

Article

# $\beta$ -Aminobutyric Acid Pretreatment Confers Salt Stress Tolerance in *Brassica napus* L. by Modulating Reactive Oxygen Species Metabolism and Methylglyoxal Detoxification

Jubayer Al Mahmud<sup>1</sup>, Mirza Hasanuzzaman<sup>2\*</sup>, M. Iqbal R. Khan<sup>3</sup>, Kamrun Nahar<sup>4</sup> and Masayuki Fujita<sup>5\*</sup>

<sup>1</sup> Department of Agroforestry and Environmental Science, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, Bangladesh; jamahmud\_bd@yahoo.com (J.A.M.)

<sup>2</sup> Department of Agronomy, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, Bangladesh; mhzsauag@yahoo.com (M.H.)

<sup>3</sup> Plant Systems Biology Laboratory, Department of Botany, Jamia Hamdard, New Delhi-110062, India; amu.iqbal@gmail.com (M.I.R.K.)

<sup>4</sup> Department of Agricultural Botany, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, Bangladesh; knahar84@yahoo.com (K.N.)

<sup>5</sup> Laboratory of Plant Stress Responses, Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, Miki-cho, Kita-gun, Kagawa 761-0795, Japan; fujita@ag.kagawa-u.ac.jp (M.F.)

\* Correspondence: mhzsauag@yahoo.com (M.H.); fujita@ag.kagawa-u.ac.jp (M.F.)

**Abstract:** Salinity is a serious environmental hazard which limits world agricultural production by adversely affects plant physiology and biochemistry. Hence increase tolerance against salt stress is very important. In this study, we explored the function of  $\beta$ -aminobutyric acid (BABA) in enhancing salt stress tolerance in rapeseed (*Brassica napus* L.). After pretreatment with BABA, seedlings were exposed to NaCl (100 mM and 150 mM) for 2 days. Salt stress increased Na content and decreased K content in shoot and root. It disrupted the antioxidant defense system by producing reactive oxygen species (ROS; H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup>), methylglyoxal (MG) content and causing oxidative stress. It also reduced the growth and photosynthetic pigments of seedlings but increased proline (Pro) content. However, BABA pretreatment in salt-stressed seedlings increased ascorbate (AsA) and glutathione (GSH) contents; GSH/GSSG ratio; and the activities of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT), glyoxalase I (Gly I), and glyoxalase II (Gly II) as well as growth and photosynthetic pigments of plants. In addition, compared to salt stress alone BABA increased Pro content, reduced the H<sub>2</sub>O<sub>2</sub>, MDA and MG contents and decreased Na content in root and increased K content in shoot and root of rapeseed seedlings. Our findings suggest that BABA plays a double role in rapeseed seedlings by reducing Na uptake and enhancing stress tolerance through upregulating the antioxidant defense and glyoxalase systems.

**Keywords:** Abiotic stress; Antioxidant defense; Glyoxalase; Ion homeostasis; Organic acid; Osmotic stress

## 1. Introduction

Salinity is among the most detrimental stresses in plants, governing agricultural yield. When plants get exposed to excessive saline conditions, the metabolism gets debilitated, growth and development are adversely affected. It has been estimated that salinity is affecting more than 20% of the cultivated land worldwide [1, 2]. The occurrence of salt stress cause characteristic changes in plant from the time of occurrence until plant maturity [3]. Salt stress induces an initial water-deficit, due to the relatively high solute concentrations in the soil, and also ion-specific stresses resulting from changes in K<sup>+</sup>/Na<sup>+</sup> ratios [4], which leads to elevated levels of Na<sup>+</sup> and Cl<sup>-</sup> in the plants that

hampers its growth and development [5]. It also impacts physiological and molecular functioning of the photosynthetic components like chlorophyll, PSII and carotenoids gets degraded thereby decreasing photosynthetic efficiency of the plants [4]. Reduced photosynthesis with increased salinity consequently may result in stomatal closure, reduction in intracellular CO<sub>2</sub> partial pressure and non-stomatal factors [6, 7], reduction in protein concentration [8], reactive oxygen species (ROS) production is most prevalent during salinity stress and can damage cellular components such as protein, lipids and DNA thusly, compromising with vital cellular functions [9].

Maintenance of plant homeostasis is vital for the survival of plants during salt stress which is achieved by ion uptake and compartmentalization for normal plant growth and development. To cope up with adverse effects of salinity signaling molecules have been identified [10]. Among these, the non-proteinogenic amino acid,  $\beta$ -amino butyric acid (BABA) is a xenobiotic compound which has evidently demonstrated its potentiality to induce resistance against abiotic and biotic stresses.  $\beta$ -aminobutyric acid -induced resistance through priming has been proposed as a way of generating crop varieties with an enhanced defensive capacity against abiotic stresses [11].  $\beta$ -aminobutyric acid equips in enhancing plants resistance to abiotic stresses without compromising with the natural yield and productivity of the plant either through exogenous application [12] or through priming [13]. Potentiality of BABA in plant defense was further determined by subjection of plants under salinity and submergence, where BABA levels were found to show simultaneous elevation in stressed plants [14]. Experimental demonstration of role of BABA in response to salt stress was suggested with *Arabidopsis* as the model organism. Expression patterns of marker genes SA pathway (PR-1, PR-5), the ABA pathway (RAB18, RD29A) [15]. It has also been made clear that induction of salt stress tolerance in *Arabidopsis* is independent of functional SA signaling. Experimental evidences demonstrated that priming of BABA induce ABA signalling which is responsible for water stress tolerance in *Arabidopsis* [15]. Further role of BABA in plant during abiotic stress conditions were illustrated in rice which were exposed to seed priming with BABA. Consequently, priming of rice seedling with BABA resulted in increased photosynthetic efficiency, increased mitochondrial activity and elevated levels of activity of nitrate reductase and antioxidant enzymes [16].

