011) reported that Glutathione *S*-transferases are multifunctional proteins involved in diverse intracellular events such as primary and secondary metabolisms, stress metabolism, herbicide detoxification and plant protection against ozone damages, heavy metals and xenobiotics. The plant glutathione S-transferase superfamily have been subdivided into eight classes. Phi, tau, zeta, theta, lambda, dehydroascorbate reductase and tetrachlorohydroquinone dehalogenase classes are soluble and one class is microsomal. Glutathione S-transferases are mostly soluble cytoplasmic enzymes. To date, the crystal structures of over 200 soluble glutathione S-transferases, present in plants, animals and bacteria have been resolved. The structures of glutathione *S*-transferase influence its function. Phylogenetic analysis suggests that all soluble glutathione *S*-transferases have arisen from an ancient progenitor gene, through both convergent and divergent pathways.

Szalai *et al.* (2011) investigated the effect of abscisic acid (ABA) treatment on chilling tolerance and salicylic acid (SA)-related responses in young maize seedlings. Although the pre-treatment of plants with ABA slightly decreased the chlorophyll content, it also reduced the level of chilling injury caused by 6 days of cold treatment at 5°C. Under normal growth conditions increased levels of bound SA and of bound ortho-hydroxycinnamic acid (oHCA) were observed in the leaves during ABA treatment. In the roots ABA did not affect the free and bound SA levels, but increased the amount of free and bound oHCA. The activity of glutathione-S-transferase (GST) increased on the 3rd day of ABA treatment, while it did not change when followed by cold stress, compared to the control leaves. In the roots the activities of glutathione reductase, GST and ascorbate peroxidase (APX) increased during the ABA treatment, and those of GST and APX were also stimulated when ABA pre-treatment was followed by cold stress, compared to the control roots. They suggested that an overlap may exist between the ABA-induced cold acclimation and the SA-related stress response.

Gallé (2005) reported that Glutathione S-transferase (GST) isoenzymes represent a large and variable group of antioxidative enzymes, with several different activities and sequence patterns. The GST activities of drought-tolerant *Triticum aestivum* L. cv. Kobomugi and cv. Plainsman were measured after one week 400 mOsm polyethylene glycol (PEG) treatment. The GST activities were much higher in the root than in the shoot and were induced by PEG especially in root. They sort out the drought stress related wheat GST genes. Phylogenetic analysis of wheat *GSTs* was performed *in silico* and using the tentative consensus sequences a dendogram was composed. According to the conserved sequences used for classification of GST proteins, they identified six groups of wheat GSTs. The phi GSTs are the most heterologous group, containing 25 sequences. The zeta, theta and tau GSTs are represented by 10, 9 and 8 TCs respectively. There are two other unidentified groups containing 8 and 6 sequences. They found homology between the osmotic stress upregulated sequences and the GST coding TCs were identified.

Alcazar *et al.* (2006) reported that environmental stresses are the major cause of crop loss worldwide. Polyamines are involved in plant stress responses. However, the precise role(s) of polyamine metabolism in these processes remain ill-defined. Transgenic approaches demonstrate that polyamines play essential roles in stress tolerance and open up the possibility to exploit this strategy to improve plant tolerance to multiple environmental stresses. The use of *Arabidopsis* as a model plant enables us to carry out global expression studies of the polyamine metabolic genes under different stress conditions, as well as genome-wide expression analyses of insertional-mutants and plants over-expressing these genes. They concluded that their studies are essential to dissect the polyamine mechanism of action in order to design new strategies to increase plant survival in adverse environments.

Galston and Sawhney (1990) studied that the diamine putrescine, the triamine spermidine, and the tetramine spermine are ubiquitous in plant cells, while other polyamines are of more limited occurrence. Their chemistry and pathways of biosynthesis and metabolism are well characterized. They occur in the free form as cations, but are often conjugated to small molecules like phenolic acids and also to

various macromolecules. Their titer varies from approximately micromolar to more than millimolar, and depends greatly on environmental conditions, especially stress. In cereals, the activity of one of the major polyamine biosynthetic enzymes, arginine decarboxylase, is rapidly and dramatically increased by almost every studied external stress, leading to 50-fold or greater increases in putrescine titer within a few hours. The physiological significance of this increase is not yet clear, although most recent work suggests an adaptive, protective role. Polyamines produced through the action of ornithine decarboxylase, by contrast, seem essential for DNA replication and cell division. The application of exogenous polyamines produces effects on patterns of senescence and morphogenesis, suggesting but not proving a regulatory role for polyamines in these processes. The evidence for such a regulatory role is growing.

Gill and Tuteja (2010) examined that environmental stresses including climate change, especially global warming, are severely affecting plant growth and productivity worldwide. It has been estimated that two-thirds of the yield potential of major crops are routinely lost due to the unfavorable environmental factors. On the other hand, the world population is estimated to reach about 10 billion by 2050, which will witness serious food shortages. Therefore, crops with enhanced vigour and high tolerance to various environmental factors should be developed to feed the increasing world population. Maintaining crop yields under adverse environmental stresses is probably the major challenge facing modern agriculture where polyamines can play important role. Polyamines (putrescine, spermidine and spermine) are group of phytohormone-like aliphatic amine natural compounds with aliphatic nitrogen structure and present in almost all living organisms including plants. Evidences showed that polyamines are involved in many physiological processes, such as cell growth and development and respond to stress tolerance to various environmental factors. ın many cases the relationship of plant stress tolerance was noted with the production of conjugated and bound polyamines as well as stimulation of polyamine oxidation. Therefore, genetic manipulation of crop plants with genes encoding enzymes of polyamine biosynthetic pathways may provide better stress tolerance to

crop plants. Furthermore, the exogenous application of PAs is also another option for increasing the stress tolerance potential in plants. Here, they described the synthesis and role of various polyamines in abiotic stress tolerance in plants.

Roychoudhury *et al.* (2011) presented the comparative protective potentiality of exogenously applied polyamines (PAs), namely spermidine (Spd) and spermine (Spm), in mitigating NaCl toxicity and inducing short-term salinity tolerance in three indica rice varieties, namely M-1-48 (salt-sensitive), Nonabokra (salt-tolerant) and Gobindobhog (highly sensitive). The retardation in root length or shoot length and toxic Na+ accumulation or K+ loss, the considerable increment in malondialdehyde/H_2O_2 accumulation or lipoxygenase activity, all of which were particularly noteworthy in M-1-48 and Gobindobhog during salinity stress, was appreciably reduced by co-treatment with Spd or Spm. Both the PAs also inhibited the extent of salt-induced protein carbonylation in all the varieties and enhanced protease activity, especially in Gobindobhog. The prevention of chlorophyll degradation was better with Spd in Nonabokra and Gobindobhog. While the salt-induced increase in anthocyanin or reducing sugar level was further prompted by Spd or Spm in all the varieties, the proline content was elevated by Spd particularly in Gobindobhog. During salinity stress, both the PAs were effective in lowering the putrescine accumulation in M-1-48 and Gobindobhog, and strikingly increasing the Spm level in all the varieties, the highest being in Gobindobhog. In addition, they enhanced the activity of peroxidases and compensated for the decreased catalase activity in all the varieties. Thus the two PAs could recuperate all the three varieties from salt-induced damages to different degrees. The salt injuries, encountered in M-1-48 and Gobindobhog, both of which showed greater susceptibility to salinity stress, were more pronouncedly alleviated and counteracted by the PAs, than the salt-tolerant Nonabokra. The reversal of inhibitory effect of salinity stress was conferred by preventing growth inhibition or various forms of cellular damages, maintaining proper K+/Na+ balance or triggering the level of osmolytes and activity of antioxidant enzymes. Their communication offers a referenced evidence for an

understanding of the mechanism by which higher PAs relieve the damages particularly in salt-sensitive rice varieties.

Kasukabe *et al.* (2004) studied that Polyamines play pivotal roles in plant defense to environmental stresses. However, stress tolerance of genetically engineered plants for polyamine biosynthesis has been little examined so far. They cloned spermidine synthase cDNA from *Cucurbita ficifolia* and the gene was introduced to *Arabidopsis thaliana* under the control of the cauliflower mosaic virus 35S promoter. The transgene was stably integrated and actively transcribed in the transgenic plants. As compared with the wild-type plants, the T_2 and T_3 transgenic plants exhibited a significant increase in spermidine synthase activity and spermidine content in leaves together with enhanced tolerance to various stresses including chilling, freezing, salinity, hyperosmosis, drought, and paraquat toxicity. During exposure to chilling stress (5°C), the transgenics displayed a remarkable increase in arginine decarboxylase activity and conjugated spermidine contents in leaves compared to the wild type. A cDNA microarray analysis revealed that several genes were more abundantly transcribed in the transgenics than in the wild type under chilling stress. These genes included those for stress-responsive transcription factors such as DREB and stress-protective proteins like rd29A. These results strongly suggest an important role for spermidine as a signaling regulator in stress signaling pathways, leading to build-up of stress tolerance mechanisms in plants under stress conditions.

Chunthaburee *et al.* (2014) studied to elucidate the spermidine (Spd) and gibberellic acid (GA_3) priming-induced physiological and biochemical changes responsible for induction of salinity tolerance in two rice (*Oryza sativa* L.) cultivars, namely 'Niewdam Gs. no. 00621' (salt tolerant) and 'KKU-LLR-039' (salt sensitive). The seeds of the two cultivars were primed separately with distilled water, 1 mM Spd or 0.43 mM GA_3. Primed seeds were germinated and the resultant seedlings were hydroponically grown for 14 days before being exposed to salinity stress (150 mM NaCl) for 10 days. Seed priming with Spd or GA_3 slightly improved salt-induced reductions in growth, anthocyanin and chlorophyll contents of the seedlings. Salt stress induced pronounced increases in Na+/K+ ratio, proline and H_2O_2 contents,

particularly in the sensitive cultivar. The levels of these salt sensitivity physiological indicators tended to be mitigated by priming with Spd and GA_3. Salt-stressed seedlings grown from seeds primed with these growth regulators also possessed higher phenolic contents and greater antioxidant capacity than the control seedlings. Based on all growth and physiological data, they concluded that Spd tended to be more effective than GA_3 in improving salt tolerance in both rice cultivars.

Li *et al.* (2014) identified the physiological effect and elucidate the possible mechanism caused by exogenous Spd (0.05 mM) in white clover under water stress induced by 20% polyethylene glycol 6000 for 12 days. Water stress elevated significantly the accumulation of reactive oxygen species and malonaldehyde, and resulted in the decrease of cell membrane stability, relative water content and relative growth rate. Spd effectively alleviated the damage effect from water stress. Spd-treated plants showed a promoted the ascorbate–glutathione cycle and maintained greater antioxidant enzyme activities (superoxide dismutase, peroxidase and catalase), as well as higher transcript level of genes encoding antioxidant enzymes than untreated plants. Additionally, the plants treated with Spd under water stress exhibited more accumulated organic solutes including soluble sugar, reducing sugar, betaine and free proline. Spd also accelerated proline catabolism and biosynthesis proceeding from glutamate pathway during water stress, but had no effect on the ornithine pathway of proline biosynthesis. They suggested that exogenous Spd under water stress may directly or indirectly regulate antioxidant defense system, organic solutes accumulation and proline metabolism.

Hsu and Kao (2007) investigated the protective effect of polyamines against Cd toxicity of rice (Oryza sativa) leaves. Cd toxicity to rice leaves was determined by the decrease in protein content. $CdCl_2$ treatment results in (1) increased Cd content, (2) induction of Cd toxicity, (3) increase in H_2O_2 and malondialdehyde (MDA) contents, (4) decrease in ascorbic acid (ASC) and reduced glutathione (GSH) contents, and (5) increase in the activities of antioxidative enzymes (superoxide dismutase, glutathione reductase, ascorbate peroxidase, catalase, and peroxidase). Spermidine (Spd) and spermine (Spm), but not putrescine (Put), were effective in

reducing $CdCl_2$-induced toxicity. Spd and Spm prevented $CdCl_2$-induced increase in the contents of H_2O_2 and MDA, decrease in the contents of ASC and GSH, an increase in the activities of antioxidative enzymes. Spd and Spm pretreatments resulted in a decrease in Cd content when compared with H_2O pretreatment, indicating that Spd and Spm may reduce the uptake of Cd. Finally they suggested that Spd and Spm are able to protect Cd-induced oxidative damage and this protection is most likely related to the avoidance of H_2O_2 generation and the reduction of Cd uptake.

Ndayiragije and Lutts (2006) analyzed the putative impact of polyamines (PAs) on the plant response to salt, seedlings from the salt-sensitive rice cultivar I Kong Pao (IKP) were exposed for 5, 12 and 19 days to 0, 50 or 100 mM NaCl in the absence, or in the presence of exogenous PAs (putrescine (Put), spermidine (Spd) or spermine (Spm) 1 mM) or inhibitors of PA synthesis (methylglyoxalbis-guanyl hydrazone (MGBG) 1 mM, cyclohexylammonium (CHA) 5 mM and D-arginine (D-Arg) 5 mM). The addition of PAs in nutritive solution reduced plant growth in the absence of NaCl and did not afford protection in the presence of salt. PA-treated plants exhibited a higher K+/Na+ ratio in the shoots, suggesting an improved discrimination among monovalent cations at the root level, especially at the sites of xylem loading. The diamine Put induced a decrease in the shoot water content in the presence of NaCl, while Spd and Spm had no effects on the plant water status. In contrast to Spd, Spm was efficiently translocated to the shoots. Both PAs (Spd and Spm) induced a decrease in cell membrane stability as suggested by a strong increase in malondialdehyde content of PA-treated plants exposed to NaCl. They discussed the results in relation to the putative functions of PAs in stressed plant metabolism.

Amooaghaie and Moghym (2011) investigated the role of polyamines in the heat-shock protection of soybean seedling by application of exogenous polyamines (putrescine, spermidine and spermine) and their biosynthetic inhibitors involving CHA (cyclohexylamine) and DFMO (D, L α-diflouromethylornithine). Application of polyamines, as a pre-treatment at 28°C for 2 h prior to heat-shock (45°C for 2 h), enhanced the recovery growth of both roots and hypocotyls. Treatment with

polyamine biosynthetic inhibitors, that is, CHA and DFMO resulted in thermo sensitization, making seedlings vulnerable to heat-shock. Treatment of polyamines plus inhibitors reduced inhibitory effects and enhanced the growth recovery of seedling. Application of polyamines decreased electrolyte leakage and malondialdehyde from different tissue sections, suggesting protection of membrane integrity. Their resuts showed that in comparisons with the heat-shock control, $CaCl_2$ application in similar to PAs decreased electrolyte leakage and lipid peroxidation from root and hypocotyl tissue sections, and in contrast, in the presence of EGTA (a chelator of calcium), the stress injury intensified and growth were severely inhibited, and electrolyte leakage and MDA content of roots were significantly increased. Therefore they suggested that under stress conditions, polyamines may replace calcium in maintaining membrane integrity by binding to phospholipids of the membrane.

Duan *et al.* (2006) investigated the effects of exogenous spermidine (Spd) on lipid peroxidation, relative plasma membrane permeability, photosystem II (PSII) gene expression and PSII photochemical activity in water-stressed wheat seedlings. The decrease in relative water content (RWC), Chl content, and 2, 6-dichlorophenol indophenols (DCIP) photoreduction of PSII, and increases in electrolyte leakage of plasma membranes and malonyldialdehyde (MDA) in water-stressed leaves was alleviated by Spd pretreatment. Furthermore, Western and Northern blot analysis showed that decreases in the PSII major proteins D1, D2 and LHCII and the transcripts of corresponding genes *psbA*, *psbD* and *cab* were also alleviated by Spd pretreatment under water stress. They concluded that the application of exogenous Spd protects PSII against water stress at both the transcriptional level and the translational level, and allows PSII to retain a higher activity level during water stress and also discussed about the protective role of Spd in the photosynthetic apparatus.

