Australian Journal of Crop Science AJCS
AJCS 8(4):631-639 (2014) ISSN:1835-2707

Exogenous salicylic acid alleviates salt stress-induced oxidative damage in *Brassica napus* by enhancing the antioxidant defense and glyoxalase systems

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Abstract

Regulatory roles of exogenous salicylic acid (SA) on the antioxidant defense and methylglyoxal (MG) detoxification systems were investigated in rapeseed seedlings (*Brassica napus* L. cv. BINA Sharisha 3) grown under salinity. Ten-day-old seedlings, grown in petri dishes, were supplemented with SA and salt (100 and 200 mM NaCl) separately and in combination for 48 h. After treatment, MDA and H_2O_2 content, non-enzymatic and enzymatic components of antioxidant and glyoxalase enzymes were measured. The ascorbate (AsA) content of the seedlings was decreased significantly with increased salt stress. Salt stress resulted marked raise in the levels of H_2O_2 and lipid peroxidation (MDA). The amount of glutathione (GSH) and glutathione disulfide (GSSG) were increased with an increase in the level of salt stress, while the GSH/GSSG ratio was decreased. Imposition of salt stress causes decrease in most of the antioxidant enzymes except for the ascorbate peroxidase (APX) and glutathione *S*-transferase (GST). However, compared to salt stressed seedlings alone, exogenous SA treatment in combination with salt stress enhanced AsA and GSH contents; GSH/GSSG ratio; and activities of antioxidant enzymes such as monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione *S*-transferase (GST), glutathione peroxidase (GPX), catalase (CAT), glyoxalase I (Gly I), and glyoxalase II (Gly II). This study indicates that exogenous application of SA is an effective protectant in improving the activities of both antioxidant defense and glyoxalase enzymes in coffering salt stress tolerance in *B. napus*.

Keywords: Abiotic stress; Antioxidants defense; Methylglyoxal; Oxidative stress; Phytohormones, Reactive oxygen species.
Abbreviations: AO_ ascorbate oxidase; APX_ ascorbate peroxidase; AsA_ ascorbic acid (ascorbate); BSA_ bovine serum albumin; CAT_ catalase; CDNB_ 1- chloro-2, 4-dinitrobenzene; Chl_ chlorophyll; DHA_ dehydroascorbate; DHAR_ dehydroascorbate reductase; DTNB_ 5,5´-dithio-bis (2-nitrobenzoic acid); EDTA_ ethylenediaminetetraacetic acid; Gly I_ glyoxalase I; Gly II_ glyoxalase II; GR_ glutathione reductase; GSH_ reduced glutathione; GSSG_ oxidized glutathione; GPX_ glutathione peroxidase; GST_ glutathione *S*-transferase; LOX_ Lipoxygenase; MDA_ malondialdehyde; MDHA_ monodehydroascorbate; MDHAR_ monodehydroascorbate reductase; MG_ methylglyoxal; NADPH_ nicotinamide adenosine dinucleotide phosphate; NTB_ 2-nitro-5-thiobenzoic acid; ROS_ reactive oxygen species; SLG_ *S*-D-lactoylglutathione; TBA_ thiobarbituric acid; TCA_ trichloroacetic acid.

Introduction

Global climate change is currently viewed as the most devastating threat to the environment and gaining considerable attention from farmers, researchers, and policy makers because of its major influences on agriculture. Abiotic stresses are the greatest restriction for crop production worldwide and account for yield reductions of as much as 50% (Rodríguez et al., 2005; Acquaah, 2007). Crop plants, as sessile organisms, encounter unavoidable abiotic stresses during their life cycles, including salinity, drought, extreme temperatures, metal toxicity, flooding, UV-B radiation, ozone, etc., which all pose serious challenges to plant growth, metabolism, and productivity (Hasanuzzaman et al., 2012, 2013, 2014). Worldwide, more than 45 million ha of irrigated land which account to 20% of total land have been damaged by salt, and 1.5 million ha are taken out of production each year due to high salinity levels in the soil (Pitman and Läuchli, 2002; Munns and Tester, 2008). On the other hand, increased salinity of agricultural land is expected to have destructive global effects, resulting in up to 50% loss of cultivable lands by the middle of the 21st century (Mahajan and Tuteja, 2005). The situation is becoming more serious due to gradual increases in the complex nature of the environment and due to the unpredictability of environmental conditions and global climate change. In most of the cases,