Priming of non-proteinogenic amino acids (BABA) is gaining attention these days, for their efficient role in abiotic stress tolerance with cost effective defense mechanism eliminating costly energy requirements. The exact mode of BABA priming and their molecular, physiological, and ecological aspects in plants are still under shadow and is expected to emerge as a great tolerance system by plants in near future. Brassicaceae are important contributor to total oilseed production and most of them are classified as moderately salt tolerant which would be more suitable for saline and dry lands in years to come. So, it is very important to develop strategy to improve production of Brassicaceae plant under saline condition. *Brassica napus* L. is important and familiar member of Brassicaceae family. So in this study we tried to evaluate the effect of BABA on Na accumulation, growth and biomass, water status, photosynthetic efficiency, cellular damage, and performance of the antioxidant and glyoxalase systems of *B. napus* L. under NaCl stress during the early seedling stage.

## 2. Results

### 2.1 Salt accumulation and Na/K ratio

Due to salt exposure, Na content of *B. napus* in the shoot and root amplified in a concentration-dependent approach, and the roots confirmed elevated accumulation than the shoots (Table 1). Potassium content under same condition showed the reverse result of Na content. In contrast to control plants 100 mM and 150 mM NaCl stress decrease K content by 19 and 27% in shoot and 15 and 38% in root, respectively. Pretreatment of seedlings with BABA notably decreased only root Na content but decreased both shoot and root K content. Consequently, supplementation of BABA before salt exposure lessened the ratio of Na and K by 15 and 23% in shoot and 18 and 36%

in root under 100 mM and 150 mM NaCl stress, respectively, in contrast to their respective stress alone (Table 1).

**Table 1.** Effect of BABA on Na content, K content, ratio of Na and K in both shoot and root of rapeseed (*Brassica napus* L.) seedlings exposed to different concentration of salt stress. Average from three replications (n = 3) was used to determine mean ( $\pm$ SD) for each treatment and means of the same column were compared by letters where letters with different values varied significantly at  $p \leq 0.05$  due to Fisher's LSD test.

Treatment	Shoot Na ( $\mu$ mol g <sup>-1</sup> DW)	Root Na ( $\mu$ mol g <sup>-1</sup> DW)	Shoot K ( $\mu$ mol g <sup>-1</sup> DW)	Root K ( $\mu$ mol g <sup>-1</sup> DW)	Shoot Na/K ratio	Root Na/K ratio
Control	11 $\pm$ 1.5 d	97 $\pm$ 13 d	118 $\pm$ 4.3 a	302 $\pm$ 10 a	0.10 $\pm$ 0.01 d	0.32 $\pm$ 0.05 d
BABA	11 $\pm$ 1.4 d	95 $\pm$ 12 d	115 $\pm$ 3.9 ab	301 $\pm$ 7 a	0.09 $\pm$ 0.01 d	0.31 $\pm$ 0.05 d
S1	990 $\pm$ 23 bc	1950 $\pm$ 94 b	96 $\pm$ 2.1 c	256 $\pm$ 4 c	10.29 $\pm$ 0.46 b	7.34 $\pm$ 0.30 bc
BABA+S1	977 $\pm$ 24 c	1727 $\pm$ 29 c	111 $\pm$ 3.1 b	294 $\pm$ 11 ab	8.79 $\pm$ 0.42 c	6.00 $\pm$ 0.34 c
S2	1062 $\pm$ 57 a	2272 $\pm$ 195 a	86 $\pm$ 1.1 d	185 $\pm$ 10 d	12.37 $\pm$ 0.71 a	12.29 $\pm$ 1.36 a
BABA+S2	1050 $\pm$ 53 ab	1941 $\pm$ 179 b	110 $\pm$ 3.3 b	252 $\pm$ 40 c	9.57 $\pm$ 0.71 bc	7.87 $\pm$ 1.70 b

Here, BABA, S1 and S2 indicate 150  $\mu$ M  $\beta$ -aminobutyric acid, 100 mM NaCl and 150 mM NaCl, respectively.

## 2.2 Plant growth and biomass

Salt toxicity harshly damages the growth and development of *B. napus* seedlings including plant height, fresh weight (FW) and dry weight (DW) (Table 2). In contrast to control seedlings, plant height lessened by 9 and 13% due to 100 and 150 mM NaCl concentration, respectively. Seedling pretreated with BABA considerably restored the plant height by 4% under 100 mM NaCl and 6% under 150 mM NaCl. Parallel tendency was obtained in case of fresh and dry weight under the same treatment condition (Table 2).

**Table 2.** Effect of BABA on plant height, FW, DW, leaf RWC, Pro content, chl *a*, chl *b* and chl (*a+b*) content of rapeseed (*Brassica napus* L.) seedlings exposed to different concentration of salt stress. Average from three replications (n = 3) was used to determine mean ( $\pm$ SD) for each treatment and means of the same column were compared by letters where letters with different values varied significantly at  $p \leq 0.05$  due to Fisher's LSD test.