Liu *et al.* (2006) examined the effects of polyamines (Putrescine, Put; Spermidine, Spd; and Spermine, Spm) on salt tolerance of seedlings of two barley (*Hordeum vulgare* L.) cultivars (J4, salt-tolerant; KP7, salt-sensitive). The results showed that,

the salt-tolerant cultivar J4 seedlings accumulated much higher levels of Spd and Spm and lower Put than the salt-sensitive cultivar KP7 under salt stress. At the same time, the dry weight of KP7 decreased significantly than that of J4. After methylglyoxal bis (guanylhydrazone) [MGBG, an inhibitor of S-adenosylmethionine decarboxylase (SAMDC)] treatment, Spd and Spm levels together with the dry weight of both cultivars were reduced, but the salt-caused dry weight reduction in two cultivars could be reversed by the concomitant treatment with Spd. MGBG decreased the activities of tonoplast H^+-ATPase and H^+-PPase too, but the experiments in vitro indicated that MGBG was not able to affect the above two enzyme activities. However, the polyamines, especially Spd, promoted their activities obviously. Thus they suggested that the conversion of Put to Spd and Spm and maintenance of higher levels of Spd and Spm were necessary for plant salt tolerance.

Wang *et al.* (2007) studied the protective effects of polyamines (PAs) against copper (Cu) toxicity in the leaves of Nymphoides peltatum. Cu treatment increased the putrescine (Put) level and lowered spermidine (Spd) and spermine (Spm) levels, thereby reducing the (Spd+Spm)/Put ratio in leaves. Exogenous application of Spd or Spm markedly reversed these Cu-induced effects for all three PAs and partially restored the (Spd+Spm)/Put ratio in leaves. It also significantly enhanced the level of proline, retarded the loss of soluble protein, decreased the rate of $O_2^{\bullet-}$ generation and H_2O_2 content, and prevented Cu-induced lipid peroxidation. Furthermore, exogenous Spd and Spm reduced the accumulation of Cu and effectively maintained the balance of nutrient elements in plant leaves under Cu stress. Finally they suggested that exogenous application of Spd or Spm can enhance the tolerance of *N. peltatum* to Cu by increasing the levels of endogenous Spd and Spm as well as the (Spd+Spm)/Put ratio.

Minocha *et al.* (2014) investigated that the physiological relationship between abiotic stress in plants and polyamines was reported more than 40 years ago. Ever since there has been a debate as to whether increased polyamines protect plants against abiotic stress (e.g., due to their ability to deal with oxidative radicals) or

cause damage to them (perhaps due to hydrogen peroxide produced by their catabolism). The observation that cellular polyamines are typically elevated in plants under both short-term as well as long-term abiotic stress conditions is consistent with the possibility of their dual effects, i.e., being protectors from as well as perpetrators of stress damage to the cells. The observed increase in tolerance of plants to abiotic stress when their cellular contents are elevated by either exogenous treatment with polyamines or through genetic engineering with genes encoding polyamine biosynthetic enzymes is indicative of a protective role for them. However, through their catabolic production of hydrogen peroxide and acrolein, both strong oxidizers, they can potentially be the cause of cellular harm during stress. In fact, somewhat enigmatic but strong positive relationship between abiotic stress and foliar polyamines has been proposed as a potential biochemical marker of persistent environmental stress in forest trees in which phenotypic symptoms of stress are not yet visible. Such markers may help forewarn forest managers to undertake amelioration strategies before the appearance of visual symptoms of stress and damage at which stage it is often too late for implementing strategies for stress remediation and reversal of damage. Therefore they provided a comprehensive and critical evaluation on interactions between abiotic stress and polyamines in plants, and examined the experimental strategies used to understand the functional significance of this relationship with the aim of improving plant productivity, especially under conditions of abiotic stress.

Yang *et al.* (2010) examined the effects of increasing concentrations of cadmium (Cd) on the ultrastructure, Cd accumulation, generation of $O_2^{\bullet-}$, contents of ascorbate (AsA), reduced glutathione (GSH) and polyamines (PAs), as well as the activities of polyamine oxidase (PAO) (EC 1.5.3.3) and diamine oxidase (DAO) (EC 1.4.3.6) in the leaves of *Potamogeton crispus*. Cd exposure resulted in significant damage in chloroplasts and mitochondria, suggesting that Cd hastened the senescence of the tested plants. The accumulation of Cd was found to increase in a concentration-dependent manner, accompanied by increased production of $O_2^{\bullet-}$. AsA content increased progressively up to 70 μM Cd, followed by a decline at higher

concentration. GSH content slightly increased up to 70 µM Cd and then declined. In addition, Cd treatment increased the putrescine (Put) content, while decreasing spermidine (Spd) and spermine (Spm) contents, which reduced the ratio of free (Spd + Spm)/Put in the leaves. PS-conjugated PAs changed in the same pattern as free PAs, while PIS-bound PAs was different. PIS-bound Put content enhanced with the increase of Cd concentration up to 50 µM and then decreased, and PIS-bound Spd and Spm contents decreased to a lesser extent. Moreover, the activities of PAO and DAO increased significantly with the increase of the Cd concentrations, reaching the peak values at 70 µM Cd. their results suggested that certain PAs and PAs forms could play a significant role in the adaptation mechanism of *P. crispus* under Cd stress.

Yang *et al.* (2007) investigated whether and how polyamines (PAs) in rice (*Oryza sativa* L.) plants are involved in drought resistance. Six rice cultivars differing in drought resistance were used and subjected to well-watered and water-stressed treatments during their reproductive period. The activities of arginine decarboxylase, *S*-adenosyl-L-methionine decarboxylase, and spermidine (Spd) synthase in the leaves were significantly enhanced by water stress, in good agreement with the increase in putrescine (Put), Spd, and spermine (Spm) contents there. The increased contents of free Spd, free Spm, and insoluble-conjugated Put under water stress were significantly correlated with the yield maintenance ratio (the ratio of grain yield under waterstressed conditions to grain yield under well-watered conditions) of the cultivars. Free Put at an early stage of water stress positively, whereas at a later stage negatively, correlated with the yield maintenance ratio. No significant differences were observed in soluble-conjugated PAs and insoluble-conjugated Spd and Spm among the cultivars. Free PAs showed significant accumulation when leaf water potentials reached 20.51 MPa to 20.62 MPa for the drought-resistant cultivars and 20.70 MPa to 20.84 MPa for the drought-susceptible ones.By their results they suggested that rice has a large capacity to enhance PA biosynthesis in leaves in response to water stress. The role of PAs in plant defence to water stress varies with PA forms and stress stages. In adapting to drought it would be good for rice to have

the physiological traits of higher levels of free Spd/free Spm and insoluble-conjugated Put, as well as early accumulation of free PAs, under water stress.

Ahmad *et al.* (2009) conducted a hydroponic experiment to assess the possible involvement of polyamines (PAs), abscisic acid (ABA) and anti-oxidative enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) in adaptation of six populations of *Panicum antidotale* Retz. to selection pressure (soil salinity) of a wide range of habitats. Plants of six populations were collected from six different habitats with ECe ranging from 3.39 to 19.23 dSm^{-1} and pH from 7.65 to 5.86. Young tillers from 6-month-old plants were transplanted in plastic containers each containing 10 l of half strength Hoagland's nutrient solution alone or with 150 $molm^{-3}$ NaCl. After 42 days growth, contents of polyamines (Put, Spd and Spm) and ABA, and the activities of anti-oxidative enzymes (SOD, POD and CAT) of all populations generally increased under salt stress. The populations collected from highly saline habitats showed a greater accumulation of polyamines and ABA and the activities of anti-oxidative enzymes as compared to those from mild or non-saline habitats. However, the populations from highly saline environments had significantly higher Spm/Spd and Put/(Spd + Spm) ratios as compared to those from mild or non-saline environments. Similarly, the populations adapted to high salinity accumulated less Na^+ and Cl^- in culm and leaves, and showed less decrease in leaf K^+ and Ca^{2+} under salinity stress. They concluded that higher activities of anti-oxidative enzymes and accumulation of polyamines and ABA, and increased Spm/Spd and Put/(Spm + Spd) ratios are highly correlated with the degree of adaptability of *Panicum* to saline environment.

Jimenez-Bremont *et al.* (2007) reported that the maize plants tend to maintain or accumulate the levels of spermidine and spermine, while putrescine levels fluctuate depending on the NaCl concentration. The effect of salt stress on the expression of the main genes involved in polyamine biosynthesis was also assessed. Their data showed a time and NaCl dependent regulation of the Zmspds2 and Zmspds1 genes, suggesting that the former might be hyperosmotic responsive while the later NaCl responsive.

Above findings suggested that polyamines (Pas) including spermedine (spd) has important protective role in cell under different abiotic stresses.

CHAPTER III

MATERIALS AND METHODS

The study was conducted at molecular breeding laboratory of Bangladesh Agricultural Research Institute (BARI), Gazipur during the period from October 2012 to March 2013 to examine the protective role of spermidine (Spd) in onion seedling (BARI Piaj - 3) from oxidative damage under saline (dSm^{-1}) condition.

3.1 Plant materials and stress treatments

Seedlings of 'BARI Piaj- 3', were used as plant material for stress responses. Onion bulb was used for GST purification. One month old seedlings were planted in backets in green house of Plant Breeding Division. After establishment of seedlings, 16 dSm^{-1} were set up by adding NaCl solution or water for several days. Salinity level was measured by a digital EC meter (HI993310). Reaching the salinity level to 16 dS/m was counted as stress treatment, Then 100 µM of Spd were used twice daily as foliar spray. A control set was also maintained side by side. Therefore, the treatments like control, salinity and spermedine were maintained. The seedlings were observed for 7 days. Data were taken after 1, 3, 5 and 7 day of stress implementation. For GST purification fresh onion bulb tissue was used.

3.2 Chlorophyll and carotenoid determination

Extraction and determination of chlorophyll (Chl) and carotenoid (Car) were performed according to the method of Arnon (1949). Five hundred milligrams (mg) of fresh leaf material (from each treatment) was ground with 10 ml of 80% acetone at 4°C and centrifuged at 5000 rpm for 10 minutes at 4°C. The absorbance was read at 645, 663 and 470 nm for Chl *a*, Chl *b* and Car, respectively with a spectrophotometer (UV-1800, Shimadzu, Japan) against 80% acetone as blank. Chl and Car were calculated using following formulas and expressed in mg g^{-1} FW.

Chl *a* (mg g^{-1}) = $(0.0127) \times (A_{663}) - (0.00269) \times (A_{645})$

Chl *b* (mg g^{-1}) = $(0.0229) \times (A_{.645}) - (0.00468) \times (A_{663})$

Car (mg g^{-1}) = $A_{470} + (0.114 \times A_{663} - 0.638 \times A_{645})$

3.3 Determination of proline

Proline colorimetric determination proceeded according to Bates et al. (Bates et al., 1973) based on proline's reaction with ninhydrin. For proline colorimetric determination, a 1:1:1 solution of proline, ninhydrin acid and glacial acetic acid was incubated at 100ºC for 1 hour. The reaction was arrested in an iced bath and the chromophore was extracted with 4 ml toluene and its absorbance at 520 nm was determined spectrophotometrically

3.4 Extraction and measurement of ascorbate and glutathione

Onion leaves (0.5 g fresh weight) were homogenized in 3 ml ice-cold acidic extraction buffer (6% meta-phosphoric acid containing 1 mM EDTA) using a mortar and pestle. Homogenates were centrifuged at 11,500×g for 15 min at 4°C, and the supernatant was collected for analysis of ascorbate and glutathione.

Ascorbate content was determined following the method of Huang *et al.* (2005). The supernatant was neutralized using 0.5 M K-phosphate buffer (pH 7.0). The reduced ascorbate was assayed spectrophotometrically at 265 nm in 100 mM K-phosphate buffer (pH 5.6) with 0.5 U of ascorbate oxidase (AO). A specific standard curve with ASA was used for quantification.

The glutathione pool was assayed according to Yu *et al.* (2003), utilizing 0.4 ml of aliquots of supernatant neutralized with 0.6 ml of 0.5 M K-phosphate buffer (pH 7.0). Based on enzymatic recycling, glutathione is oxidized by 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of GR, and glutathione content is evaluated by the rate of absorption changes at 412 nm of 2-nitro-5-thiobenzoic acid (NTB) generated from the reduction of DTNB. GSSG was determined after removal of GSH by 2-vinylpyridine derivatization. Specific standard curves with GSH and GSSG were used.

3.5 Determination of protein

The protein concentration in the leaf extracts was determined according to the method of Bradford (1976) using BSA as a protein standard.

3.6 Enzyme extraction and assays

Using a pre-cooled mortar and pestle, 0.5 g of leaf tissue was homogenized in 1 ml of 50 mM ice-cold K-phosphate buffer (pH 7.0) containing 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 procedures were performed at 0°C to 4°C.

The Diamine oxidase (DAO, EC*:* 1.4.3.6) and Polyamine oxidae (PAO, EC: 1.5.3.11) activities were determined according to the procedure described by Gao *et al.* with some modifications. Fresh samples were homogenized in 100 mM potassium phosphate buffer (pH- 6.5). The homogenate was centrifuged at 10,000×g for 20 min at 4°C. The supernatant was used for enzyme assay. The reaction mixture contained 2.5 mL of potassium phosphate buffer (100 mM, pH- 6.5), 0.2 mL of 4-aminoantipyrine/N,N-dimethylaniline reaction solution, 0.1 mL of horseradish peroxidase (250 U/mL), and 0.2 mL of the enzyme extract. The reaction was initiated by the addition of 0.1 mL of Putrescine (at a final concentration of 20 mM) for DAO determination and 0.1 mL of Spd (at a final concentration of 20 mM) for PAO determination. The change in the optical density at 550 nm per minute by 0.001 absorbance unit was considered one a unit of enzyme activity.

The Superoxide dismutase (SOD, EC 1.15.1.1) activity of whole cell homogenates prepared on ice in 50 mM potassium phosphate buffer (pH- 7.8, with 1.34 mM diethylenetriaminepentaacetic acid) was determined using an indirect competitive inhibition assay (Spitz and Oberley, 1989). This assay is based on the competition between SOD and an indicator molecule NBT for superoxide production from xanthine and xanthine oxidase. One unit of activity was defined as that amount of protein required to inhibit NBT reduction by 50%. Incubation for at least 45 min with 5 mM sodium cyanide was used to inhibit Cu, Zn-SOD activity to measure Mn-SOD activity. Cu, Zn-SOD activity was determined by subtracting Mn-SOD activity from total SOD activity.

The Ascorbate peroxidase (APX, EC: 1.11.1.11) activity was assayed following the method of Nakano and Asada (1981). The reaction buffer solution contained 50 mM K-phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2, 0.1 mM EDTA, and enzyme extract in a final volume of 0.7 ml. The reaction was started by the addition of H_2O_2, and the activity was measured by observing the decrease in absorbance at 290 nm for 1 min using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$.

The Mono-dehydroascorbate reductase (MDHAR, EC: 1.6.5.4) activity was determined by the method of Hossain *et al.* (2010). The reaction mixture contained 50 mM Tris–HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, 0.5 U of AO and enzyme solution in a final volume of 0.7 ml. The reaction was started by the addition of AO. The activity was calculated from the change in ascorbate at 340 nm for 1 min using an extinction coefficient of 6.2 mM^{-1} cm^{-1}.

The Dehydroascorbate reductase (DHAR, EC: 1.8.5.1) activity was determined by the procedure of Nakano and Asada (1981). The reaction buffer contained 50 mM K-phosphate buffer (pH 7.0), 2.5 mM GSH, and 0.1 mM DHA. The reaction was started by adding the sample solution to the reaction buffer solution. The activity was calculated from the change in absorbance at 265 nm for 1 min using extinction coefficient of 14 $mM^{-1}cm^{-1}$.

The Glutathione reductase (GR, EC: 1.6.4.2) activity was measured by the method of Hossain *et al.* (2010). The reaction mixture contained 0.1 M K-phosphate buffer (pH- 7.8), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, and enzyme solution in a final volume of 1 ml. The reaction was initiated with GSSG, and the decrease in absorbance at 340 nm due to NADPH oxidation was recorded for 1 min. The activity was calculated using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

The Glutathione-*S*-Transferase (GST, EC: 2.5.1.18) activity was determined spectrophotometrically by the method of Rohman *et al.* (2010). The reaction mixture contained 100 mM Tris–HCl buffer (pH- 6.5), 1.5 mM GSH, 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB), and enzyme solution in a final volume of 0.7 ml. The enzyme reaction was initiated by the addition of CDNB, and the increase in

absorbance was measured at 340 nm for 1 min. The activity was calculated using the extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

The Glutathione peroxidase (GPX, EC: 1.11.1.9) activity was measured as described by Elia *et al.* (2003) using H_2O_2 as a substrate. The reaction mixture consisted of 100 mM Na-phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM NaN3, 0.12 mM NADPH, 2 mM GSH, 1 U GR, 0.6 mM H_2O_2, and 20 µl of sample solution. The reaction was started by the addition of H_2O_2. The oxidation of NADPH was recorded at 340 nm for 1 min, and the activity was calculated using the extinction coefficient of 6.62 $mM^{-1}cm^{-1}$.