the negative effects of salinity have been attributed to increase in Na^+ and Cl^- ions in different plants hence these ions produce the critical conditions for plant survival by intercepting different plant defense mechanisms. Salinity at higher levels causes both hyperionic and hyperosmotic stress and can lead to plant demise. The outcome of these effects may cause membrane damage, nutrient imbalance, altered levels of growth regulators, enzymatic inhibition and metabolic dysfunction, including photosynthesis which ultimately leading to plant death (Mahajan and Tuteja, 2005; Hasanuzzaman et al., 2012). A key sign of salt stress at the molecular level is the accelerated production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH•). The excess production of ROS is common in many abiotic stresses, including salt stress, and results from impaired electron transport processes in the chloroplasts and mitochondria (Hasanuzzaman and Fujita, 2013). Methylglyoxal (MG) is another highly reactive cytotoxic α-oxoaldehyde compound and there are several reports indicating the overproduction of MG under abiotic including salinity (Yadav et al., 2008; Kumar and Yadav, 2009) which also lead to proteins, lipids and DNA damage (Yadav et al., 2005). By nature, plant possesses antioxidant defense system, both antioxidant enzymes and non-enzymatic metabolites those largely play significant roles in ROS signaling in plants (Mantri et al., 2012). The enzymatic system includes the four enzymes of the ascorbate–glutathione (AsA–GSH) cycle: ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) as well as other enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione *S*-transferase (GST). The non-enzymatic antioxidant includes ascorbic acid (AsA), glutathione (GSH), phenolic compounds, alkaloids, non-protein amino acids, and α-tocopherols. These antioxidant and glyoxalase systems function in concert to control oxidation and to protect plant cells from oxidative damage by scavenging ROS (Hasanuzzaman et al., 2012; Hasanuzzaman and Fujita, 2013). In plants, the MG is detoxified mainly by the maintenance of GSH homeostasis via glyoxalase system (Yadav et al., 2005) which consists of two enzymes: glyoxalase I (Gly I) and glyoxalase II (Gly II). The Gly I uses one molecule of reduced GSH to convert MG to *S*-D-lactoylglutathione (SLG). Then Gly II converts SLG to D-lactate and one molecule of reduced glutathione is recycled back into the system (Mustafiz et al., 2010). Therefore, besides detoxification of MG, the glyoxalase system could also play a role in providing tolerance under stress by recycling GSH that would be "trapped" spontaneously by MG to form hemithioacetal, thereby maintaining GSH homeostasis (Creighton et al., 1988; El-Shabrawi et al., 2010). Enzymes of the glyoxalase system in plants are found to regulate environmental stresses including salinity as reported in many plant studies (Hasanuzzaman et al., 2011a, b; Hasanuzzaman and Fujita, 2011; 2013). It was reported that the coordinated inductions or regulations of both the antioxidant and glyoxalase pathway enzymes are necessary to obtain substantial tolerance against oxidative stress (Hasanuzzaman et al., 2011a, 2012). Since the mechanism of salt stress is not yet elucidated due to its complex nature (Hasegawa et al., 2000), a well-focused approach combining the molecular, physiological, biochemical and metabolic aspects of salt tolerance is essential to develop salt-tolerant crop varieties. Exploring suitable ameliorants or stress alleviant is one of the tasks of plant biologists. In recent decades, exogenous protectant such as plant hormone antioxidants, signaling molecules, polyamines, trace elements etc. have been found effective in mitigating the salt induced damage in plant (Hasanuzzaman et al., 2011a, b; 2013). These protectants showed the capacity to enhance the plants' growth, yield as well as stress tolerance under salinity. Salicylic acid (SA) is a common plant-produced phenolic compound and a potential endogenous plant hormone that plays an important role in plant growth and development (Khan et al., 2012; Alam et al., 2013). The role of SA is intensively studied in plant responses to biotic stress. In recent years, the involvement of SA in the response to abiotic stresses has come into light. Several studies support a major role of SA in plant adaptation to the changing environment, and induce plant tolerance to various abiotic stresses including elevated NaCl (Stevens et al., 2006; Arfan et al., 2007; Gunes et al., 2007). It is a well observed fact that SA potentially generates a wide array of metabolic responses in plants and also affects plant water relations (Hayat et al., 2010). There are plenty of evidences on the role of SA in enhancing the endogenous antioxidant levels in plants which resulted in better protection again oxidative stress (Hayat et al., 2008; 2010 Kadioglu et al., 2011). In a recent study, Syeed et al. (2011) observed SA-induced enhanced antioxidant metabolism in *Brassica juncea* which conferred better tolerance to salt stress. Although there are several reports on the role of SA in salt stress tolerance, few studies have investigated the effects of exogenously applied SA on both antioxidant defense and the glyoxalase system of plants. Therefore, the present study focuses on the effects of exogenous SA on the antioxidant defense and glyoxalase systems in rapeseed (*B. napus*) seedlings grown under saline media.

Results

Lipid peroxidation and H_2O_2 level

The oxidative stress-induced lipid membrane damage in salt-affected plants was determined by lipid peroxidation, in terms of quantification of malondialdehyde (MDA). Upon exposure to 100 and 200 mM NaCl the seedlings showed 118 and 198% increase in MDA content compared to control (Fig. 1A). No effect of SA on MDA content was observed in seedlings grown under normal condition, but exogenous application of SA diminished the MDA content in stressed plants which was 29 and 39% lower than the seedlings those were grown under salt stress without SA (Fig. 1A). Salt stress accelerated the H_2O_2 production over the controls (103 and 144% higher at 100 and 200 mM NaCl, respectively); while the application of SA inhibited the H_2O_2 generation in NaCl-stressed plants (Fig. 1B).

Endogenous levels of ascorbic acid and glutathione

Marked decline in AsA content was observed in the seedlings exposed to salt stress and compared to the control it was 30 and 46% lower at 100 and 200 mM NaCl, respectively (Fig. 2A). The application of SA did not cause any significant change in AsA content of seedlings under normal condition, but it effectively ameliorated the salt stress effect by enhancing AsA content of NaCl-stressed plants (Fig. 2A).