Treatment	Plant height (cm)	Fresh weight,FW (mg seedling <sup>-1</sup> )	Dry weight,DW (mg seedling <sup>-1</sup> )	Leaf relative water content, RWC (%)	Proline, Pro content ( $\mu$ molg <sup>-1</sup> FW)	Chl <i>a</i> (mg g <sup>-1</sup> FW)	Chl <i>b</i> (mg g <sup>-1</sup> FW)	Chl ( <i>a+b</i> ) (mg g <sup>-1</sup> FW)
Control	5.28 $\pm$ 0.14 a	69.2 $\pm$ 1.9 ab	7.98 $\pm$ 0.26 a	91.6 $\pm$ 0.9 a	1.33 $\pm$ 0.05 e	0.68 $\pm$ 0.03 a	0.28 $\pm$ 0.01 a	0.95 $\pm$ 0.016 a
BABA	5.31 $\pm$ 0.07 a	70.5 $\pm$ 1.5 a	8.16 $\pm$ 0.24 a	91.3 $\pm$ 0.6 a	1.34 $\pm$ 0.06 e	0.67 $\pm$ 0.03 a	0.27 $\pm$ 0.01 a	0.95 $\pm$ 0.022 a
S1	4.83 $\pm$ 0.06 c	64.1 $\pm$ 2.3 c	7.17 $\pm$ 0.26 cd	86.2 $\pm$ 0.8 c	2.78 $\pm$ 0.08 d	0.42 $\pm$ 0.01 d	0.11 $\pm$ 0.004 d	0.53 $\pm$ 0.004 d
BABA+S1	5.04 $\pm$ 0.06 b	68.1 $\pm$ 2.0 ab	7.78 $\pm$ 0.23 ab	88.7 $\pm$ 0.5 b	3.46 $\pm$ 0.13 c	0.59 $\pm$ 0.01b	0.27 $\pm$ 0.005 b	0.85 $\pm$ 0.008 b
S2	4.60 $\pm$ 0.08 d	60.0 $\pm$ 1.8 d	6.78 $\pm$ 0.47 d	81.4 $\pm$ 0.9 d	3.78 $\pm$ 0.11 b	0.26 $\pm$ 0.02 e	0.08 $\pm$ 0.008 e	0.34 $\pm$ 0.013 e
BABA+S2	4.88 $\pm$ 0.06 c	66.0 $\pm$ 2.1 bc	7.41 $\pm$ 0.25 bc	86.2 $\pm$ 0.4 c	4.93 $\pm$ 0.19 a	0.47 $\pm$ 0.02 c	0.18 $\pm$ 0.01 c	0.65 $\pm$ 0.013 c

Here, BABA, S1 and S2 indicate 150  $\mu$ M  $\beta$ -aminobutyric acid, 100 mM NaCl and 150 mM NaCl, respectively.

## 2.3 Water status and osmotic adjustment

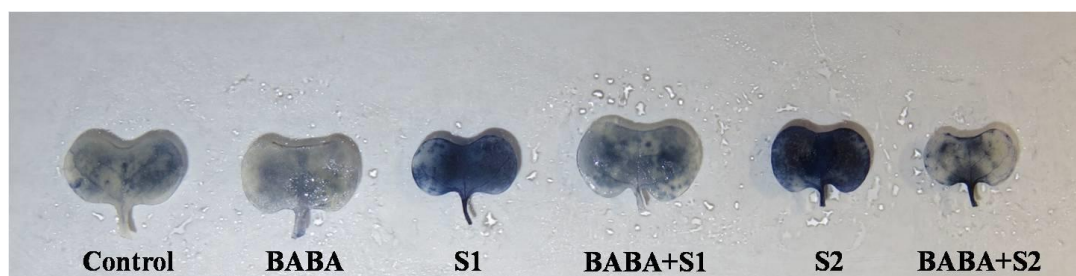
Salt stress negatively affected water status of *B. napus* seedlings. Reduction of leaf relative water content (RWC) content and a drastic enhancement of proline (Pro) content owing to salt stress established the water shortage situation of *B. napus*. In present experiment, leaf RWC reduced by 6 and 11% in the seedlings under 100 mM NaCl and 150 mM NaCl stress, respectively, compared to control plants, whilst Pro level augmented by 109 and 184%, respectively (Table 2). In contrast, pretreatment of seedlings with BABA before salt exposure conspicuously improved the water status through adjusting the osmotic potential by enhancing leaf RWC and increasing the concentration of Pro in comparison with respective stress alone (Table 2).

#### 2.4 Photosynthetic pigments

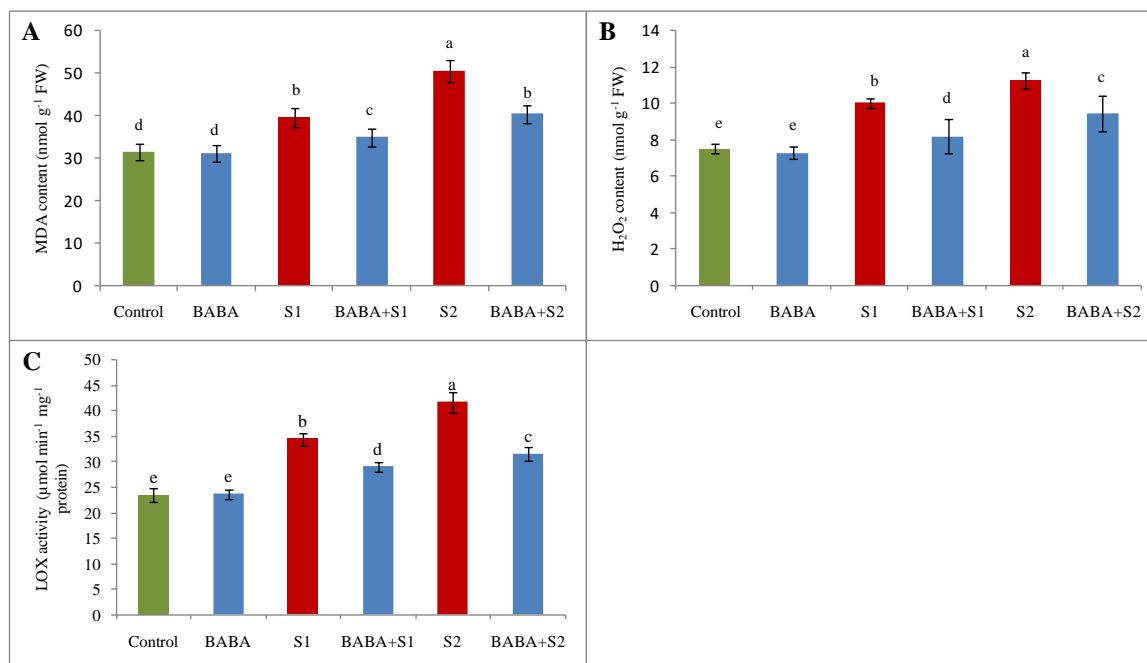
Salt stress hampered the photosynthesis process as it lessened levels of important photosynthetic pigments including chl *a* and chl *b*. As a result in comparison with control seedlings chl (*a+b*) content reduced by 44 % due to 100 mM NaCl concentration and 64% due to 150 mM NaCl concentration. Pretreatment with BABA reinstates the chl (*a+b*) level by 60 and 91% under 100 and 150 mM NaCl concentration, respectively, in contrast to respective stress alone (Table 2).