The Catalase (CAT, EC: 1.11.1.6) activity was measured according to the method of Csiszár *et al.* (2007) by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition of H_2O_2. The reaction mixture contained 50 mM K-phosphate buffer (pH 7.0), 15 mM H_2O_2, and enzyme solution in a final volume of 0.7 ml. The reaction was initiated with enzyme extract, and the activity was calculated using the extinction coefficient of 39.4 $M^{-1}cm^{-1}$.

Glyoxalase I (Gly-I, EC: 4.4.1.5) assay was carried out according to Yadav *et al.* (2005). Briefly, the assay mixture contained 100 mM K-phosphate buffer (pH- 7.0), 15 mM magnesium sulphate, 1.7 mM reduced glutathione, and 3.5 mM methylglyoxal in a final volume of 0.7 ml. The reaction was started by the addition of MG, and the increase in absorbance was recorded at 240 nm for 1 min. The activity was calculated using the extinction coefficient of 3.37 $mM^{-1}cm^{-1}$.

Glyoxalase II (Gly-II, EC: 3.1.2.6) activity was determined according to the method of Principato *et al.* (1987) by monitoring the formation of GSH at 412 nm for 1 min. The reaction mixture contained 100 mM Tris–HCl buffer (pH- 7.2), 0.2 mM DTNB, and 1 mMS-D-lactoylglutathione (SLG) in a final volume of 1 ml. The reaction was started by the addition of SLG, and the activity was calculated using the extinction coefficient of 13.6 $mM^{-1}cm^{-1}$.

3.7 Measurement of the $O_2^{\bullet-}$ generation rate

Superoxide radical was determined according to the method of Elstner and Heupel (1976) with modifications. Leaves (0.3 g) were homogenized in 3 mL of 65 mmol L-1 phosphate buffer (pH- 7.8) on an ice bath and were then centrifuged at 4°C and 5,000 × g for 10 min. The supernatants (0.75 mL) were mixed with 0.675 mL of 65 mmol L-1 phosphate buffer (pH- 7.8) and 0.07 mL of 10 mmol L-1 hydroxylamine chlorhydrate and were placed at 25°C. After 20 min, 0.375 mL of 17 mmol L-1 sulfanilamide and 0.375 mL of 7 mmol L-1 α-naphthylamine were added, and the mixture was placed at 25°C for another 20 min before it was mixed with 2.25 mL of ether. The absorbance was measured t 530 nm and the $O_2^{\bullet-}$ concentration was calculated from a standard curve of $NaNO_2$.

3.8 Measurement of H_2O_2

H_2O_2 was assayed according to the method described by Yu *et al.* (2003). H_2O_2 was extracted by homogenizing 0.5 g of leaf tissue with 3 ml of 50 mM K-phosphate buffer (pH- 6.5) at 4° C. The homogenate was centrifuged at 11,500×g for 15 min. The supernatant (3 ml) was mixed with 1 ml of 0.1% TiCl4 in 20% H_2SO_4 (v/v), and the mixture was then centrifuged at 11,500×g for 15 min at room temperature. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the H_2O_2 content (ϵ= 0.28 $\mu M^{-1}cm^{-1}$) and expressed as micromoles per gram fresh weight.

3.9 Measurement of lipid peroxidation

The level of lipid peroxidation was measured by estimating MDA (Melondialdehyde), a decomposition product of the peroxidized polyunsaturated fatty acid component of the membrane lipid, using thiobarbituric acid (TBA) as the reactive material following the method of Heath and Packer (1968). Briefly, the leaf tissue (0.5 g) was homogenized in 3 ml 5% (w/v) trichloroacetic acid (TCA), and the homogenate was centrifuged at 11,500×g for 10 min. The supernatant (1 ml) was mixed with 4 ml of TBA reagent (0.5% of TBA in 20% TCA). The reaction mixture was heated at 95°C for 30 min in a water bath and then quickly cooled in an ice bath and centrifuged at 11,500×g for 15 min. The absorbance of the colored supernatant

was measured at 532 nm and was corrected for non-specific absorbance at 600 nm. The concentration of MDA was calculated by using the extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed as nanomole of MDA per gram fresh weight.

3.10 Measurement of methyl glyoxalase

Sample preparation for methylglyoxalase (MG) estimation in plants about 0.3 g tissue was extracted in 3 mL of 0.5M perchloric acid. After incubating for 15 min on ice, the mixture was centrifuged at 4°C at 11,000×g for 10 min. A colored supernatant was obtained in some plant extracts that was decolorized by adding charcoal (10 mg/mL), kept for 15 min at room temperature, and centrifuged at 11,000×g for 10 min. Before using this supernatant for MG assay, it was neutralized by keeping for 15 min with saturated solution of potassium carbonate at room temperature and centrifuged again at 11,000×g for 10 min. Neutralized supernatant was used for MG estimation following the method of Wild *et al.* (2012). An aqueous 500-mM N-acetyl-L-cysteine solution was freshly prepared. The reaction was carried out in 100 mM sodium dihydrogen phosphate buffer (adjusted to pH- 7.0 with 10 M NaOH) at 25°C. First, the MG solutions (5, 10, 15, 20 and 25 μL) equating to 0.5, 2 and 5 mM were added up to a volume of 980 μL with sodium dihydrogen phosphate buffer and the spectrophotometer set to zero. The reaction was started by adding 20 μL of the N-acetyl-L-cysteine solution (final concentration up to 10 mM), and the formation of the product N-α-acetyl-S-(1-hydroxy-2-oxo-prop-1-yl)cysteine was recorded for 10 min at a wavelength of 288 nm.

3.11 Extraction of crude protein for GST purification

Eighty gram of onion seedlings were homogenized in an equal volume of 25 mM Tris–HCl buffer (pH- 8.0) containing 1 mM EDTA, 1% (w/v) ascorbate and 10% (w/v) glycerol with Waring blender. The homogenates squeezed in a nylon cloth and was centrifuged at 11,500×g for 15 min, and the supernatant was used as crude enzyme solution.

3.12 DEAE-cellulose chromatography

Proteins were precipitated by ammonium sulfate at 65% saturation from the supernatant and centrifuged at 11,500×g for 10 minutes. The proteins were dialyzed against 10 mM Tris-HCl buffer (pH 8) containing 0.01% (w/v) β-mercaptoethanol and 1 mM EDTA (buffer A) overnight to completely remove low molecular inhibitors. The dialyzate (crude enzyme solution) was applied to a column (1.77 cm i.d. × 20 cm) of DEAE-cellulose (DE-52, Whatman, UK) that had been equilibrated with buffer A and eluted with a linear gradient of 0 to 0.4 M KCl in 750 ml of buffer A. The high active eluted peak at around 157 mM KCl was collected for further purification.

3.13 Hydroxyapatite chromatography

The pooled sample of high active GST, separated by DEAE-cellulose column chromatography, was applied on a hydroxyapatite column (1.5 cm i.d. × 5.5 cm) that had been equilibrated with buffer A. The column was eluted with a 300 ml linear gradient of potassium phosphate buffer (K-P buffer; 0-20 mM, pH- 7.0) in buffer A. The high active fraction (5 ml) was found to elute which was collected and further purified by affinity chromatography.

3.14 Affinity chromatography

The collected sample was applied to a column (0.76 cm i.d. × 4.0 cm) of S-hexyl glutathione agarose (Sigma, St. Louis, MO) that had been equilibrated with 10 mM Tris-HCl buffer (pH- 8.0) containing 0.01% (v/v) β-mercaptoethanol (buffer B). The column was washed with buffer B containing 0.2 M KCl and eluted with buffer B containing 1.2 mM *S*-hexyl glutathione. The high active protein fractions eluted with *S*-hexyl glutathione were combined and dialyzed against buffer B and the dialyzate was used as the purified and named as GSTa.

3.15 Rabbit treatment for antibody production

A rabbit (weighing about 2.5 kg) received subcutaneous injections of a 0.5 mg of purified GSTa protein in Freund's complete adjuvant at several sites. After two weeks, the rabbit was given a first booster injection of 0.5 mg of the purified GSTa

protein in incomplete adjuvant, and then a second booster injection of 0.5 mg of the purified protein in incomplete adjuvant was given two weeks after the first booster injection. Blood was taken from the ear vein one week after the second booster injection.

3.16 Extraction of soluble protein for GST and CAT

Fresh plants were extracted by homogenizing in an equal volume of 25 mM Tris–HCl buffer (pH- 8.0) containing 1 mM EDTA, 1% (w/v) ascorbate and 10% (w/v) glycerol with mortar pestle. The homogenate was centrifuged at 11,500×g for 15 min, and the supernatant was used as a soluble protein solution for enzyme assay and Western blot analysis.

3.17 Extraction for H_2O_2 assay

Fresh plants were extracted by homogenizing in 6X volume of 50 mM K-P buffer (pH- 6.5). The homogenate was centrifuged at 11,500×g for 15 min, and 2.5 ml supernatant was used for further centrifugation at 11,500×g for 15 min with 833µl reaction mixture containing 5 ml H_2SO_4 and 15µl $TiCl_4$. The 2^{nd} supernatant was used for the assay of H_2O_2 spectrophotometrically.

3.18 SDS-PAGE and Western Blotting

To check the accumulation of GST SDS-PAGE was done in 12.5% (w/v) gel containing 0.1% (w/v) SDS by the method of Laemmli (1970) followed by western blotting.

Blotting procedure

1. After electrophoresis, the gel was cut from glass plates and dipped into a plastic box having transblot buffer and was shaken for 30 min.

2. Filter paper and nitrocellulose membrane was cut with scissors and the measurement of the paper and the membrane were taken in cm.

3. Rest of all transblot buffers was poured into the steel tray. The paper and the membrane was lifted with forceps and kept in the steel tray containing

transblot solution. The paper and the membrane were wetted completely with no air bubbles.

4. The gel was taken into the tray to set in the sequence: filter paper-gel-membrane-filter paper.

5. The arrangements were placed in Semi-dryer-apparatus chamber and connected with power @ 0.8 mA/cm^2 of gel for 20 min.

Treatments of the membrane

1. After the transfer of protein to the membrane, the membrane was washed for 5 min using 20 ml PBS-T solution.

2. Twenty (20) ml PBS-T solution was taken and 5% (w/v) nonfat milk was added and the membrane was shaken into it for 1 hr.

3. The membrane was washed with PBS-T solution 2 times for 5 min each.

4. The membrane was treated with PBS-T solution containing Anti Pug A (Primary antibody, Rabbit IgG serum) solution for 20 min. Pug A antibody was 400 times dilute (The dilution was determined earlier).

5. The membrane was washed with PBS-T solution for 15 min and then 2 times for 5 min each.

6. The membrane was treated with PBS-T solution containing Anti Rabit IgG (Secondary antibody, Goat serum) solution for 20 min. Rabit IgG antibody was 1000 times diluted.

7. Again, the membrane was washed with PBS-T solution for 15 min and then 2 times for 5 min each.

8. The membrane was incubated with the solution mixture containing luminol reagent and oxidizing reagent @ 0.0625 ml/cm^2 for 1 min. Before incubation, all buffer solution was soaked by using tissue paper.

9. After incubation, the membrane was transferred to a polythene wrapped paper base and then it was covered with polythene properly to ensure as bubble free.
10. After all these steps, it was ready for film development.

Film development

1. Before at least 30 min. of film development, the developing reagents were brought from the cold room to keep it in room temperature.
2. Three different plastic trays were used for reagents and poured them accordingly. The X-ray film and forceps were taken to the dark room.
3. The X-ray film was placed on the membrane for 3-5 sec. and very quickly transferred to the developing reagents.
4. The film was developed 1 to 5 min in RENDOL, 1 min. in RENFIX and 4-5 min in Acetic acid.
5. The film was kept in water sink for 30 min and hanged for proper drying.

3.17 Statistical Analysis

All data obtained was analyzed by General Linear Model (GLM) and the mean differences were compared by Turkey tests by using SAS program. Value presented in table and figures are mean of three indepented experiments (each experiment consists of three replications). Comparisons with $P \leq 0.05$ were considered significantly different.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Effect of Spd on chlorophyll and carotenoid

The NaCl induced salinity decreased the chlorophyll (Chl) and carotenoid (Car) continuously with stress duration (Table 4.1). In control condition, Chl*a* content was 0.704 mg g^{-1} FW in onion leaf. Chl*a* content were decreased significantly upon exposure to 3 day salinity stress. Salinity decreased the Chl*a* content by 12, 21, 51 and 58% as compared to control at 1, 3, 5 and 7 day after stress implementation, respectively. In application of 100μmol of Spd as flioar spray in salinity stress seedlings, the reduction of Chl*a* was delayed. Application of Spd increased the Chl*a* by 6, 12, 38 and 28% in salinity stress seedlings at 1, 3, 5 and 7 day after stress implementation, respectively. Like Chl*a*, Chl*b* was also decreased by salinity by 20, 37, 79 and 82% over control. Spd also reduced the loss of Chl*b* and increased the content by 9, 26, 180 and 128% in salinity stressed seedlings at 1, 3, 5 and 7 day after stress implementation, respectively. The reduction of Chl*a* and Chl*b* under salinity ultimately reduced the total Chlorophyll. At 5 day and 7 day after stress implementation, total Chlorophyll content was decreased significantly (Table 4.1). Application of Spd improved the chlorophyll content significantly at these stages. Under salinity, Car content decreased gradually and significant reduction was observed at 3, 5 and 7 day after stress implementation, respectively. Upon application of Spd, Car content increased by 6, 41, 79 and 61% when compared to the content in salinity stressed seedlings.

Table 4.1. Effect of Spd on Chlorophyll and Carotenoid contents in leaves of onion seedlings under salinity stress.

Stress duration	Treatment	Chl*a*	Chl*b*	Chl*a* + Chl*b*	Car
1 day	Control	0.704^{ab}±0.057	0.475^{ab}±0.123	1.189^{ab} ±0.222	0.223^{a} ±0.068
	Saline	0.621^{ab}±0.112	0.379^{a-c} ±0.046	0.988^{bc}±0.053	0.196^{ab} ±0.049
	Saline +Spd	0.656^{ab}±0.051	0.413^{bc} ±0.055	1.077^{ac} ±0.118	0.207^{a} ±0.009
3 day	Control	0.704^{ab}±0.043	0.482^{ab}±0.047	1.182^{ab} ±0.271	0.211^{a} ±0.029
	Saline	0.556^{bc}±0.031	0.305^{cd}±0.035	0.864^{bc}±0.023	0.129^{bc} ±0.011
	Saline +Spd	0.622^{ab}±0.041	0.383^{a-c} ±0.036	1.005^{a-c} ±0.100	0.182^{ab} ±0.017
5 day	Control	0.795^{a}±0.020	0.493^{a}±0.048	1.272^{a}±0.112	0.22^{a} ±0.021
	Saline	0.388^{c} ±0.035	0.102^{e} ±0.003	0.486^{d}±0.047	0.062^{d}±0.006
	Saline +Spd	0.536^{bc}±0.018	0.286^{c-d}±0.035	0.823^{bd} ±0.035	0.111^{cd} ±0.008
7 day	Control	0.816^{a}±0.027	0.534^{a}±0.036	1.360^{a} ±0.170	0.212^{a}±0.021
	Saline	0.340^{c}±0.045	0.099^{e} ±0.001	0.436^{e}±0.060	0.059^{d}±0.005
	Saline +Spd	0.436^{bc}±0.028	0.226^{de} ±0.034	0.664^{c-e} ±0.029	0.095^{cd} ±0.007

(The data presented are the mean values of three independent experiments each containing three replications. Similar letters within a column are not significant at 5% level)

Here, salinity caused a significant reduction in chlorophyll (Chl) and carotenoid (Car) contents in onion seedlings (Table 4.1). Salt stress generally causes the inhibition of pigment accumulation in plants owing to the instability of protein complex and destruction of chlorophyll by inducing the activity of chlorophyllase, chlorophyll-degrading enzyme (Reddy and Vora, 1986). In this study, application of Spd enhanced the accumulation of Chl*a* and Chl*b* and Car (Table 4.1). This phenolmenon enhanced by Spd was similar to the results obtained by Chattopadhayay *et al.* (2002). They demonstrated that at physiological concentrations, Spd and Spm significantly prevented chlorophyll loss and down-regulated chloroplast-encoded genes, such as *psb*A, *psb*B, *psb*E and *rbc*L. Similarly, it was reported that Spd mitigated the degradation of chlorophyll in salt-stressed rice (Roychoudhury *et al.,* 2011). They also disclosed that salt-induced accumulation of

anthocyanins in seedlings of three rice cultivars was substantially enhanced by exogenously applied Spd. Chunthaburee *et al.* (2014) also reported the accumulation of chlorophyll in black glutinous rice by seed priming with Spd. On the other hand, Radhakrishnan and Lee (2014) demonstrated the increment of Car in cucumber. Similarly, Sharma *et al. (*2014) reported protective role of Spd in *Adiantum capillus-veneris.*

4.2 Effect of Spd on Relative Water Content (RWC)

The salinity induced drought reduced the leaf RWC gradually with stress duration and at 5 and 7 day after stress implementation the RWC was significantly lower as compared to control condition (Fig. 4.1). Application of Spd in leaf reduced the loss of water and restored the RWC by 7, 15, 14 and 14 % in salinity stress seedlings at at 3, 5 and 7 day after stress implementation, respectively.