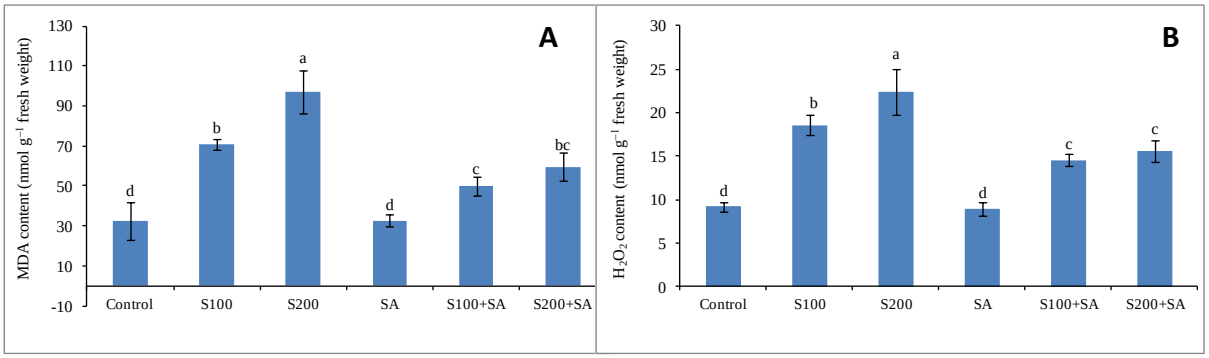

Fig 1. Levels of MDA (A) and H_2O_2 (B) in salt stressed *Brassica napus* seedlings supplemented with SA. S_{100}, S_{200}, SA, S_{100}+SA and S_{200}+SA indicates 100 mM NaCl, 200 mM NaCl, Salicylic acid (100 µM), 100 mM NaCl+ SA, 200 mM NaCl+ SA treatment, respectively. Bars represent means± standard deviation (n = 3). Means followed by the same letter are not significantly different (P≤0.05) as determined by Fisher's LSD test

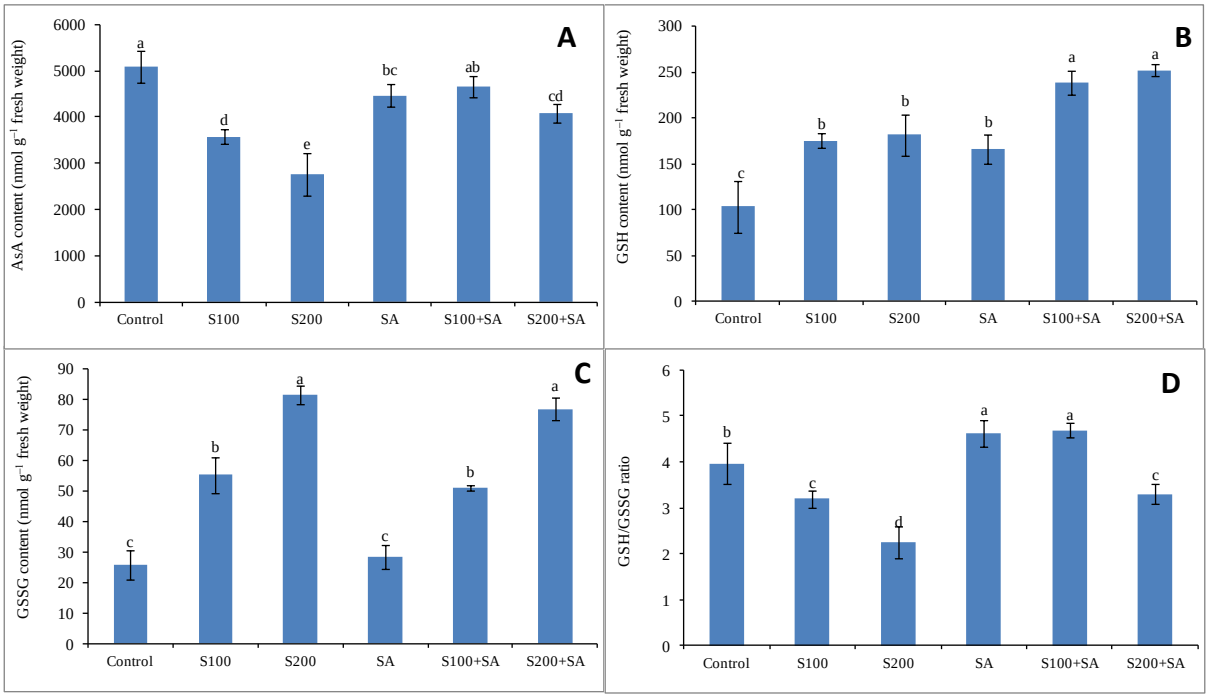

Fig 2. Contents of AsA (A), GSH (B), GSSG (C), and GSH/GSSG ratio (D) in salt stressed *Brassica napus* seedlings supplemented with SA. S_{100}, S_{200}, SA, S_{100}+SA and S_{200}+SA indicates 100 mM NaCl, 200 mM NaCl, Salicylic acid (100 µM), 100 mM NaCl+ SA, 200 mM NaCl+ SA treatment, respectively. Bars represent means± standard deviation (n = 3). Means followed by the same letter are not significantly different (P≤0.05) as determined by Fisher's LSD test

Sharp increase of GSH content was observed under salt stress which was 103 and 175% higher at 100 and 200 mM, respectively compared to control (Fig. 2B). Application of SA also slightly increased the GSH content in seedlings grown under normal condition but when SA-supplemented seedlings exposed to salt they exhibited 36 and 39% increase of GSH content compared to salt stress alone. The GSSG content was increased by 114 and 216% at 100 and 200 mM NaCl, respectively (Fig. 2C). However, SA spray could not change the GSSG content but it maintained the level similar to salt stress alone. The ratio of GSH/GSSG decreased by salt stress in dose dependent manners which was 20 and 44% lower than the control when exposed to 100 and 200 mM NaCl, respectively (Fig. 2D). The application of SA caused slight increase in GSH/GSSG ratio of seedlings under normal condition, but it effectively enhanced the ratio which was 47% higher than the seedlings exposed to 100 and 200 mM of NaCl, respectively (Fig. 2D).