#### 2.5 Reactive oxygen species generation, oxidative stress, and membrane damage

In present study salt stress contributed in causing oxidative stress due to over generation of ROS. Higher  $O_2^{\cdot-}$  was noticed in the leaves of *B. napus* by histochemical detection using nitroblue tetrazolium chloride (NBT). Salt stress affected leaves showed significantly more deep blue spots/patches of  $O_2^{\cdot-}$  anions in comparison with control seedlings. But pretreatment with BABA followed by salt stress reduce the spots/patches of  $O_2^{\cdot-}$  anions (Figure 1). Hydrogen peroxide ( $H_2O_2$ ), another reactive free radical, enhanced by 33 and 50% under 100 and 150 mM NaCl stress, respectively, in contrast to control plants, whereas BABA pretreatment reduced  $H_2O_2$  by 18 and 19% under 100 and 150 mM NaCl stress, respectively, in comparison with their respective stress treatment (Figure 2B). Moreover LOX activity enhanced by 46% under 100 mM NaCl and 77% under 150 mM NaCl, compared to control seedlings. Applying BABA reduced LOX activity by 16 and 24% under 100 and 150 mM NaCl stress, respectively, compared to their respective stress (Figure 2C). Consequently exposure of *B. napus* seedling to salt stress augmented malondialdehyde (MDA a major index of lipid peroxidation) content by 26 and 605 at 100 and 150 mM NaCl stress, respectively but BABA pretreatment decreased MDA content notably under stress condition (Figure 2A).



**Figure 1.** Effect of BABA on superoxide ( $O_2^{\cdot-}$ ) accumulation of rapeseed (*Brassica napus* L.) seedlings exposed to different concentration of salt stress. Here, BABA, S1 and S2 indicate 150  $\mu$ M  $\beta$ -aminobutyric acid, 100 mM NaCl and 150 mM NaCl, respectively.