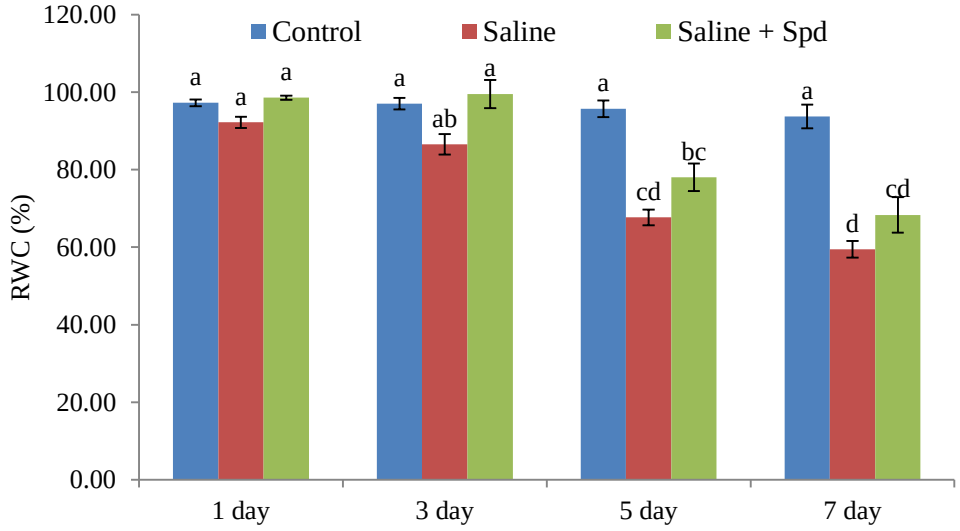

Figure 4.1. Effect of Spd on Relative Water Content (RWC) in leaves of onion seedlings under salinity stress. (Values present in the bars are mean ± standard error (SE). Similar letters between the bars are not significant at 5% level)

The function of osmotic adjustment in plants could be involved in two ways in response to water deficit: the one is to improve absorbing water under moderate water deficit; another is to maintain structural and function of cell components and enhance osmoprotection when water stress is severe (Hasegawa *et al.*, 2000; Lambers *et al.*, 2006). This study clearly showed that exogenous Spd was directly

correlated with the leaf water content (Fig. 4.1). However, the osmotic adjustment might also be due to proline sinthesys also and it is duscused in next paragraph.

4.3 Effect of Spd on proline content

Proline content increased sharply and continuously under salinity stress in onion seedlings (Fig. 4.2). In leaves of salinity treated seedlings, the content was was 1.3, 2.3, 4.7 and 7.5 fold higher over control at 1, 3, 5 and 7 day after stress implementation, respectively. Exogenous foliar spray of Spd increased the proline by 46, 22, 29 and 7% over salinity stress at 1, 3, 5 and 7 day after stress implementation, respectively.

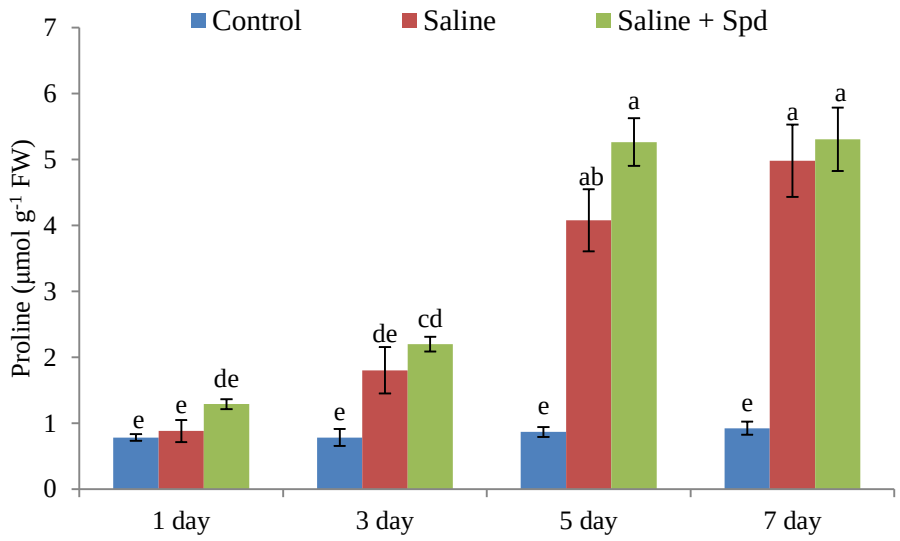

Figure 4.2. Effect of Spd on proline content in leaves of onion seedlings under salinity stress. (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

It was interesting that proline accumulation in onion seedlings had a similar patern like RWC (Fig. 4.2). It has been reported that exogenous Spd strongly promoted reducing sugar and proline content in different plants during salinity stress, which proved that osmolyte levels are regulated by PAs synthesis or absorption in alleviating stress injury (Duan *et al.*, 2008; Roychoudhry *et al.*, 2011). Proline function was considered as an osmoprotectant for the detoxification of reactive oxygen species (ROS), where it can accumulate a huge concentration without detrimental ionic strength effects (Yancey *et al.*, 1982), and as compatible solute for osmotic adjustment (Kocsy *et al.*, 2005; Ashraf and Foolad, 2007) and also as a

source of nitrogen or energy (Verslues and Sharp, 1999). Hence, its metabolism is very important for plants to survive the salinity. Proline biosynthesis in higher plants may proceed not only from glutamate but also from ornithine pathway. Δ1-pyrroline-5-carboxylate synthetase (P5CS) and ornithine aminotransferase (OAT) are key enzymes involved in glutamate pathway and ornithine pathway, respectively (Sanchez *et al.*, 2001; Szabados and Savoure, 2009). Previously, under drought stress, Li *et al.* (2014) reported that exogenous Spd only promoted P5CS activity and failed in OAT activity, which demonstrated that the significantly elevated proline accumulation in Spd-treated plants was dependent upon the up-regulated P5CS activity, while OAT was not essential for this change. Therefore, proline metabolism under salinity is important which needs more clarification. In this study, higher proline metabolism caused by exogenous Spd may be involved in osmotic adjustment under salinity stress, which explains the better membrane stability and higher RWC in Spd-treated plants regarding the response of salinity mediated water stress.

4.4 Effect of Spd on Reactive Oxygen Species (ROS) production

The formation rate of $O_2^{\bullet-}$ increased continuously and significantly with duration of

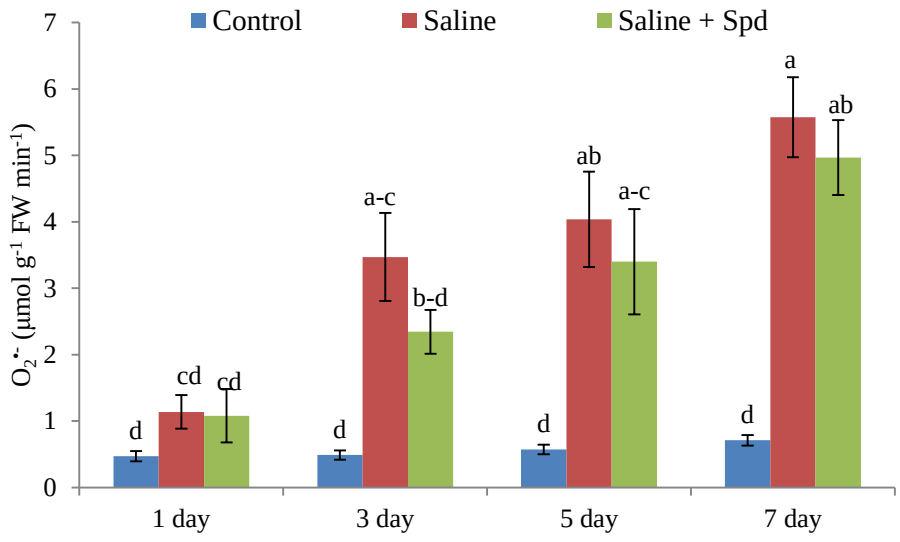

Figure 4.3. Effect of Spd on $O2^{\bullet-}$ generation rate in leaves of onion seedlings under salinity stress. (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

saline stress (Fig. 4.3). The $O_2^{\bullet-}$ production rates were 2.4, 7.1, 7.1 and 7.8 fold higher in saline stress over control at 1, 3, 5 and 7 day after stress implementation, respectively. Lower generation rates were observed upon application of Spd in salinity stressed seedlings. Spd treatment reduced the formation rate of $O_2^{\bullet-}$ by 5, 32, 16 and 11%, at 1, 3, 5 and 7 day after stress implementation, respectively.

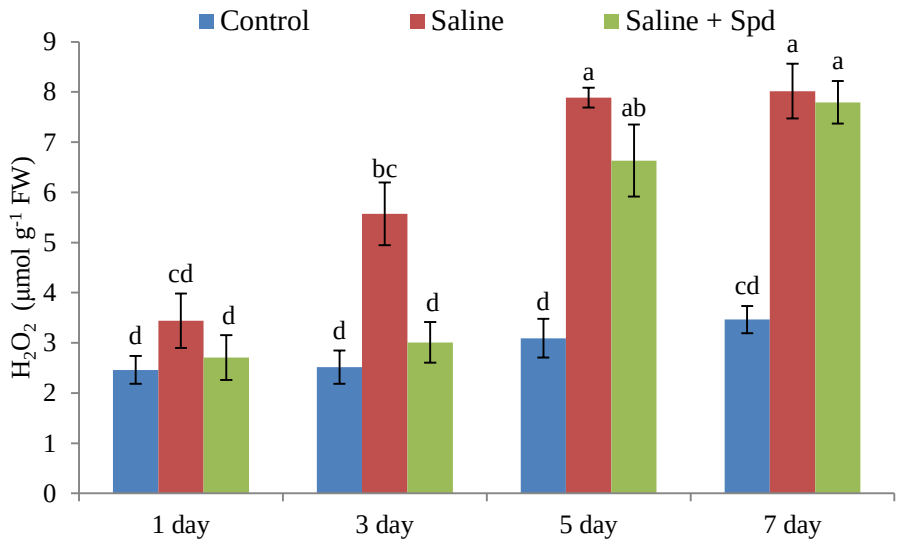

Figure 4.4. Effect of Spd on H_2O_2 content in leaves of onion seedlings under salinity stress. (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level.)

In control seedlings, the concentration of H_2O_2 ranged from 2.5 to 3.5 µmol min^{-1} mg^{-1} protein (Fig. 4.4). Like $O_2^{\bullet-}$, H_2O_2 increased sharply under saline condition. The content of H_2O_2 were 1.4, 2.2, 2.5 and 2.3 fold higher in saline treated seedlings compared to control at 1, 3, 5 and 7 day after stress implementation, respectively (Fig. 4.4). Spd reduced the content by 21, 46, 16 and 28% over saline stress at 1, 3, 5 and 7 day after stress implementation, respectively.

Reactive Oxygen Species (ROS), including singlet oxygen ($^1O_2$), superoxide($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$), are highly reactive causing damage to major cellular components like proteins, lipids, carbohydrates, DNA which ultimately results in cell death (Foyer *et al.*, 1994; Foyer & Noctor, 2005). To protect themselves against the toxic ROS, plant cells and its organelles like

chloroplast, mitochondria and peroxisomes employ antioxidant defense systems. ROS generation is a common phenomenon in crop under salinity (Noctor *et al.*, 2002; Hernández *et al.*, 2000; Huang *et al.*, 2005). In this study, we observed profound increase in $O_2^{\bullet-}$ and H_2O_2 contents in onion seedlings (Fig. 4.3 and 4.4). The tolerance of plants to ROS requires the adaptation of many complex and multifaceted processes. For example, ROS-scavenging enzymes and antioxidant molecules in plants prevent or alleviate the damage from $O_2^{\bullet-}$ and H_2O_2, and $O_2^{\bullet-}$ can be dismutated into H_2O_2 by SOD in chloroplasts, mitochondria, the cytoplasm and peroxisomes (Bowler *et al.*, 1992).

4.5 Effect of Spd on lipid peroxidation

Lipid peroxidation was measured as melondialdehyde (MDA) which sharply increased at any duration of saline stress in onion leaves which was significantly higher as compared to respective control (Fig. 4.5). The increments of MDA were 1.5, 4.5, 5.7 and 8.8 fold higher over control at 1, 3, 5 and 7 day after stress implementation, respectively. The supplementation of Spd reduced the contents of MDA by 39, 51, 18 and 14% at 1, 3, 5 and 7 day of salinity stress, respectively.

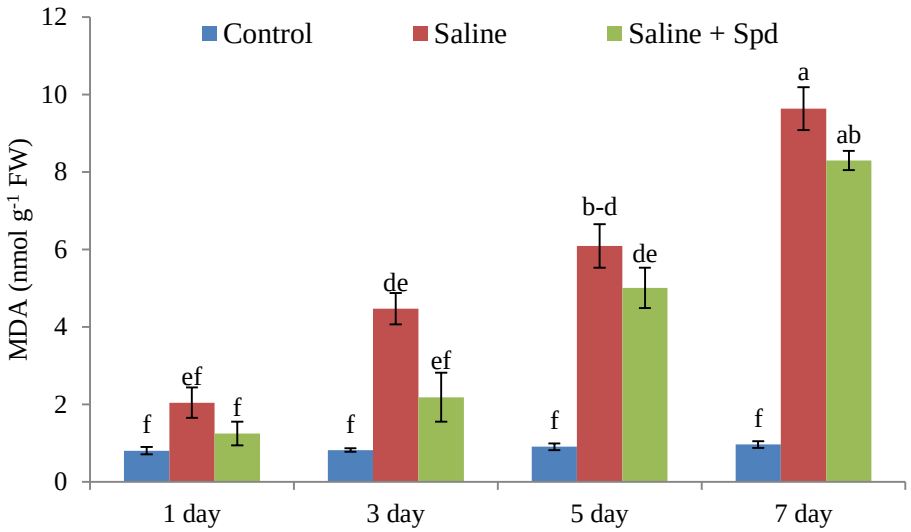

Figure 4.5. Effect of Spd on Melondialdehyde (MDA) content in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

MDA is regarded as a marker for evaluation of lipid peroxidation or damage to plasmalemma and organelle membranes that increases with environmental stresses

including salinity (Garg and Manchanda, 2009). In our experiment, both MDA and H_2O_2 were found to be increased with stress duration under salt stress (Fig. 4.5), which was in agreement with several previous reports (Saleethong *et al.*, 2011; Moschou *et al.*, 2008).

4.6 Effect of Spd on Methylglyoxal (MG) content

As a cytotoxic stress indivator MG was measured in onion seedlings. Continuous increase was observed in the contents of MG in onion leaves under salinity stress (Fig. 4.6). After 3 day of stress, the content was significantly higher as compared to control. At 1, 3, 5 and 7 day of saline stress, MG contents increased by 38, 186, 233 and 538% over control. Application of Spd reduced the MG contents in saline treated seedlings by 30, 48, 14 and 13% at 1, 3, 5 and 7 day after stress implementation, respectively.