Activities of antioxidant enzymes

The influence of SA on the activities of AsA-GSH cycle enzymes (APX, MDHAR, DHAR and GR) in salt-stressed *B. napus* seedlings is shown in Fig. 3. The activity of APX was increased by 22 and 19% upon exposure to 100 and 200 mM of NaCl, respectively (Fig. 3A). No further changes in the

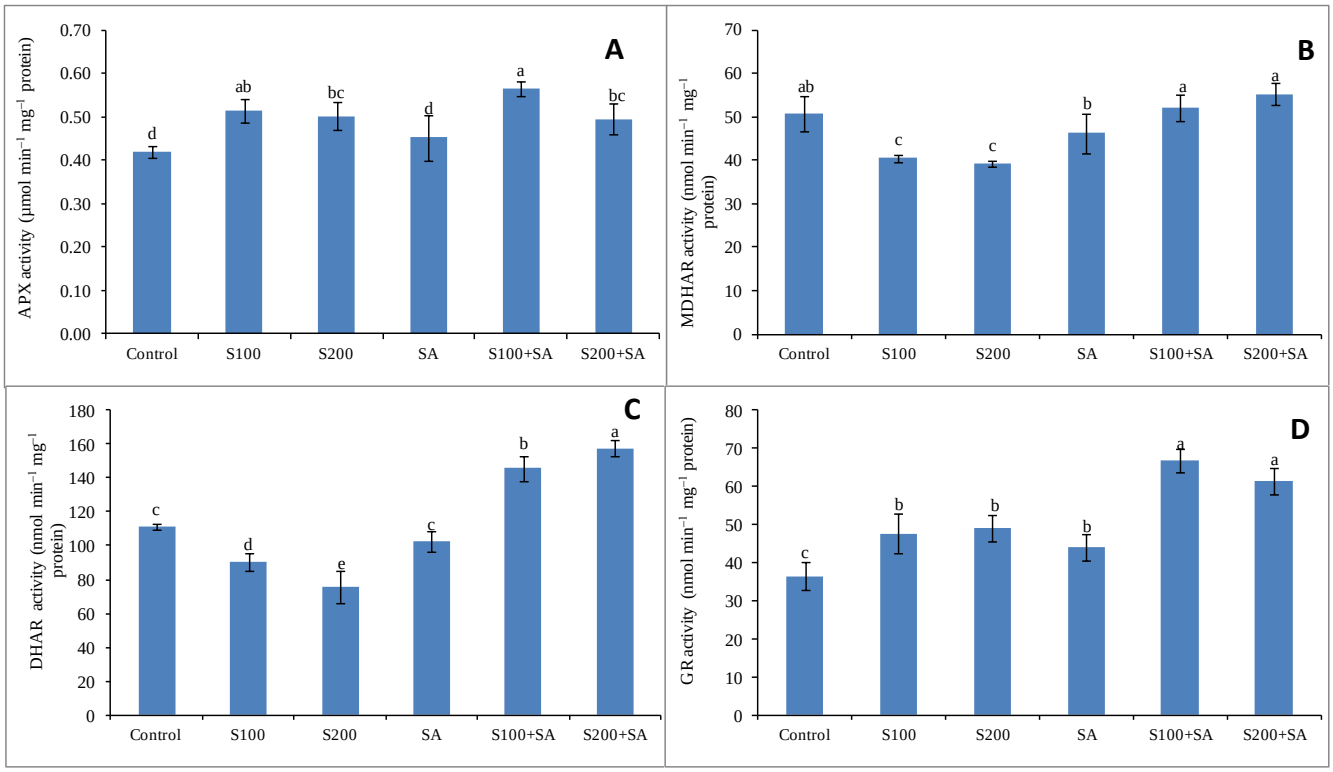

Fig 3. Activities of APX (A), DHAR (B), DHAR (C), and GR (D) in salt stressed *Brassica napus* seedlings supplemented with SA. S_{100}, S_{200}, SA, S_{100}+SA and S_{200}+SA indicates 100 mM NaCl, 200 mM NaCl, Salicylic acid (100 µM), 100 mM NaCl+ SA, 200 mM NaCl+ SA treatment, respectively. Bars represent means± standard deviation (n = 3). Means followed by the same letter are not significantly different (P≤0.05) as determined by Fisher's LSD test