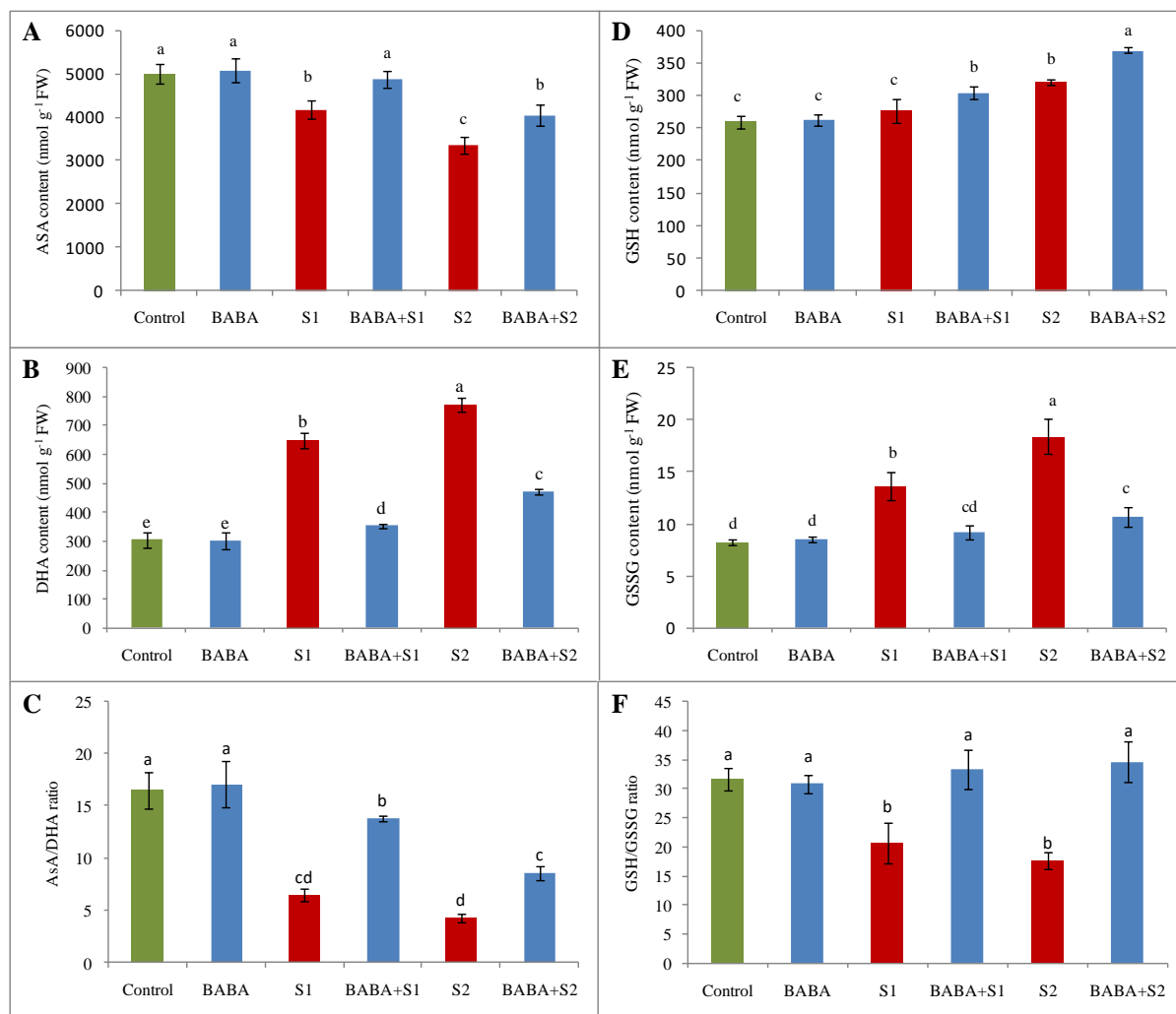


**Figure 2.** Effect of BABA on MDA content (A), H<sub>2</sub>O<sub>2</sub> content (B), and LOX activity (C) of rapeseed (*Brassica napus* L.) seedlings exposed to different concentration of salt stress. Means ( $\pm$ SD) were calculated from three replications (n = 3) for each treatment. Average from three replications (n = 3) was used to determine mean ( $\pm$ SD) for each treatment. Letters with different values varied significantly at  $p \leq 0.05$  due to Fisher's LSD test. Here, BABA, S1 and S2 indicate 150  $\mu$ M  $\beta$ -aminobutyric acid, 100 mM NaCl and 150 mM NaCl, respectively.

### 2.6 Ascorbate-glutathione cycle

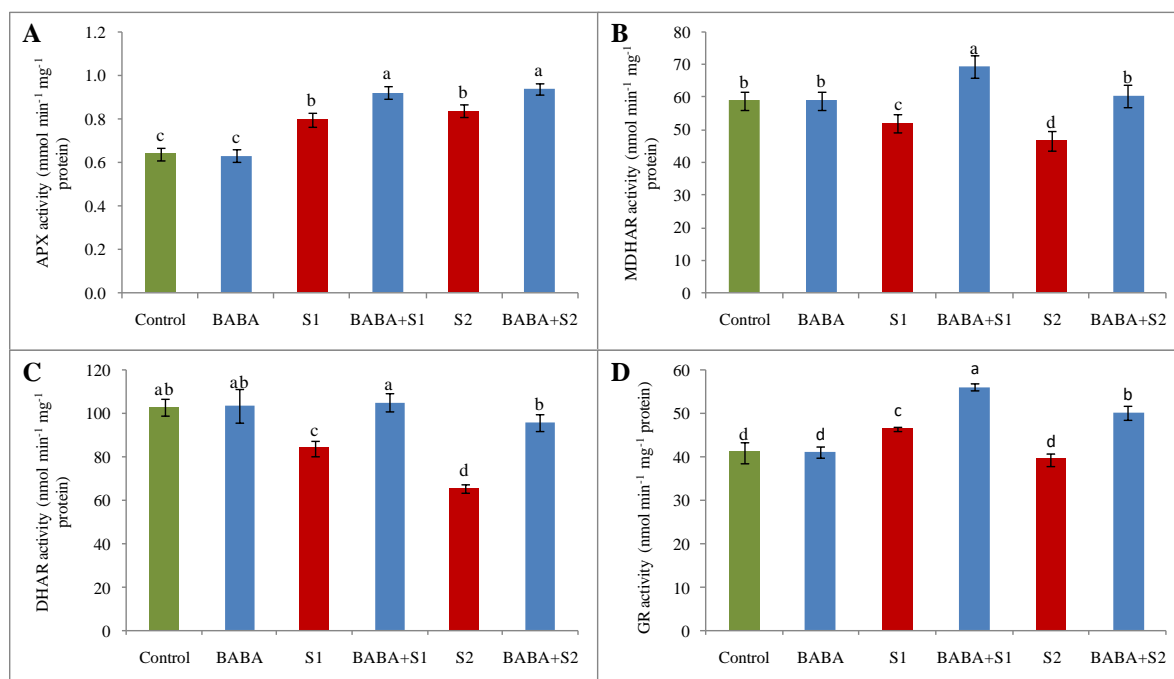
Activity of ascorbate (AsA) decreased by 17% in the *B. napus* seedlings under 100 mM NaCl stress and 33% under 150 mM NaCl stress, at the same time, DHA content augmented by 113 and 153% under 100 mM and 150 mM NaCl stress, in contrast to the control seedlings. Accordingly, ratio between AsA and dehydroascorbate (DHA) notably reduced due to both concentration of salt stress. However, in comparison with respective stress, BABA supplementation enhanced AsA content by 17 and 21%; decreased DHA content by 46 and 39 %; increased AsA/DHA ratio by 115 and 98% under 100 mM and 150 mM NaCl concentrations, respectively (Figure 3A-C).

Glutathione (GSH), the key non-enzymatic antioxidant enhanced by 7% due to 100 mM and 24% due to 150 mM NaCl stress, respectively, whilst oxidized GSH (GSSG) content enhanced by 65% due to 100 mM and 123% due to 150 mM NaCl stress. Consequently the ratio between GSH and GSSG reduced by 35 and 44% under under 100 and 150 mM NaCl concentration, respectively. Conversely, in contrast to respective stress treatments BABA supplementation enhanced GSH content further by 10 and 13%; reduced GSSG content by 32 and 42%; enhanced ratio of GSH and GSSG by 62 and 97% under 100 mM and 150 mM NaCl stress, respectively (Figure 3D-F).



**Figure 3.** Effect of BABA on AsA (A) and DHA (B) contents, AsA/DHA ratio (C), GSH (D) and GSSG (E) contents, and GSH/GSSG ratio (F) of rapeseed (*Brassica napus* L.) seedlings exposed to different concentration of salt stress. Average from three replications ( $n = 3$ ) was used to determine mean ( $\pm$ SD) for each treatment. Letters with different values varied significantly at  $p \leq 0.05$  due to Fisher's LSD test. Here, BABA, S1 and S2 indicate 150  $\mu$ M  $\beta$ -aminobutyric acid, 100 mM NaCl and 150 mM NaCl, respectively.