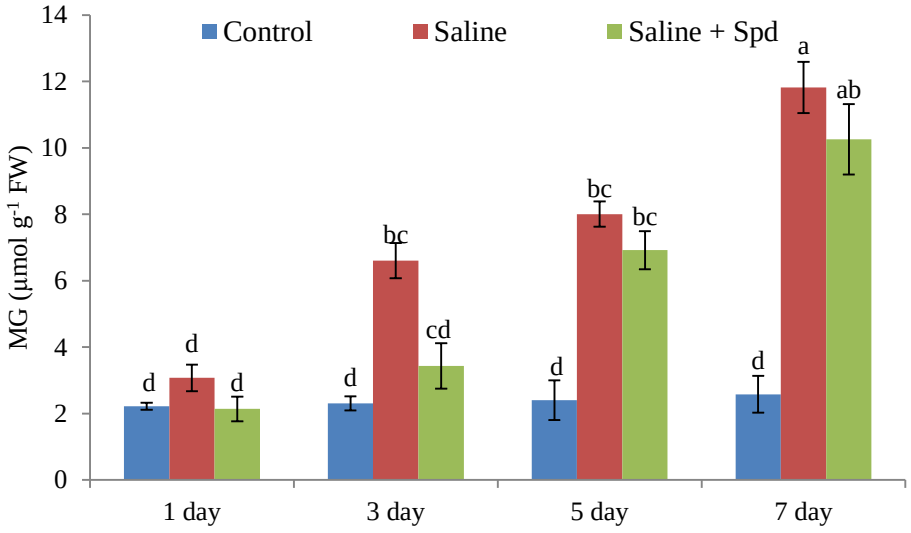

Figure 4.6. Effect of Spd on Methylglyoxal (MG) content in leaves of onion seedlings under salinity stress. (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

The present investigation demonstrated that salt stress caused significant accumulation of MG in onion cell (Fig. 4.6). Supply of Spd increased the activities of glyoxalases decreased ultimately the MG contents. However, Spd seemed to be more effective in MG detoxification system for short duration (Upto 3 day) of salinity.

4.7 Effect of Spd on ascorbate and glutathione

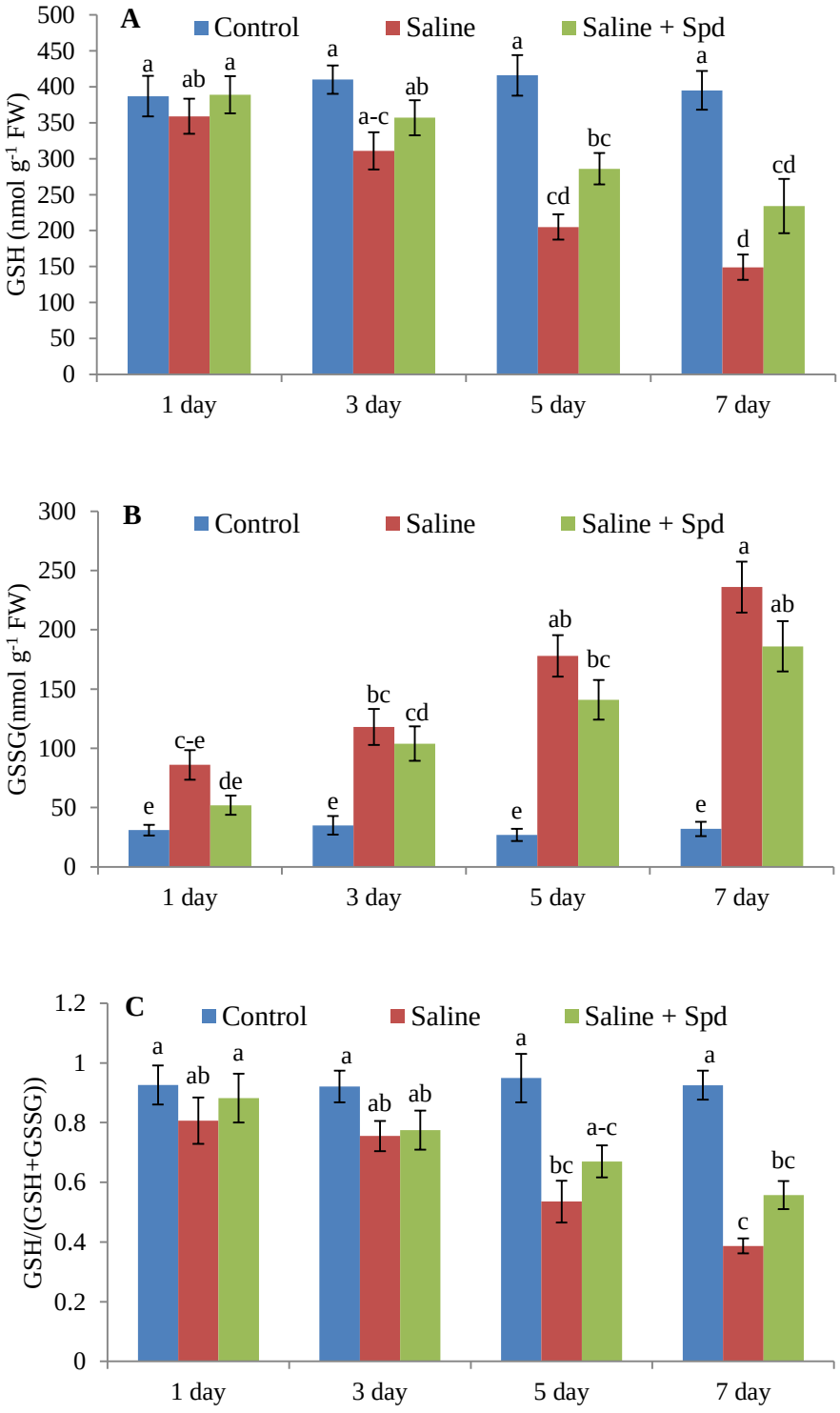

Figure 4.7. Effect of Spd on reduced glutathione [GSH] (A), oxidized glutathione [GSSG] (B) and glutathione redox [GSH/(GSH+GSSG)] (C) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

Saline stress caused significant decrease in GSH content in onion leaves, while GSH contents decreased by 7, 24, 51 and 62% at 1, 3, 5 and 7 day after stress implementation, respectively {Fig. 4.7(A)}. In presence of Spd, the saline treated seedlings showed 8, 14, 40 and 57% higher GSH compared to saline treated seedlings without Spd at 1, 3, 5 and 7 day after stress implementation, respectively. On the other hand, salinity increased GSSG content significantly and alter the glutathione redox state (Fig. 4.7). In saline stressed seedlings, GSSG contents were 1.8, 2.4, 5.6 and 6.4 folds higher compared to control at 1, 3, 5 and 7 day after stress implementation, respectively {Fig. 4.7(B)}. Application of Spd in saline stress seedlings decreased 40, 12, 20 and 21% GSSG compared to the content in saline stressed seedlings without Spd at 1, 3, 5 and 7 day after stress implementation, respectively. Saline stress also caused a significant decrease in glutathione redox state, where, importantly, application of Spd significantly contributed to the maintenance of the NaCl-reduced redox state (Fig. 4.7).

GSH is a key component of the antioxidant network that scavenges ROS either directly or indirectly by participating in the ascorbate-glutathione cycle (Noctor *et al.,* 2002). The central role of GSH in the antioxidant defense system is due to its ability to regenerate ascorbate through reduction of DHA via the ascorbate-glutathione cycle (Apel and Hirt, 2004). GSH also plays a vital role in the antioxidant defense system as well as glyoxalase system by acting as a substrate or cofactor for some enzymes. Furthermore, GSH plays a protective role in salt tolerance by the maintenance of the redox state (Saha *et al.,* 2015). The increased level of GSH pool is generally regarded as a protective response against oxidative stress, although defense against stress situations sometimes occurs irrespective of the GSH concentration (Potters *et al.,* 2004). Salinity stress caused a significant decrease in GSH levels at 3, 5 and 7 day saline stress, and at the same time, GSSG levels also increased significantly at 1, 3, 5 and 7 day saline stress (Fig. 4.7). Salinity stress also altered the glutathione redox state during stress period, and Spd treatment significantly decreased the level of GSSG at 1, 3, 5 and 7 day after stress implementation. However, the results suggested the contribution of Spd to the

maintenance of the glutathione redox state during the stress period. Conversion of DHA to ASA requires GSH (Apel and hirt, 2004). The slow increment of GSH content in onion cells in presence of Spd under salt stress may be attributed to increasing activity of DHAR (Saha *et al*., 2015), which utilizes GSH as an electron donor in the reduction of DHA.

A continual decrease was observed in ascorbic acid (ASA) content under saline stress {Fig. 4.8(A)}. Salinity reduced the ASA content by 15, 36, 43 and 60%, over control at 1, 3, 5 and 7 day after stress implementation, respectively. Application of Spd maintained the ASA content higher by 7, 16, 16 and 17% in stressed seedlings. Contrary, the DHA contents were observed to increase continuously with duration of saline stress {Fig. 4.8(B)}, where 32, 34, 33 and 52% higher DHA content was found at 1, 3, 5 and 7 day after stress implementation, respectively. The presence of Spd in saline stressed seedlings reduced the oxidation of ASA and decreased the contents of DHA (15, 17, 16 and 23% at 1, 3, 5 and 7 day after stress implementation, respectively) as well. Under saline stress, the ascorbate redox reduced by 16, 28, 34 and 52% at 1, 3, 5 and 7 day after stress implementation, respectively. Importantly, Spd maintained the ascorbate redox in saline stressed seedlings by 11, 18, 20 and 31% at 1, 3, 5 and 7 day after stress implementation, respectively.

In ascorbate-glutathione cycle, ASA have vital roles in development of plant stress tolerance to adverse environmental conditions (Apel and Hirt, 2004; Nakano & Asada, 1981). ASA can effectively reduce ROS produced under stress conditions including salt stress and thus prevents oxidative stress (Shalata & Neumann, 2001). In our study, the content of ASA reduced gradually and significantly with duration of salt stress and ascorbate redox suggested more oxidation of ASA with stress duration (Fig. 4.8). In ascorbate-glutathione cycle, APX, MDHAR and DHAR are important enzymes involved in maintaining the ASA. In plant cells, the most important reducing substrate for H_2O_2 detoxification is ascorbate (Mehlhorn *et al.*, 1996; Nakano & Asada, 1987). The higher contents of DHA might be resulted from oxidation of ASA (Fig. 4.8).

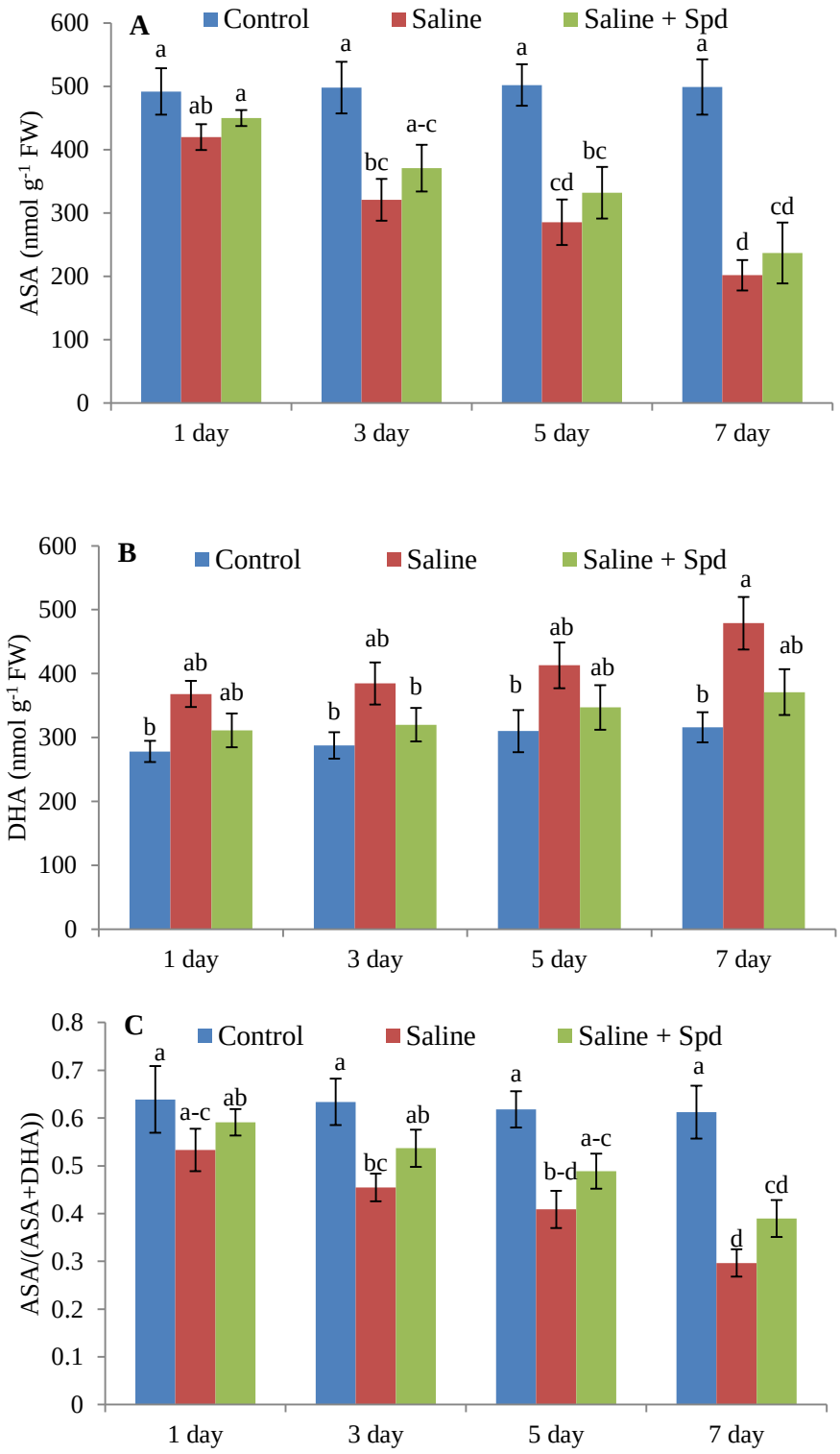

Figure 4.8. Effect of Spd on Ascorbic acid [ASA] (A), dehydroascorbate [DHA] (B) and Ascorbate redox [ASA/(ASA+DHA)] (C) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

4.8 Effect of Spd on polyamine related enzymes

Under saline stress, the activity of polyamine oxidase (PAO) increased under stress duration, and 7, 38, 33 and 38% of increment was found at 1, 3, 5 and 7 day of stress (Fig. 4.9). Application of Spd in saline stressed seedlings increased the activity over salinity. However, the activity decreased after 3 day of stress implementation.