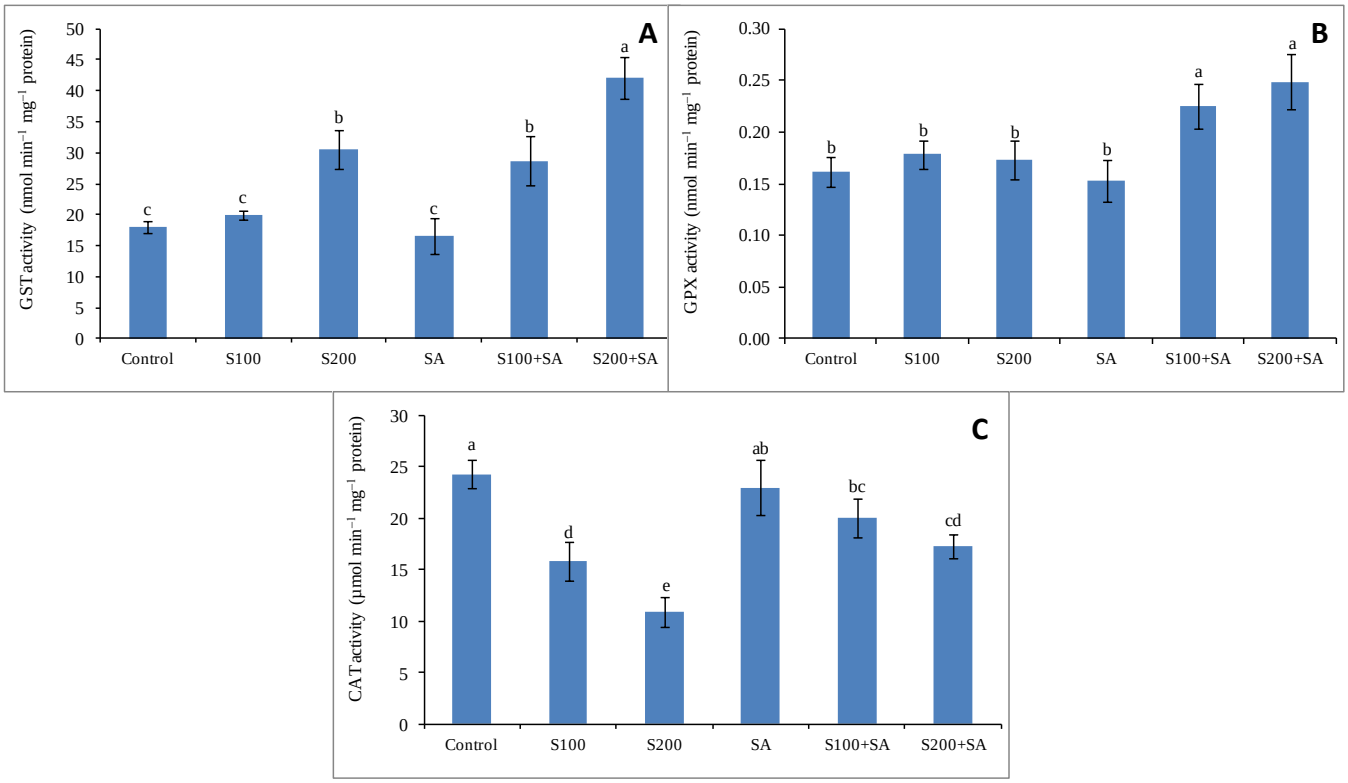

Fig 4. Activities of GST (A), GPX (B), and CAT (C) in salt stressed *Brassica napus* seedlings supplemented with SA. S_{100}, S_{200}, SA, S_{100}+SA and S_{200}+SA indicates 100 mM NaCl, 200 mM NaCl, Salicylic acid (100 µM), 100 mM NaCl+ SA, 200 mM NaCl+ SA treatment, respectively. Bars represent means± standard deviation (n = 3). Means followed by the same letter are not significantly different (P≤0.05) as determined by Fisher's LSD test

activities were observed in salt-stressed seedlings when supplemented with SA. In case of MDHAR 100 and 200 mM NaCl decreased the activities by 20 and 22%, respectively over control (Fig. 3B). However, compared to salt stress alone, SA treated seedlings showed 28 and 41% increase in the activity of MDHAR. Like MDHAR, the activity of DHAR was also decreased by salt stress (18 and 32% at 100 and 200 mM NaCl; Fig. 3C). In contrary, upon addition of SA, there was a significant increase of DHAR activity compared to the salt treated seedlings without SA (61 and 108% at 100 and 200 mM NaCl, respectively; Fig. 3C). The activity of GR slightly increased upon exposure to salt stress (30 and 34% increase at 100 and 200 mM NaCl, respectively). In normal condition, SA application also showed little increase in the activity of GR. Importantly, SA-treated seedlings when exposed to salt stress significantly increased the GR activity which was 40 and 25% higher compared to the salt stressed seedlings grown without SA (Fig. 3D). The activity of GST significantly increased only at 200 mM NaCl which was 70% higher than the control (Fig. 4A). The SA treated salt stressed seedlings also showed significant increase in GST activity, compared with the seedlings subjected to salt stress alone. Salt stress could not impose any change in GPX activity in *B. napus* seedlings. However, when SA treated seedlings exposed to salt solution a marked increase was observed in the activity of GPX. At 100 and 200 mM of NaCl, the activities were 26 and 44% higher in SA treated seedlings compared to the seedlings grown without SA (Fig. 4B). The marked decreased in CAT activity was observed in salt-stressed plants than in their controls. At 100 and 200 mM NaCl the activity of CAT was 35 and 55% lower than the control (Fig. 4C). However, the application of SA to salt-stressed seedlings increased the activity of CAT to a great extent compared to the salt stressed seedlings grown without SA (Fig. 4C).

Activities of glyoxalase system enzymes

A slight decrease in Gly I activity was observed under salt stress, which was measured as 21 and 33% lower with 100 and 200 mM NaCl, respectively, compared with control seedlings (Fig. 5A). However, the SA treated salt-stressed seedlings had significantly higher Gly I activity, as compared with the seedling subjected to salt stress without SA treatment. The Gly I activity of the SA supplemented control seedlings was similar to that of the untreated control. Similar to Gly I, the activity of Gly II was significantly decreased in rapeseed seedlings exposed to salt stress (Fig. 5B). The activities were 30 and 37% lower at 100 and 200 mM NaCl, respectively, than the activity in the control seedlings. SA treated salt-stressed seedlings had significantly higher Gly II activities (31 and 35% higher at 100 and 200 mM NaCl, respectively, compared to the activity in the seedlings subjected to salt stress without SA) which were almost similar to control (Fig. 5B).