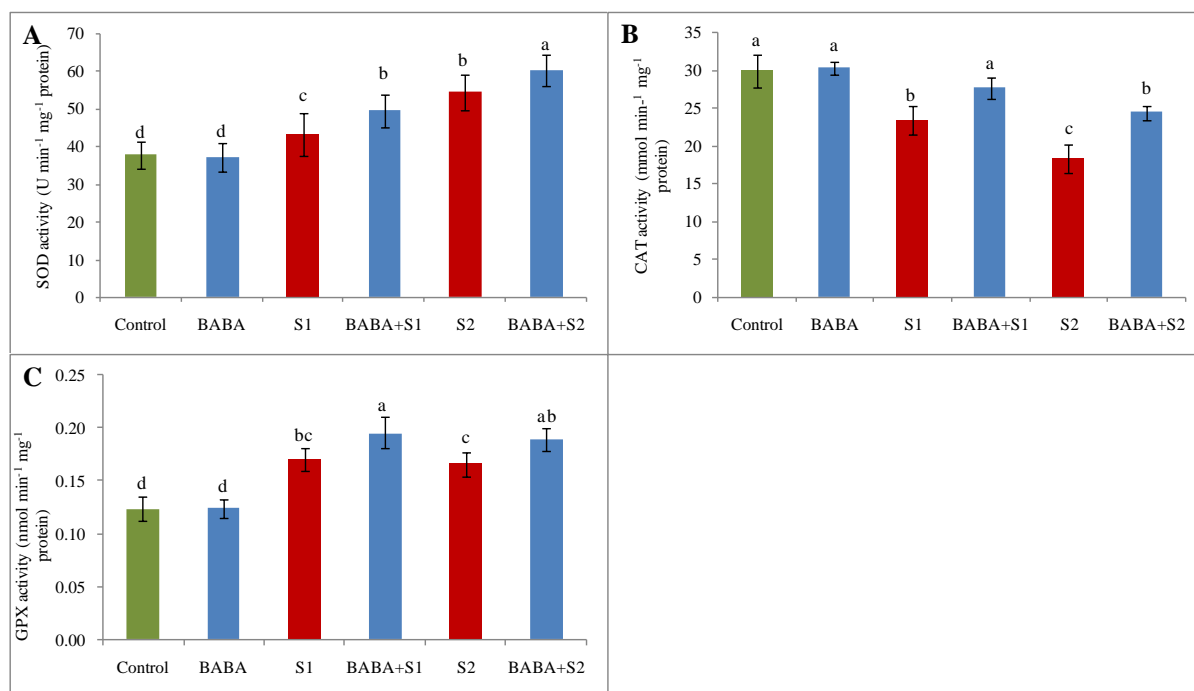
Ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and GSH reductase (GR) (four major enzymes of AsA-GSH pool) confirmed differentiable activity under salt stress condition (Figure 4). In contrast to control seedlings, function of APX enhanced by 25 and 31%; MDHAR activity reduced by 12 and 21%; DHAR activity decreases by 18 and 36%, respectively (Figure 4A-C). However action of GR enhanced by 13% under 100 mM NaCl concentration but lessened by 4% under 150 mM NaCl concentration (Figure 4D). Compared to respective stress treatment, BABA supplementation before stress application enhanced the activity of all the antioxidant enzymes in the AsA-GSH pool (Figure 4).



**Figure 4.** Effect of BABA on activity of APX (A), MDHAR (B), DHAR (C), and GR (D) of rapeseed (*Brassica napus* L.) seedlings exposed to different concentration of salt stress. Means ( $\pm$ SD) were calculated from three replications (n = 3) for each treatment. Average from three replications (n = 3) was used to determine mean ( $\pm$ SD) for each treatment. Letters with different values varied significantly at  $p \leq 0.05$  due to Fisher's LSD test. Here, BABA, S1 and S2 indicate 150  $\mu$ M  $\beta$ -aminobutyric acid, 100 mM NaCl and 150 mM NaCl, respectively.

### 2.7 Activities of superoxide dismutase, catalase, and glutathione peroxidase

Compared to control treatment superoxide dismutase (SOD) activity enhanced by 14 and 44% due to expose of seedlings to 100 and 150 mM NaCl concentration, respectively. Applying BABA considerably increased SOD activity further (Figure 5A). Catalase activity lessened steadily with enhance of salt stress intensity. On the other hand, BABA pretreated salt affected seedlings reversed and restored the activity of catalase (CAT) by 18 and 33% under 100 and 150 mM NaCl stress, respectively (Figure 5B). In comparison with control seedlings, action of GSH peroxidase (GPX) enhanced by 38% under 100 mM and 35% under 150 mM NaCl stress. Conversely, BABA pretreatment further improved the action of GPX in comparison with respective stress alone (Figure 5C).