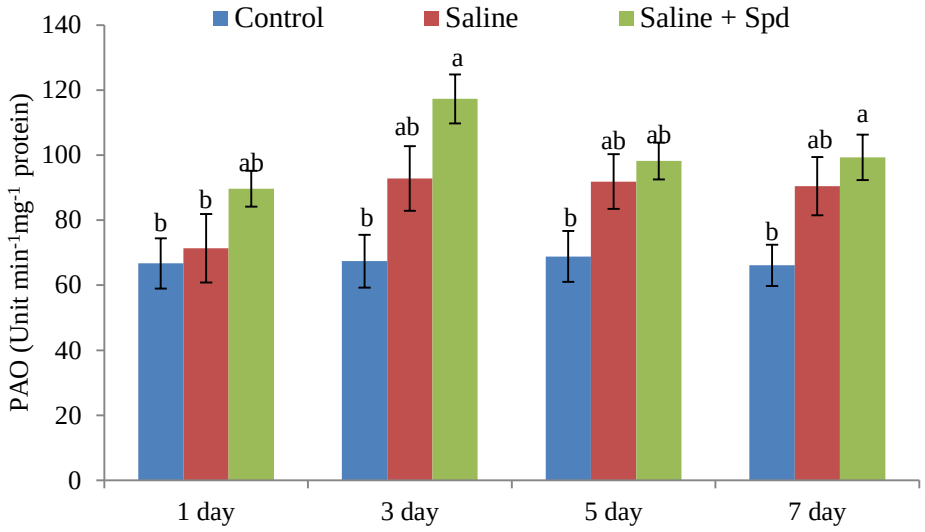

Figure 4.9. Effect of Spd on activities of Polyamine oxidase (PAO) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

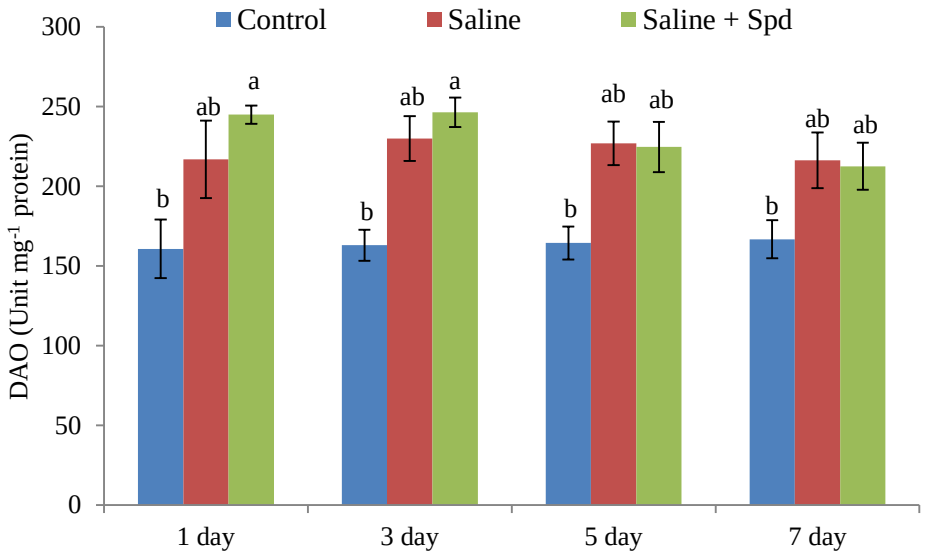

Figure 4.10. Effect of Spd on activities of Diamine oxidase (DAO) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

The activity of diamine oxidase (DAO) increased under saline stress with stress duration (Fig. 4.10). Salinity increased the activity by 35, 41, 38 and 30% at 1, 3, 5 and 7 day after stress implementation, respectively. Application of Spd in saline stressed seedlings increased the activity over salinity up to 3 days of stress. However, after that Spd failed to increase the activity.

PAO and DAO activities play an important role in increasing the stress-resistance of plants. Data from studies in recent years has shown that PAO and DAO play a role in the Polyamine (PA) catabolism and the products formed as a result of PA degradation are required in many important physiological events (Kongkiattikajorn, 2009). PA oxidation plays an essential role during PA signal transduction. Importantly, the activities of PAO and DAO enzymes are increased upon pathogen infection (Moschou *et al.*, 2009). In tobacco, oxidation induces the hypersensitive response during TMV infection and this is essential for defense against the bacterium *P. syringae* pv. *Tabaci* and the oomycete *Phytophthora parasitica var. nicotianae* (Yoda *et al.*, 2003; Moschou *et al.*, 2009) because PAO and DAO activities result in the production of H_2O_2, a process that contributes to stimulate host cell death. The activities of PAO and DAO increased under salinity stress, but this enhancement was effectively increased by Spd addition upto 3 days after stress. (Fig. 4.9 and 4.10). It has been reported that DAO or PAO activity was stimulated by salinity in oat seedlings or *Brassica campestris* (Smith, 1977; Das *et al.*, 1995).

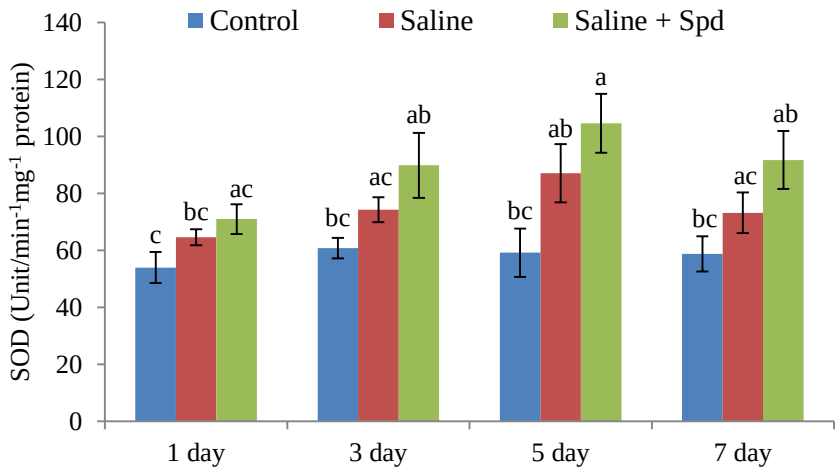

Figure 4.11. Effect of Spd on activities of superoxide dismutase (SOD) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

The activity of superoxide dismutase (SOD) increased under saline stress with stress duration (Fig. 4.11). However, after 5 day of stress it decreased. Salinity increased the activity by 20, 22, 47 and 25% at 1, 3, 5 and 7 day after stress implementation, respectively, over control. Application of Spd in saline stressed seedlings increased the activity over salinity by 10, 21, 20 and 25% at 1, 3, 5 and 7 day after stress implementation, respectively.

In this study, exogenous Spd increased SOD activity which correlate negatively with $O_2^{\bullet-}$ generation. Therefore, an increase in SOD activity can be induced by the addition of Spd, and this will enhance the dismutation of $O_2^{\bullet-}$ in NaCl-stressed onion seedlings. On the other hand, H_2O_2, produced through action of SOD, is a toxic compound which is injurious to the cell, and excessive accumulation of H_2O_2 is one of the indicators of oxidative stress (Apel and Hirt, 2004). In this study the activities of SOD increased under salinity stress, but this enhancement was effectively increased by Spd addition upto 5 days after stress.

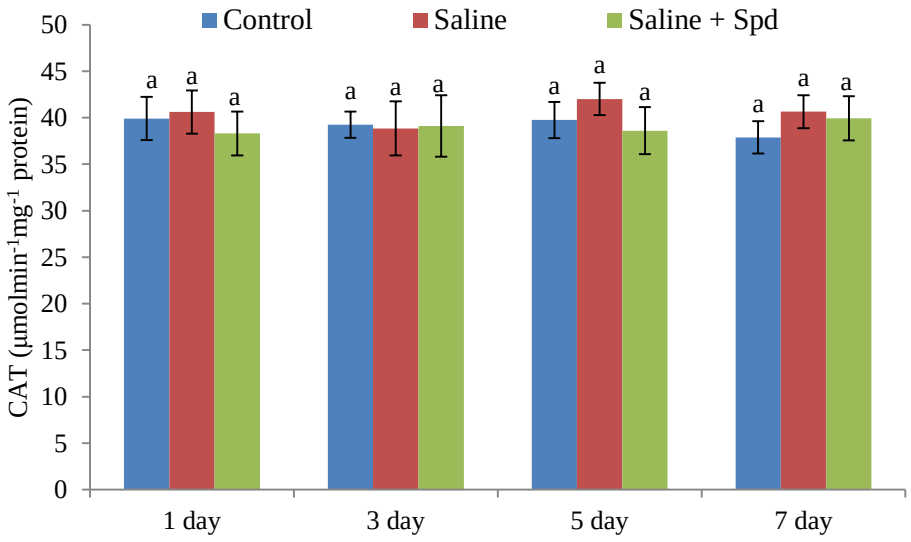

Figure 4.12. Effect of Spd on activities of Catalase (CAT) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

The strongest decomposer of H_2O_2, catalase (CAT) activity was not observed to increase in the onion seedlings under saline stress (Fig. 4.12). Foliar application of Spd also failed to increase the activity in saline stressed seedlings.

Catalase (CAT), as compared to APX, GPX and POD, with low affinity towards H_2O_2 but with a high processing rate (Scandalios, 2005), may become the principal enzymatic H_2O_2 scavenger in plants under salt-stressed conditions, where the cellular H_2O_2 level becomes several fold higher than found in plants grown under normal conditions (Nor'aini *et al.*, 1997). CAT, POD, GPX and APX are reported to scavenge H_2O_2 to water in plant species (Gill and Tujeta, 2010; Miller *et al.*, 2010) but unlike other H_2O_2 scavenging enzymes, enzymatic reaction of CAT is not saturated with increasing concentrations of the peroxide and is independent of other cellular reductants for instituting its activity (Scandalios, 2005). However, a large body of reports suggests that as compared to unstressed plants, the CAT activity is significantly down regulated in salt-stressed plants (Sharma *et al*., 2014; Demiral and Türkan, 2004; Tanaka *et al.*, 1999; Foyer and Noctor, 2000; Lee *et al.*, 2001), suggesting that the enzyme may not serve as the major scavenger of H_2O_2 under salt offence to plants. In this study, the CAT activity also remained almost unchanged under salt stress (Fig. 4.12) which might be due to ineffective enzyme synthesis or change in assembly of enzyme subunits (Gupta *et al.*, 2009).

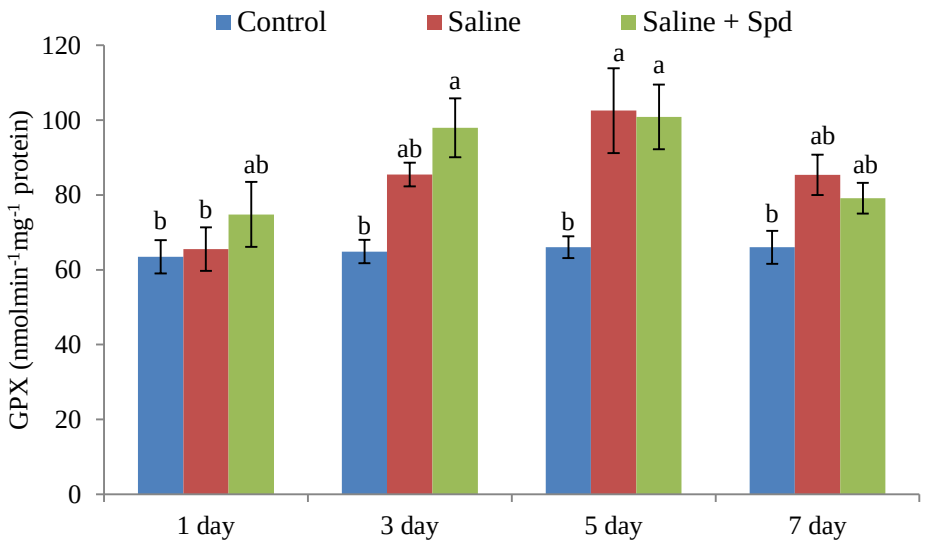

Figure 4.13. Effect of Spd on activities of Glutathion peroxidase (GPX) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

Saline stress increased the activity of glutathione peroxidase (GPX) under with stress duration (Fig. 4.13). However, after 5 day of stress, the activity decreased. Salinity

increased the activity by 3, 32, 55 and 29% over control at 1, 3, 5 and 7 day after stress implementation, respectively. Application of Spd in the seedlings increased the activity over salinity by 14 and 15% at 1 and 3 day after stress implementation, respectively. At five day, the activity was almost similar with the activity without Spd. However, at seven day, Spd reduced the activity by 7% as compared to the activity under salinity.

Application of Spd treatment increased the activities of GPX (Fig. 4.13) which reduced the H_2O_2 level and MDA. However, GPX activity down regulated after 3 days of stress which might have sensitivity with Spd for prolong interaction. GPX uses GSH in H_2O_2 decomposition.

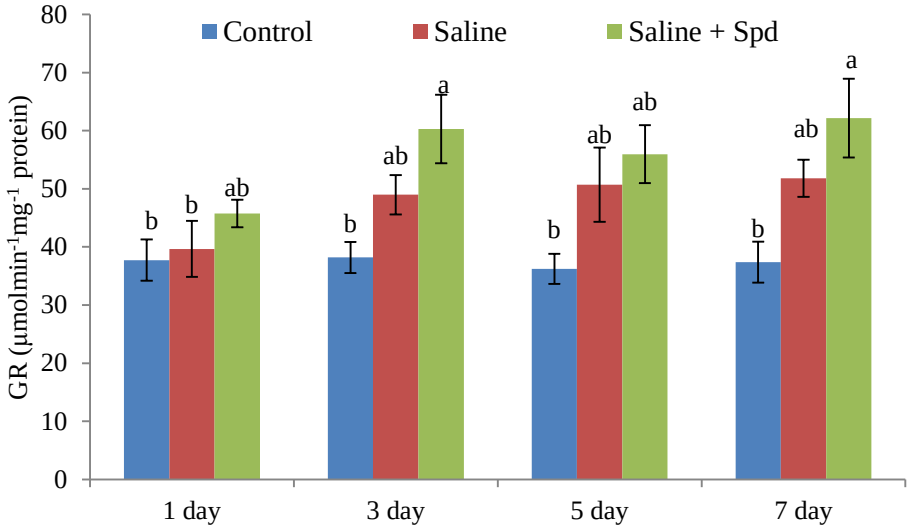

Figure 4.14. Effect of Spd on activities of Glutathion reductase (GR) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

Saline stress increased the activity of glutathione reductase (GR) under with stress duration (Fig. 4.14). Salinity increased the activity by 5, 28, 40 and 38% over control at 1, 3, 5 and 7 day after stress implementation, respectively. Notably, application of Spd increased the activity over salinity. Presence of Spd in the seedlings increased the activity over salinity by 15, 23, 10 and 20% at 1, 3, 5 and 7 day after stress implementation, respectively.

GR is an important enzyme which is important for maintaining high ratio of GSH in plant cells, also necessary for accelerating the H_2O_2 scavenging (Saha *et al.*, 2015). In this study, GR activity increased significantly in the seedlings with or without Spd (Fig. 4.14) which could maintain the GSH in salt stressed onion seedlings. This result was collaborated with other recent findings, where exogenous PAs including Spd upregulated the GR activity under salt stress (Erat *et al.*, 2008).

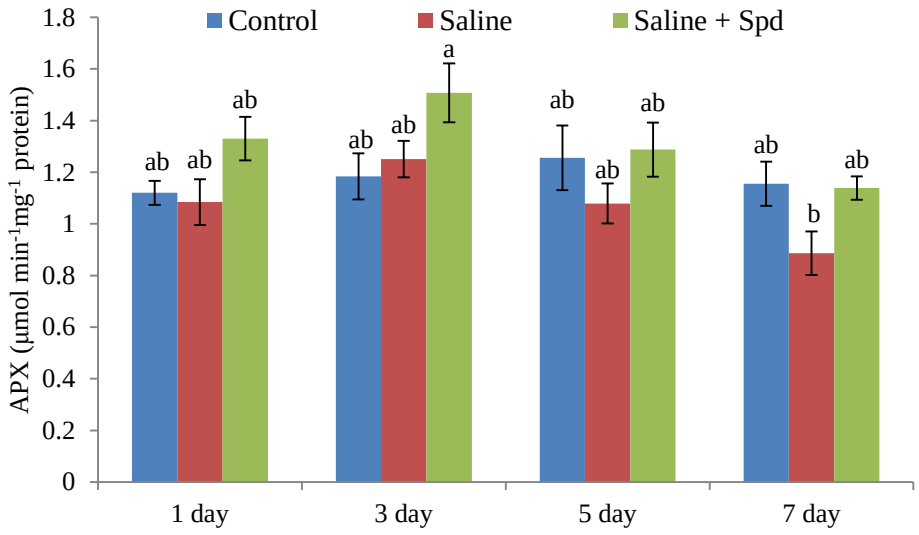

Figure 4.15. Effect of Spd on activities of Ascorbate peroxidase (APX) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

Under salinity, the ascorbate peroxidase (APX) activity decreased after 3 day of stress (Fig. 4.15). At 3 day, the activity was slightly higher (6%) over control. Application of Spd in the seedlings increased the activity over salinity, where Spd increased the activity by 23, 21, 19 and 39% over salinity at 1, 3, 5 and 7 day after stress implementation, respectively.

Like CAT, APX activity also decreased under salt stress after 3 day of stress (Fig. 4.15). The activity might be inhibited by Na toxicity. Addition of Spd improved the activity in the salinity stressed seedlings and here, Spd might reduce the uptake of Na^+ (Gupta *et al.*, 2012). APX uses two molecules of ascorbate to reduce H_2O_2 to water, with the concomitant generation of two molecules of monodehydroascorbate that disproportionates to ASA and DHA (Apel and Hirt, 2004). The decreased

content of APX activity under salinity stress might be due to inactivation under high salinity, and exogenous Spd enhanced the activity which indicated the H_2O_2 scavenging role of Spd.