Discussion

In stress response, lipid peroxidation (often measured as MDA content) is a well known index for determining the extent of oxidative stress and increased level of MDA has been found to be highly correlated with oxidative damages induced by various abiotic stress including salinity (Garg and Manchanda, 2009). Excessive accumulation of H_2O_2 is one of the indicators of oxidative stress (Hasanuzzaman et al., 2012). In our experiment, both the MDA and H_2O_2 levels significantly increased by salt stress in dose dependent manners (Fig. 1A, B). Increase in the levels of MDA and H_2O_2 under salt stress were reported in rapeseed and wheat in our previous studies (Hasanuzzaman et al., 2011a, b). Supplementation of SA in salt treated seedlings, on the other hand, reversed the effect and decreased the MDA and H_2O_2 content compared to salt treatment alone. This indicates that SA has a key role in ROS scavenging and reduction of oxidative stress in *B. napus* seedlings subjected to salt stress. Similar reductions of MDA and H_2O_2 in SA treated seedlings under salt stress were observed in other plant studies (Hayat et al., 2012; Alam et al., 2013). Ascorbic acid is one of the most abundant non-enzymatic antioxidants, serving as a major contributor to the cellular redox state and protecting plants against oxidative damage (Smirnoff, 2000). It reacts with a range of ROS, such as H_2O_2, $O_2^{\bullet-}$, and $^1O_2$, which are the bases of its antioxidant action. In present study, salt-induced oxidative stress decreased the AsA content in rapeseed seedlings and co-treatment of SA alleviated this stress effects by enhancing the level of AsA. The decrease in AsA content under salt stress was also reported in our previous study (Hasanuzzaman et al. 2011a). The decreased activities of AsA regenerating enzymes (MDHAR and DHAR) impaired the production and regeneration of AsA in salt stressed plants. On the other hand, application of SA in salt stress plants recovered the AsA level almost similar to control (Fig. 2A). Glutathione is another strong antioxidant which has diverse function including growth promotion, antioxidant defense and xenobiotic detoxification (Hasanuzzaman et al., 2012) and in most cases it was found to be increased in response to stress. The increased GSH level in *B. napus* seedlings under salt stress is supported by our previous study (Hasanuzzaman et al., 2011a). This increase of GSH content in plants subjected to salt stress that might be due to a greater rate of GSH synthesis. The increased GSH content was also largely due to increased GR activity under salt stress (Fig. 3D). However, after SA supplementation these phenomena were very active that rendered higher level of GSH in seedlings compared to the seedlings exposed to salt without SA (Fig. 2B) which is in consistent with other reports (He and Zhu, 2008; Nazar et al., 2011). The increased level of GSSG (Fig. 2C) in salt-treated seedlings may be attributed to the reaction of GSH with oxyradicals generated by oxidative stress or decreased GR activity (Shalata et al., 2001; Aravind and Prasad, 2005). In our investigation, salt stress caused a significant reduction in the GSH/GSSG ratio. However, SA-treated, salt-stressed seedlings showed higher GSH/GSSG ratio than the seedlings grown in salt solution alone (Fig. 2D). This increase in the GSH/GSSG ratio in SA-supplemented salt-stressed seedlings also provides a clear demonstration of the role of SA toward redox regulation (Alam et al., 2013). The AsA–GSH cycle involves 4 enzymes (APX, MDHAR, DHAR and GR) as well as AsA, GSH and NADPH which work together to detoxify H_2O_2 in a series of cyclic reactions and further regenerate AsA and GSH (Hasanuzzaman et al., 2012). In our study, a slight increase in APX activity was observed in leaves of salt treated seedlings which were supported by Gusman et al. (2013). However, SA supplementation could not enhance the activity further (Fig. 3A). In our experiment both the MDHAR and DHAR decreased due to salt treatment. These results were well agreed with other reports (Keyster et al., 2012; Tanou et al., 2013). Since these enzymes are responsible to regenerate AsA and are necessary in order to maintain the antioxidative capacity of AsA, the increased activities of these enzymes in SA supplemented seedlings proved the role of SA in AsA regeneration under salt stress (Fig. 2A, 3B, C). Under stressful condition GR helps in