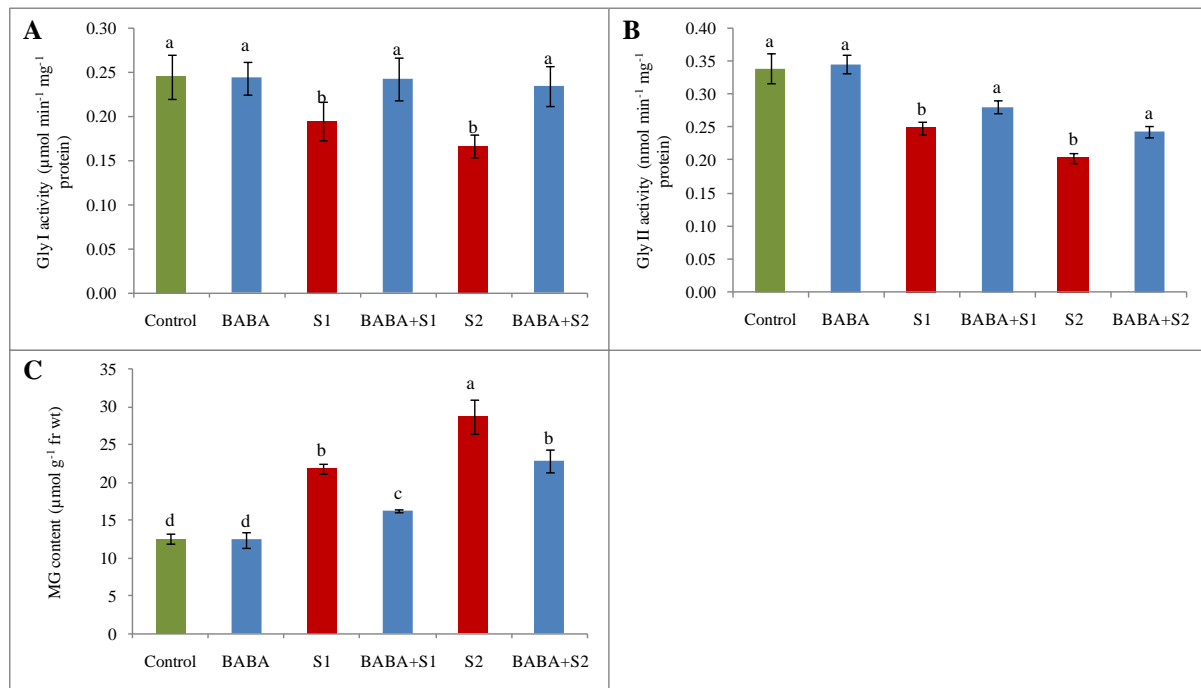


**Figure 5.** Effect of BABA on activity of SOD (A), CAT (B), and GPX (D) of rapeseed (*Brassica napus* L.) seedlings exposed to different concentration of salt stress. Average from three replications (n = 3) was used to determine mean ( $\pm$ SD) for each treatment. Letters with different values varied significantly at  $p \leq 0.05$  due to Fisher's LSD test. Here, BABA, S1 and S2 indicate 150  $\mu$ M  $\beta$ -aminobutyric acid, 100 mM NaCl and 150 mM NaCl, respectively.

### 2.8 Glyoxalase system and methylglyoxal content

Glyoxalase (Gly) I and Gly II activity decreased under salt stress. Activity of Gly I decreased by 20 and 32% and activity of Gly II decreased by 27 and 40% under 100 mM NaCl and 150 mM NaCl stress, respectively. However, pretreatment with BABA followed by 100 mM and 150 mM NaCl concentration increased activity of Gly I by 25 and 40% and activity of Gly II by 13 and 19%, respectively, in comparison with their respective stresses (Figure 6A,B). Probably due to action of BABA, activity of both Gly I and Gly II were involved in scavenging toxic methylglyoxal (MG). Methylglyoxal increased 74 and 129% under both 100 mM and 150 mM NaCl stress, respectively. However enhanced efficiency of Gly I and Gly II due to BABA pretreatment was involved in reduction of MG content by 24 and 20% under 100 and 150 mM NaCl stress, respectively (Figure 6C).





**Figure 6.** Effect of BABA on Gly I activity (A), Gly II activity (B) and MG content (C) of rapeseed (*Brassica napus* L.) seedlings exposed to different concentration of salt stress. Average from three replications (n = 3) was used to determine mean ( $\pm$ SD) for each treatment. Letters with different values varied significantly at  $p \leq 0.05$  due to Fisher's LSD test. Here, BABA, S1 and S2 indicate 150  $\mu$ M  $\beta$ -aminobutyric acid, 100 mM NaCl and 150 mM NaCl, respectively.

### 3. Discussion

Soil salinity has emerged as one of the most serious factors limiting plant growth and development ultimately soil health [17]. Salt stress disturbs normal physiological, biochemical and molecular processes, most commonly manifested in stunted seed germination, seedling growth, that ultimately cause poor plant productivity and distinctly changed concentration of key biomolecules [18, 19, 20,]. Salinity stress causes enormous alterations in the signaling pathways as well as the regulation of gene expression that have effects on the growth and development of plants [21]. In order to illustrate the impact of BABA-induced plant tolerance to salt stress, study has been focusing on physiological mechanisms to exhibit the reduction of plant sensitivity to stress upon BABA treatment. However, exogenous application of BABA can induce resistance against several biotic and abiotic stressors like salinity, drought, heat, cold and metal toxicity [22].

In the present study, salt treatment resulted in higher  $\text{Na}^+/\text{K}^+$  ratio in comparison to control. It has been shown that salinity induced ROS formation can lead to programmed cell death, and a high cytosolic  $\text{K}^+/\text{Na}^+$  ratio is essential for triggering salinity-induced PCD [23]. The used BABA against salt stress decreased  $\text{Na}^+$  and  $\text{K}^+$  content might be involved in the salt tolerance. The reduction in growth and dry matter of the salt-treated plants as observed in this study could be ascribed to the changes in plant water relation resulting reduction in meristem activity as well as cell elongation [24], thereby inhibiting leaf expansion [25]. Similarly, the noticed decrease in the plant height and dry weight under salt stress might be due to  $\text{Na}^+$  and  $\text{Cl}^-$  ions accumulation in leaf tissues could have resulted in growth hormone alteration, enzyme inactivation and stomatal closure and/or damage of photosynthetic machinery that in turn resulted in lower  $\text{CO}_2$  assimilation [26].  $\beta$ -aminobutyric acid induced salt tolerance in *Arabidopsis* through enhanced ABA accumulation [15]. In the present study, BABA has shown to induce growth attributes under salinity and optimal conditions suggested that the BABA promotes accumulation of biomass. Such enhancement of growth has also been observed in soybean where plant treated with BABA under stressed and non-stressed environment [27]. Similar results were also observed in inducing stress tolerance upon salt stress [28].