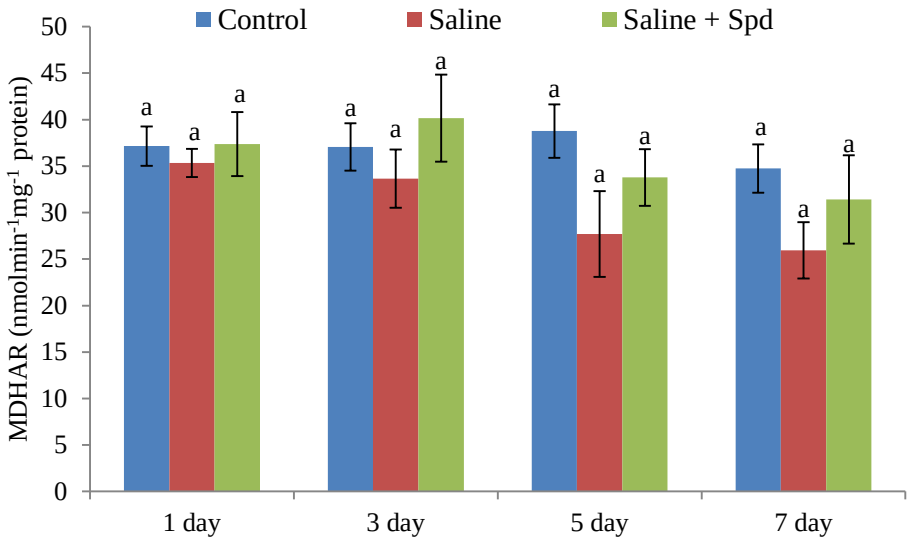

Figure 4.16. Effect of Spd on activities of Monodehydroascorbate reductase (MDHAR) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

Saline stress decreased the activity of monodehydroascorbate reductase (MDHAR) under with stress duration (Fig. 4.16). However, significant variation was not found between the activities under control and salinity. Salinity decreased the activity by 5, 29, 40 and 25% over control at 1, 3, 5 and 7 day after stress implementation, respectively. Notably, application of Spd increased the activity over salinity, where the increases were by 6, 19, 22 and 21% at 1, 3, 5 and 7 day after stress implementation, respectively.

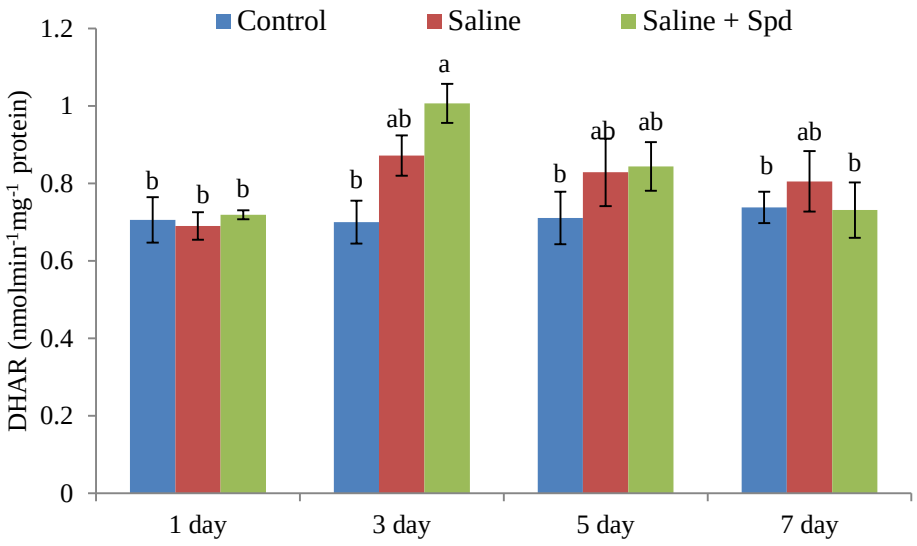

Figure 4.17. Effect of Spd on activities of Dehydroascorbate reductase (DHAR) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

Under salinity stress, the activity of dehydroascorbate reductase (DHAR) was higher after 3 day (Fig. 4.17). However, significant variation was not found between the activities under control and salinity. Spd increased the activity by 25, 17 and 9% over salinity at 1, 3, 5 and 7 day after stress implementation, respectively. Notably, in application of Spd, the highest induced activity was found at 3 day of salinity stress.

MDHAR and DHAR are two important enzymes related to the regeneration of ASA which is a strong antioxidant. Both enzymes are equally important in regulating ASA level and its redox state under oxidative stress condition (Eltayeb *et al.*, 2007; Wang *et al.*, 2010). In our study, under saline stress, the activity of MDHAR decreased continuously (Fig. 4.16). On the other hand, DHAR activity increased upto 3 days and slightly decreased (Fig. 4.17). The decreased activity which was supposed to decrease ASA in onion leaves might be used in H_2O_2 decomposition. Exogenous Spd enhanced the activities which helped in regeneration of ASA (Fig. 4.16).

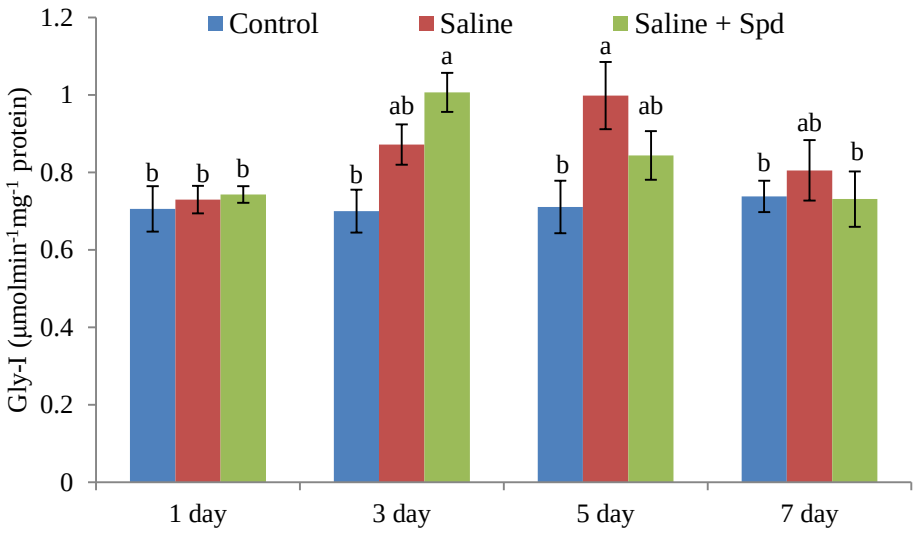

Figure 4.18. Effect of Spd on activities of Glyoxalase-I (Gly-I) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

Salinity stress increased the glyoxalase-I (Gly-I) activity gradually upto 5 day of stress (Fig. 4.18). Salinity increased the activity by 3, 25, 41 and 9% over salinity at 1, 3, 5 and 7 day after stress implementation, respectively. Notably, in application of Spd, the activity increased at 3 day salinity stress. However, Spd decreased the activity by 16 and 9% as compared to the activity level under salinity.

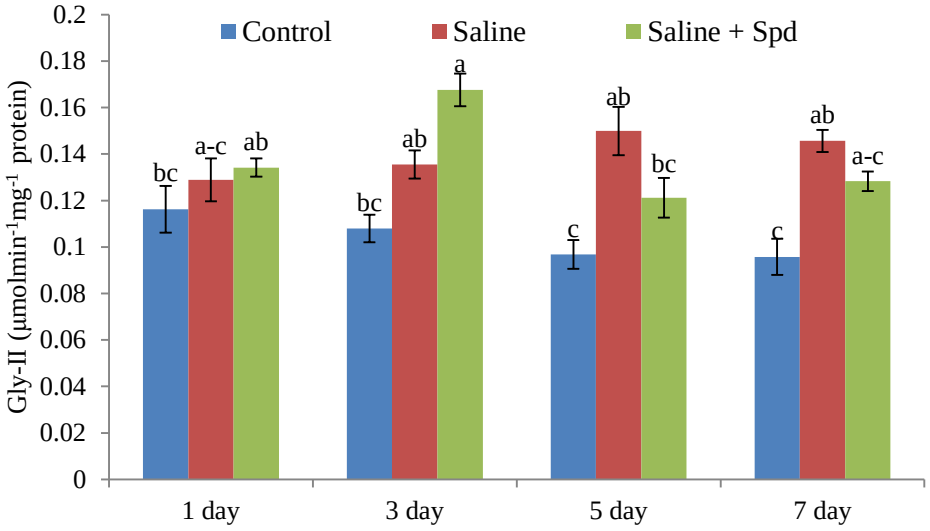

Figure 4.19. Effect of Spd on activities of Glyoxalase-II (Gly-II) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

Saline stress increased the activity of glyoxalase-II (Gly-II), where the highest activity was found at 5 day salinity stress (Fig. 4.19). Salinity increased the activity by 11, 26, 55 and 52% over control at 1, 3, 5 and 7 day after stress implementation, respectively. It was important that application of Spd increased the activity in the early stage of stress, while 4 and 24% higher activity was increased at 1 and 3 day of saline stress, respectively over salinity. Over salinity Spd decreased the activity by 19 and 12% at 5 and 7 day after stress implementation, respectively.

The glyoxalase system consists of two enzymes (Gly-I and Gly-II) acts to convert the potential cytotoxic MG to non-toxic hydroxyacids such as lactate. Gly-I use GSH to convert MG to S-D-lactoyl glutathione, while the hydrolytic reaction catalyzed by Gly-II liberates the lactic acid and free GSH (Noctor *et al.,* 2002). In several plant species, upregulation or overexpression of these enzymes increases tolerance to abiotic stresses (Hoque *et al.,* 2007a; Singla-Pareek *et al.,* 2008; Saxena *et al.,* 2011). Under salinity stress, upto 5 day of stress, Gly-I and Gly-II activities increased and decreased thereafter (Fig. 4.18 and 4.19). However, the increases in Gly-I and Gly-II activities in salinity stressed onion seedlings suggested that the detoxification of MG via the glyoxalase system as both Gly-I and Gly-II increased concomitantly with lower contents of MG. The higher GSH level with higher Gly-I and Gly-II activities with Spd suggested the evidence for protective role of Spd for glyoxalase system for conferring saline stress tolerance in onion leaves. This tolerance might be via proline accumulation, because proline was reported to maintain higher glyoxalases and GSH in other plant species (Hoque *et al.,* 2008; Rohman *et al.,* 2015).

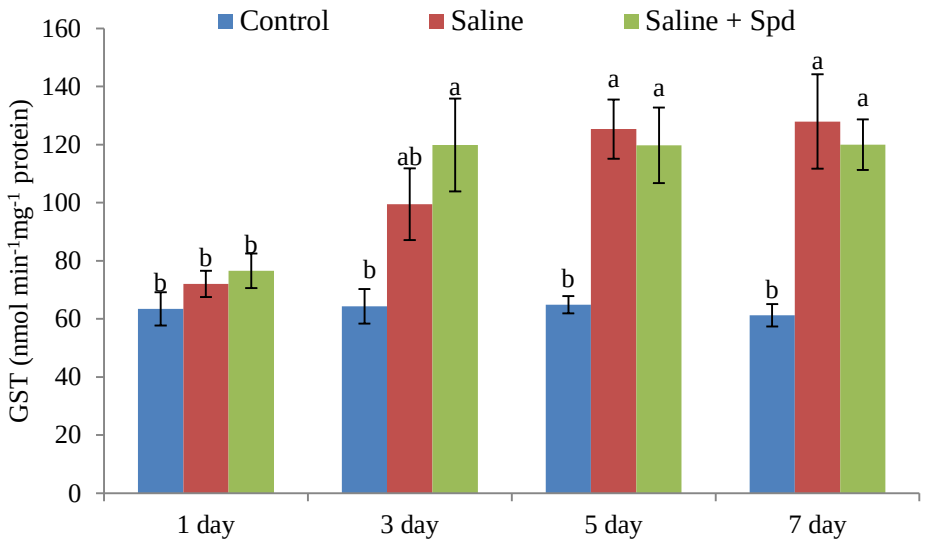

Figure 4.20. Effect of Spd on activities of Glutathion-*S*-transferase (GST) in leaves of onion seedlings under salinity stress (Values present in the bars are mean ± SE. Similar letters between the bars are not significant at 5% level)

Remarkable increase was observed in glutathione-*S*-transferase (GST) activity in leaves onion seedlings under salinity stress, where the activities increased gradually with stress duration (Fig. 4.20). Salinity increased the activity by 14, 55, 93 and 109% over control at 1, 3, 5 and 7 day after stress implementation, respectively. Application of Spd increased the activity in the early stages of stress, while 6 and 21% higher activity was increased at 1 and 3 day of salt stress, respectively over salinity. Spd decreased the activity slightly (4 and 6%) at 5 and 7 day of stress, respectively, in salinity stressed seedlings.

The GST activity increased under salinity stress in presence or absence of Spd (Fig. 4.20). Increased activity of GST in onion leaves under salinity stress used GSH for participating in detoxification of ROS, xenobiotics, and membrane lipid peroxidation (Shalata *et al.*, 2001; Mittova *et al.*, 2003b), stabilize flavonoid or transportation them to vacuole (Rohman *et al.*, 2010; Dixon *et al.*, 2010). The high GST activity might be due to regulation of flbonoid in onion (Rohman *et al.*, 2010). On the other hand, the increased GST activity also suggested its flavonoid-binding properties, and indirectly facilitating the vacuolar uptake of anthocyanins by preventing their oxidation and cross-linking in the cytoplasm (Marrs *et al.*, 1995). GST also shows GPX activity which might reduce oxidative damage in onion (Dixon *et al.*, 2010).

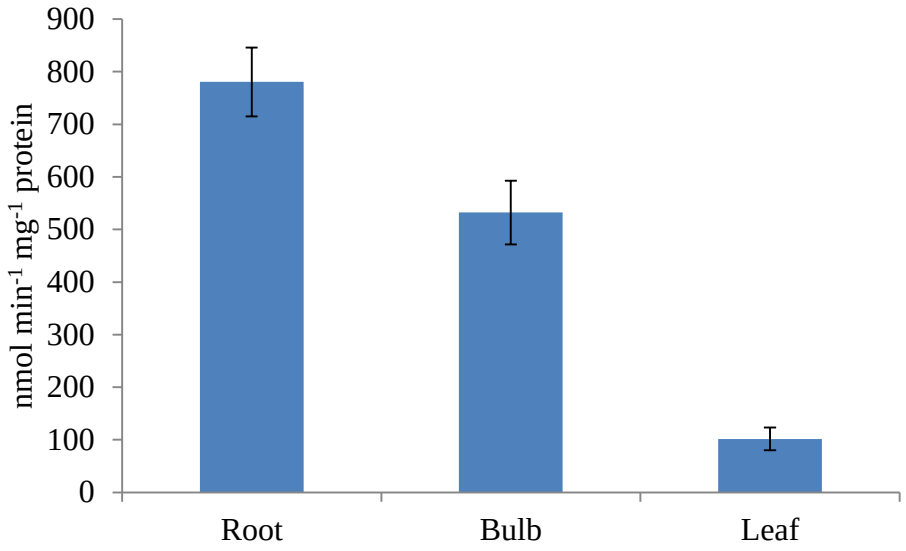

Figure 4.21. Glutathion *S*-transferase (GST) activities in different parts of onion seedlings (Values present in the bars are mean ± SE)

Since GST activity increased strongly, the activity was compared in different plant parts. It was found that both root and bulb showed higher GST activity (Fig. 4.21). Hence, an attempt was taken to purify and production of antibody to examine the expression of the protein.

Onion seedlings showed that GST activities are organ dependent (Fig. 4.21). Both root and bulb contained higher activity. The lowest activity in leaf might be due to containg much photosynthetic protein. On the other hand, the highest activity in root might be due to growing environment (Gong *et al.*, 2005) and the higher GST activity in onion bulb might regulate its physiological substrates (Rohman *et al.*, 2010).