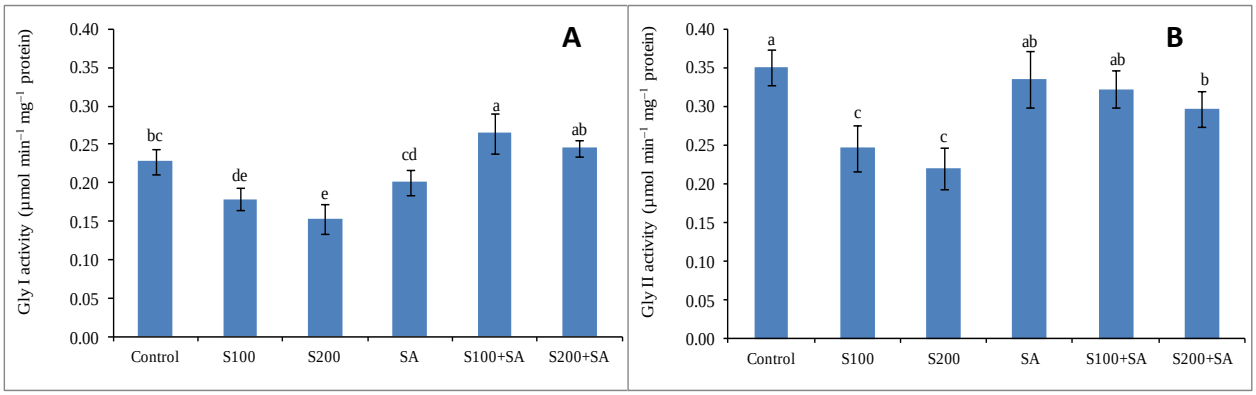

Fig 5. Activities of Gly I (A), and Gly II (B) in salt stressed *Brassica napus* seedlings supplemented with SA. S_{100}, S_{200}, SA, S_{100}+SA and S_{200}+SA indicates 100 mM NaCl, 200 mM NaCl, Salicylic acid (100 µM), 100 mM NaCl+ SA, 200 mM NaCl+ SA treatment, respectively. Bars represent means± standard deviation (n = 3). Means followed by the same letter are not significantly different (P≤0.05) as determined by Fisher's LSD test

maintaining the GSH redox state by recycling of GSSG to GSH. It also plays a vital role in maintenance of sulfhydryl (–SH) group and acts as a substrate for glutathione *S*-transferases (Yousuf et al., 2012). In our experiment salt stress could increase the GR activity to a small extent. However, when SA treated seedlings were subjected to salt stress the activity markedly increased which rendered rapid recycling of GSH in line with better synthesis of GSH under salt stress conditions (Fig. 3D). The role of SA in enhancing the activity of GR was reported by many plant studies (He and Zhu, 2008). Earlier, we reported the correlation between enhanced GR activity and better GSH levels as well as abiotic stress tolerance including salinity (Hasanuzzaman et al., 2011 a, b; Hasanuzzaman and Fujita, 2011; Hasanuzzaman and Fujita, 2013). Although GST mainly catalyzes the conjugation of electrophilic xenobiotic substrates with the tripeptide GSH it can also scavenge the ROS, especially H_2O_2. In our study, we did not observe any changes in the activity of GST under mild salt stress (100 mM NaCl) but it increased markedly at severe stress (200 mM). Different plant studies indicated significant increase in GST activities in response to salinity which is consistent with our present study (Halusková et al., 2009; Oufdou et al., 2014). Importantly, SA supplementation in salt stress seedlings increased the GST activity further compared to salt stress alone (Fig. 4A). The activity of GPX is also involves in protecting plant cells from damage due to oxidative stress (Hasanuzzaman et al., 2012) because it acts in reducing H_2O_2 using GSH as a substrate (Gill and Tuteja, 2010, Hasanuzzaman et al., 2012). In our results, the activity of GPX remained unchanged under salt stress but when SA treated seedlings were exposed to salinity the activity of GPX significantly increased (Fig. 4B). This increased activity contributed towards scavenging the excessive H_2O_2 from plant cell and rendered less oxidative damages (Fig. 1B). Salicylic-acid induced enhancement of GPX activity was also reported earlier (He and Zhu, 2008). Catalase is one of the vital enzymes in scavenging H_2O_2 in plant cells exposed to various abiotic stresses due its higher turnover rate of reaction (Garg and Manchanda, 2009). In our recent studies we observed the role of CAT in scavenging H_2O_2 (Hasanuzzaman et al., 2011a, b; Hasanuzzaman and Fujita, 2013). In our present study, CAT activity was significantly decreased upon exposure to salt stress (Fig. 4C). This decrease in CAT activity under salt stress might be due to its inactivation by the accumulated H_2O_2 induced by water shortage or ineffective enzyme synthesis or change in assembly of enzyme sub-units (Gupta et al., 2009). In contrary, SA-supplemented salt-stressed seedlings showed enhanced activity CAT than those under salt treatment without SA which suggests an unambiguous role of SA in scavenging H_2O_2 under salt stress. Similar increases in CAT activity after SA supplementation was observed under salt stress by other researchers (Yusuf et al., 2008; Noriega et al., 2012). Besides ROS, MG is another highly cytotoxic compound and hence its concentrations must be kept under strict control to protect the plants. Therefore the elevated activities of Gly I and Gly II are necessary to maintain the level of MG to the tolerable limit under salt stress because these two enzymes act together in the detoxification of MG (Veena et al., 1999; Yadav et al., 2005). Higher Gly I and Gly II activities might protect plants against MG that is formed during abiotic stresses (Jain et al., 2002; Singla-Pareek et al., 2003; Yadav et al., 2008). The efficient manipulation of glyoxalase pathway enzymes in different plants inhibits an increase in MG level under oxidative stress and confers tolerance by increasing the GSH-based detoxification system (Singla-Pareek et al., 2006; 2008). In our study both Gly I and Gly II activities were decreased upon exposure to salt stress (Fig. 5A, B). Decreased activities of Gly I and Gly II were reported in plants treated with various stressors including salinity (El-Shabrawi et al., 2010; Upadhyaya et al., 2011). However, SA supplementation in salt stressed seedlings considerably recovered the activities of Gly I and Gly II near to control which not only detoxify the elevated level of MG but and conferred efficient regeneration of GSH and higher GSH/GSSG ratio (Fig. 2B, D; 5A, B). These results were in agreement with our previous result which revealed that enhancement of the activities of glyoxalase enzymes provided better protection against oxidative stress (Alam et al., 2013).