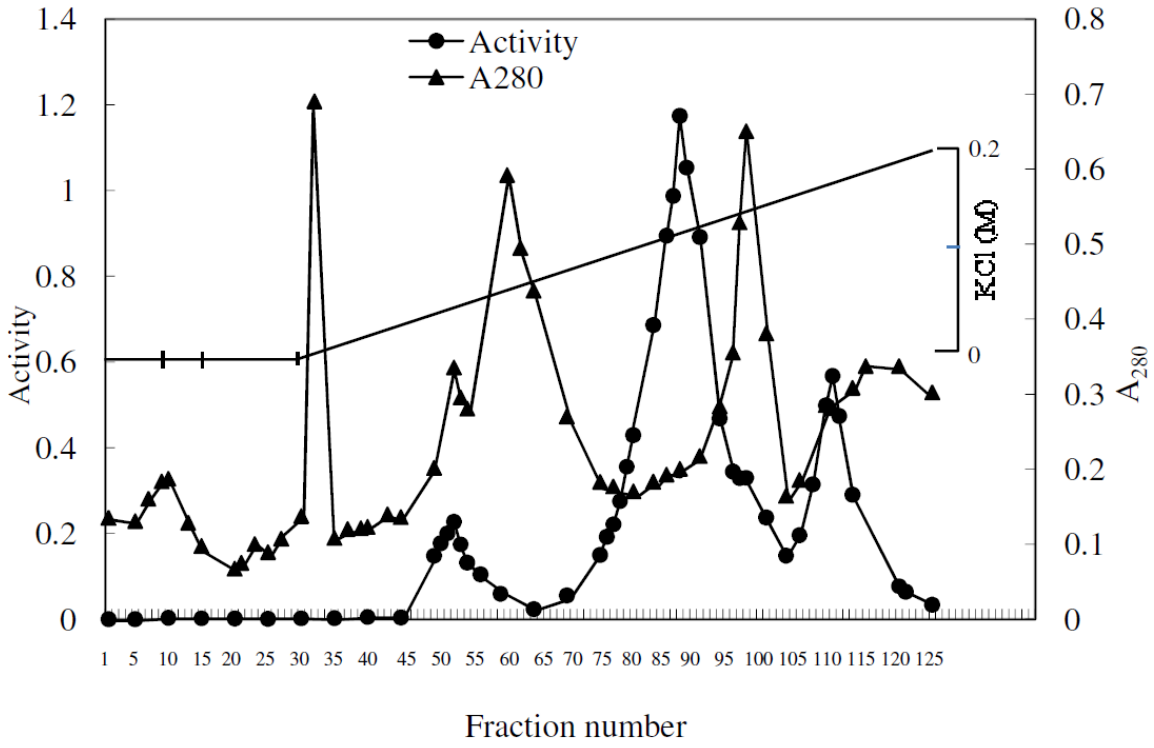

Figure 4.22. A typical column chromatography of DEAE-cellulose of soluble proteins prepared from 150 g onion seedlings {For each fraction, absorbance at 280 nm (●) and GST activity toward CDNB (♦) were determined. Activity is expressed as μmol min^{-1} ml^{-1}. The curve shows the gradient solution of KCl (0-0.2 mM)}

Onion bulb GST was separated by a DEAE-cellulose chromatography. Three GST peaks were found to elute at 56, 120 and 168 mM KCl accounting for 6.45, 63.38 and 30.14% of total activity (Fig. 4.22 and Table. 4.2). The GST eluted at 120 mM KCl was farther purified by affinity chromatography (Fig. 23). The affinity fractions were tested by SDS-PAGE, where fraction 25 showed the most concentrated solution (Fig. 4.23).

Table 4.2: Elution pattern of onion GSTs from DEAE-cellulose Chromatography

GSTs	Activity (%)	Total protein	Elution point (mM of KCl)
GST 1	6.48	4.35	56
GST 2	63.38	6.02	120
GST 3	30.14	4.88	169

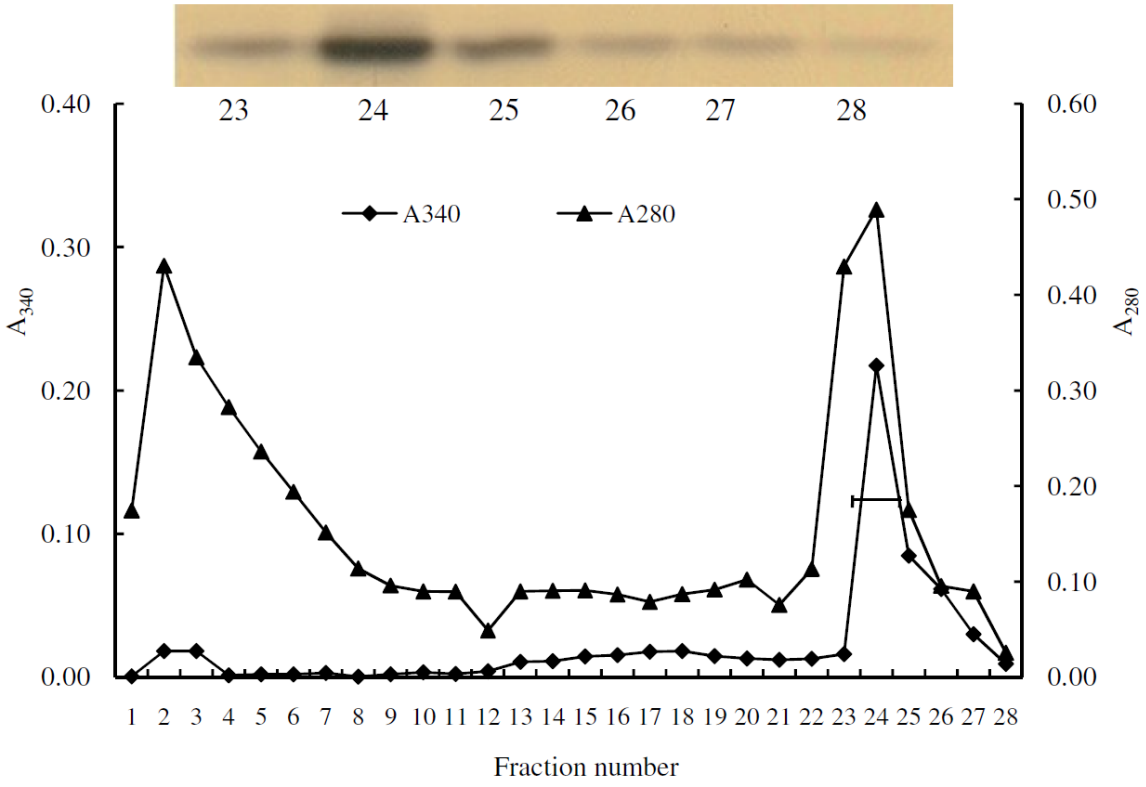

Figure 4.23. A typical affinity chromatography of *S*-hexylglutathione-agarose (For each fraction, absorbance at 280 nm (●) and GST activity toward CDNB (♦) were determined. Activity A_{340} changes for 1 min and absorbance A_{280} were measured)

The summary of the purification is presented in Table. 4.3. The purified GST had specific activity of 16407.6 nmol min^{-1} mg^{-1} protein with recovery and purification fold of 2.81 and 29.5 fold, respectively.

Table 4.3. Summary of Purification of GST from onion seedlings

Fraction	Specific Activity (nmol min^{-1} mg^{-1} protein)	Total Activity (mMol min^{-1})	Total Protein (mg)	Recovery yield (%)	Purification fold
Homogenous	556.20	200.9	361.20	100	1
$(NH_4)_2SO_4$ ppt	1539.10	162.07	105.30	80.62	2.77
DEAE-cellulose	8193.10	49.33	6.02	24.55	14.73
S-Hexyl Glutathione-agarose	16407.6	5.65	0.35	2.81	29.5

The purified GST was used to prepare polyclonal antibody in a rabbit blood serum. In examine concentration of the antibody four BSA was used in different concentration of the antibody (Fig. 4.24A). However, in this study, 100 fold dilution was used in western bloting. In western blotting of soluble protein extract from onion leaves showed significant accumulation of that GST under salinity stress in presence and absence of Spd (Fig. 4.24B).

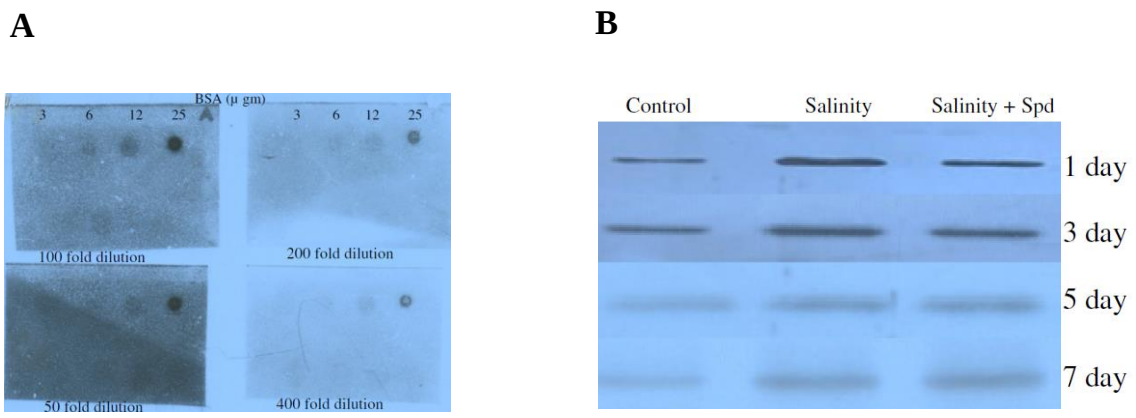

Figure 4.24. Western blot analysis. Concentration test of antibody (A) and Accumulation of onion GST(B) under salinity with or without Spd (In each lane, 65 µg protein was used for 1 day and 3 day while 45 µg protein was used for 5 day and 7 day)

In this study, three GSTs were found to be separated from onion bulb, among which one GST eluted at 120 mM KCl was found to be accumulated under salinity (Fig. 4.24B). This GST could have significant biological role in stress mitigation which thrusts more research. GSTs are an ancient and diverse group of multi-functional proteins that are widely distributed amongst living organisms. Originally defined solely as enzymes that catalyze conjugation of the tripeptide glutathione (GSH) to an electrophilic substrate (Marrs *et al*., 1995), it is now clear that GSTs catalyse a variety of reactions. Early plant GST research focused on the role of GSTs in herbicide resistance and vacuolar sequestration of anthocyanins (Edwards and Dixon, 2000). In the present study, the induced GST activity and accumulation of GST {Fig. 4.20, 4.24 (B)} under salinity might play important physiological role like vacuolar sequestration of flavonoids like quercetine (Fini *et al*., 2011.). On the other hand, high activity might be associated with recycling and stabilizing flavonoid

(Dixon *et al*., 2011; Rohman *et al*., 2009),to protect cell from toxic effect. In addition to being induced by xenobiotic-type stresses, plant GST expression is activated by abiotic stress like chilling (Seppanen *et al*., 2000), hypoxic stress (Moons, 2003), dehydration (Kiyosue *et al*., 1993; Bianchi *et al*., 2002), wounding (Vollenweider *et al*., 2000), pathogen attack (Mauch and Dudler, 1993), ethylene (Zhou and Goldsbrough, 1993), auxin (Marrs *et al*., 1995), H_2O_2 (Chen *et al*., 1996) and the defense signal salicylic acid (Chen *et al*., 1996). GSTs have been shown to possess GST activity towards 4-hydroxy-2-nonenal (HNE) (Gronwald and Plaisance, 1998), a naturally occurring lipid peroxidation product that can cause oxidation and alkylation of proteins and DNA. Potentially, GST activity allows GSTs to detoxify electrophilic compounds by catalyzing their conjugation to GSH, while GSH peroxidase (GPX) activity allows GSTs to directly detoxify lipid and DNA peroxidation products (Marrs, 1996). It is also possible that the induced GST activities (Fig. 4.20) could detoxify HNE as well as MDA, another natural lipid peroxidation product, under stress condition. In this study, we found to accumulate H_2O_2 and MDA under salinity stress concurrently with high GST activity while presence of Spd boosted up the GST activity in the onion seedlings along with lower H_2O_2 and MDA contents (Fig. 4.4 and 4.5) suggested it's detoxification role by conjugation or directly detoxification via GPX activity.

CHAPTER V

SUMMARY AND CONCLUSION

Salinity stresses caused significant reaction in the contents of Chla, Chlb and Car in onion seedlings. However the loss of Chlb was comparatively higher. Application of Spd in saline condition reduces the loss of chlorophyll and Car. Salinity mediated leaf water loss was significant. Maintenance of water was remarkable in the presence of Spd. This maintenance of water might be due to osmoprotectative role of proline as proline contents increased under salinity with or without Spd in onion leaf. Salinity increased the ROS like $O_2^{\bullet-}$ and H_2O_2 and they increased continuously and sharply. This over production of ROS might result in higher lipid peroxidation. In the onion leaf lipid peroxidation was measured as MDA, The production of MDA increased with period of salinity stress. At the same time, salinity also produced cytotoxic MG in onion cell. Application of Spd reduced the contents of ROS, MDA and MG in onion leaf. Salinity oxidized the GSH continuously with duration of stress resulting in increase of GSSG significantly. Similarly, ASA was oxidized to DHA. As a result, the redox state of glutathione and ascorbate redox. The maintenance of non-enzymatic antioxidant GSH and ASA by Spd was remarkable. Under salinity, the maintenance of higher GSH by GR activities was noticeable in presence or absence of Spd, where Spd enhanced the recycle of GSH.

The supplementation of Spd in saline stressed onion seedlings increased the PAO and DAO activities over salinity and they decreased after 3 days. The SOD activity in leaves of onion seedlings was observed to increase under saline stress condition which increased further in application of Spd. However, the strongest decomposer of H_2O_2, CAT activity was not observed to increase in the onion seedlings under saline stress and as well with foliar application of Spd. The other decomposer GPX increased under salinity and foliar application of Spd capable to increased upto 3 day. On the other hand, Spd increased the salinity reduced APX activity throughout the stress period. Saline stress decreased the activity of MDHAR with stress duration where application of Spd increased the activity by 6, 19, 22 and 21% over salinity at

1, 3, 5 and 7 day, respectively. Under salinity stress, the activity of DHAR was higher after 1 day and notably, with application of Spd, the highest induced activity was found at 3day. Salinity stress increased both the Gly-I and Gly-II activity gradually upto 5 day stress. With application of Spd, Gly-I and Gly-II activities increased upto 3 days of stress.

Remarkable increase was observed in GST activity in leaves of onion seedlings under salinity stress, where the activities increased gradually with stress duration. Salinity increased the activity by 14, 55, 93 and 109% over control at 1, 3, 5 and 7 day, respectively. Application of Spd increased the activity in the early stages of stress, while 6 and 21% higher activity was increased at 1 and 3 day, respectively over salinity. Onion GST activity was plant organ specific, where onion root and bulb showed higher GST activity and leaf showed the lowest activity. Onion bulb GST was separated by a DEAE-cellulose chromatography. Three GST peaks were found to elute at 56, 120 and 168 mM KCl accounting for 6.45, 63.38 and 30.14% of total activity. The GST eluted at 120 mM KCl was farther purified by affinity chromatography. The purified GST had specific activity of 16407.6 nmol min^{-1} mg^{-1} protein with recovery and purification fold of 2.81 and 29.5 fold, respectively. The purified GST was used to prepare polyclonal antibody in a rabbit blood serum and in this study, 100 folds dilution were used in western blotting. In western blotting of soluble protein extract from onion leaves showed significant accumulation of that GST under salinity stress in presence and absence of Spd.

Conclusion

Therefore, from this study, we can conclude that-

1. Salinity caused significant reduction in Chl, Car and RWC contents in onion seedlings. On the other hand, salinity produced higher amount of ROS, lipid peroxation, proline and MG.
2. Interestingly, Spd maintained non-enzymatic antioxidants through the stress duration studied. Whereas, enzymatic antioxidants increased mostly in between 1 to 3 days.

3. Finally, application of 100 µm of Spd as foliar spray was found to be effective to increase tolerance for short term (2-3 days) salinity to reduce the salinity induced oxidative damage.

However, the polyamine was not estimated in the plant sample. On the other hand, spectrophotometrical and Western blot analysis suggested the accumulation of GST protein in presence or absence of Spd. Therefore, further study could be recommended with the onion GST.

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