Materials and Methods

Plant materials and stress treatments

Rapeseed (*Brassica napus* L. cv. BINA Sharisha 3) seeds of uniform size were selected and surface-sterilized with 70% ethanol for 10 min followed by washing several times with sterilized distilled water. The seeds were then sown in petri plates (9 cm) lined with 6 layers of filter paper moistened with 10 mL of distilled water for germination for two days. Germinated seedlings were then allowed to grow under controlled condition (light, 100 µmol photon m^{-2} s^{-1};

temperature, 25±2°C; RH, 65–70%) that contained 10,000-fold diluted Hyponex solution (Hyponex, Japan). After 10 days, two sets of seedlings were subjected to two different levels of salt stress viz. 100 and 200 mM NaCl in Hyponex solution. First set of seedlings were grown without SA. Another set of seedlings were sprayed with 100 µM SA (Wako, Japan) containing 0.02% Tween 20 (Polyoxyethylenesorbitan monolaurate, Wako, Japan). Each set of seedlings was sprinkled twice a day. Control plants were grown with in Hyponex solution only. Data were taken after 48 hours of treatment. The experiment was repeated three times under the same conditions.

Measurement of lipid peroxidation

The level of lipid peroxidation was measured by estimating MDA, using thiobarbituric acid (TBA) as the reactive material following the method of Heath and Packer (1968) with slight modifications. 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 nmol of MDA g^{-1} fresh weight.

Measurement of H_2O_2

The H_2O_2 was quantified according to the method described by Yu et al. (2003). H_2O_2 was extracted by homogenizing 0.5 g of leaf samples with 3 ml of 50 mM potassium-phosphate (K-P) buffer (pH 6.5) at 4° C. 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 µmol g^{-1} fresh weight.

Extraction and measurement of ascorbate and glutathione

Rapeseed leaves (0.5 g fresh weight) were homogenized in 3 ml ice-cold acidic extraction buffer (5% 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) with some modifications. The supernatant was neutralized with 0.5 M K-P buffer (pH 7.0). The AsA was assayed spectrophotometrically at 265 nm in 100 mM K-P buffer (pH 7.0) with 0.5 unit of ascorbate oxidase (AO). A specific standard curve with AsA was used for quantification. The glutathione pool was assayed according to previously described methods (Yu et al. 2003) with modifications (Paradiso et al. 2008). GSSG was determined after removal of GSH by 2-vinylpyridine derivatization. Standard curves with known concentrations of GSH and GSSG were used. The content of GSH was calculated by subtracting GSSG from total GSH.

Determination of protein

The protein concentration of each sample was determined following the method of Bradford (1976) using BSA as a protein standard.

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-P 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 0−4℃. APX (EC: 1.11.1.11) activity was assayed following the method of Nakano and Asada (1981). The activity was measured by observing the decrease in absorbance at 290 nm for 1 min using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$. MDHAR (EC: 1.6.5.4) activity was determined by the method of Hossain et al. (1984). The activity was calculated from the change in ascorbate at 340 nm for 1 min using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

DHAR (EC: 1.8.5.1) activity was determined by the procedure of Nakano and Asada (1981). The activity was calculated from the change in absorbance at 265 nm for 1 min using extinction coefficient of 14 $mM^{-1}cm^{-1}$.

GR (EC: 1.6.4.2) activity was measured by the method of Hasanuzzaman and Fujita (2013). The decrease in absorbance at 340 nm was recorded for 1 min. The activity was calculated using an extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

GST (EC: 2.5.1.18) activity was determined spectrophotometrically by the method of Hasanuzzaman and Fujita (2013). The increase in absorbance was measured at 340 nm for 1 min. The activity was calculated using the extinction coefficient of 9.6 $mM^{-1}cm^{-1}$.

GPX (EC: 1.11.1.9) activity was measured as described by Elia et al. (2003) with slight modification using H_2O_2 as a substrate. The oxidation of NADPH was recorded at 340 nm for 1 min and the activity was calculated using the extinction coefficient of 6.62 $mM^{-1}cm^{-1}$.

CAT (EC: 1.11.1.6) activity was measured according to the method of Hasanuzzaman and Fujita (2013) by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition of H_2O_2. The activity was calculated using the extinction coefficient of 39.4 $M^{-1}cm^{-1}$.

Glyoxalase I (EC: 4.4.1.5) and Glyoxalase II (EC: 3.1.2.6) activities were determined according to the method of Hasanuzzaman and Fujita (2013).

Statistical analysis

All data obtained were subjected to analysis of variance (ANOVA) and the mean differences were compared by a Fisher's LSD using XLSTAT v.2013.5.03 software (Addinsoft, 2013). Differences at $P \leq 0.05$ were considered significant.

Conclusion

Based on our results we conclude that the antioxidant system and glyoxalase cycle are co-regulated to control the ROS and MG levels under salt stress condition as influenced by SA. In spite of being a well-known hormone and signal molecule, which may provide protection under biotic or abiotic stress condition still there are many gaps in grasping the basic mechanisms through which SA confer abiotic stress tolerance and its overall effects in plants should be explored. Exact mechanism of the mode of action of SA is still poorly understood, especially because it may differ in the different species, and may also depend on the environmental factors. Therefore, complete elucidation of the role of SA as well as detailed protective mechanisms would be helpful for developing stress tolerance in plants. The appropriate dose and method of SA application in plants are also matter of elucidation.

Acknowledgements

The authors thank Mr. Anisur Rahman, Laboratory of Plant Stress Responses, Faculty of Agriculture, Kagawa University, for critical reading of the manuscript.

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