Salinity enhances osmotic pressure leading to reduction in water absorbance, cell division and differentiation are inhibited, which adversely affects metabolic and physiological processes [29]. In salt stressed plant, there is a decrease in RWC, resulting in limited diffusion of H<sub>2</sub>O<sub>2</sub> from its generation site [30]. The decreased RWC in rapeseed under increased salinity is indicative of a loss of cell turgor that leads to limited water availability for the cell extension and expansion. Salinity obstructs the uptake and translocation of water due to the consequent high accumulation of Na<sup>+</sup> and Cl<sup>-</sup> ions in the cytoplasm that leads to a nutrient imbalance [31]. It has been documented that proline helps in mitigating the stresses through different mechanisms including detoxification of toxic ions, protection of membrane integrity, stabilization of proteins/enzymes and protects cell from ROS damages [32]. The proline content increased considerably with increase in salinity but BABA application moderates this effect in studied plants.  $\beta$ -aminobutyric acid has been shown to induce proline accumulation in *Arabidopsis* and potato [33, 34]. Misra and Misra [35] have reported that the up-regulation of proline biosynthesis enzymes (viz. pyrroline-5-carboxylate reductase and  $\gamma$  glutamyl kinase) and the down-regulation of proline oxidase activity directed to an increased proline status, which helped in sustaining the cell turgor in *Rauwolfia serpentina* under salinity stress.

In this study, photosynthetic pigments were decreased significantly in rapeseed due to salt stress. This also implies that reduction in photosynthetic pigments occurred under stressed conditions was significantly ameliorated on BABA application.  $\beta$ -aminobutyric acid priming might have a role in influencing the synthesis of photosynthetic pigments in the seedlings. Previously, Tabrizi et al. [36] has shown similar observation in maize seedlings. The decrease in chlorophyll content might have been due to salt induced increase in the activity of chlorophyllase [37] and the instability of pigment-protein complex brought about by the elevated salinity level [38]. The decline in photosynthesis under salinity stress may be due to inactivation of PS II complex and a loss of chlorophyll pigments [39].

The present study, the extent of oxidative stress determined by the level of superoxide, H<sub>2</sub>O<sub>2</sub>, and MDA content and LOX activity under 100 mM and 150 mM NaCl concentrations. However, in response to increasing salinity, many metabolic pathways are inhibited and proteins are catabolized. The increased ROS generation can alter normal cellular metabolism through increased oxygen-induced cellular damage, oxidative damage to DNA, proteins and membrane lipids which led to the membrane lipid peroxidation [9]. Alternatively, BABA application reduced the level of ROS, H<sub>2</sub>O<sub>2</sub> in salt-treated plants which may also be a reason for reducing lipid peroxidation of bio-membranes, as expressed by MDA content.  $\beta$ -aminobutyric acid treatment reduces H<sub>2</sub>O<sub>2</sub> accumulation that induces salt tolerance, could be mediated by a faster osmoregulation process. The decline in MDA content of seedlings raised from BABA application has been noticed in rice [40, 41]. Furthermore, Wang et al. [42] stated that BABA decreased MDA content, polygalacturonase and pectinmethylesterase activities and enhanced cell wall polysaccharide content resulting delayed senescence of cherry fruits. The emergence of this signal could induce the activation of antioxidant defense systems and ameliorate the oxidative damage initiated by ROS. Therefore, salt stress resistance may depend on the enhancement of the antioxidative defense systems. In this study, we demonstrated that BABA treatment able to protect rapeseed against salt stress by augmenting antioxidant defense system. Furthermore, BABA induced the up-regulation of antioxidant enzymes (SOD and CAT), which might play a role to remove the free radical which is correlated with increasing plant defense and reducing oxidative stress damage [28]. Moreover, numerous studies revealed that BABA is able to modulate the expression of defense-related genes involved in plant defense reactions. It was suggested that BABA induces a stress-induced morphogenic response in *Arabidopsis thaliana* that could be responsible for the BABA-induced resistance [43].

This suggests that BABA can induce the capacity to cope with salt stress in rapeseed plants. Other study showed that BABA induced greater accumulation of antioxidant defense components under salt stress [44, 45, 46], Cd stress [47] and drought stress [48]. Increase in APX and GR content in rapeseed, reduced AsA-GSH redox status accompanied by AsA-GSH cycle enzymes such as APX, MDHAR, DHAR and GR clearly evident that AsA-GSH cycle played a crucial role for scavenging ROS in the rapeseed under salinity stress. It has been reported that APX and GR genes are up-regulated in plants indicating less peroxisomal abundance of CAT activity [49]. Ascorbate

peroxidase uses ascorbate as electron donor in the first step of the ascorbate–glutathione cycle and is considered the most important plant peroxidase in H<sub>2</sub>O<sub>2</sub> detoxification [50]. Previously, Abogadallah et al. [51] reported that APX-GR is the main H<sub>2</sub>O<sub>2</sub> detoxifier that maintains ROS balancing in barnyard grass under salt stress. Recently it has been shown that APX, CAT, GR and SOD antioxidant activities boosted the protection against oxidative stress [52, 53]. The elevated levels of GR activity could increase the GSH/GSSG ratio, which is required for ascorbate regeneration and activation of several CO<sub>2</sub> fixing enzymes in the chloroplasts [54], ensuring NADP<sup>+</sup> availability to accept electrons from the photosynthetic electron transport chain. The present study showed that MG production increased around 2-4 fold in rapeseed under salt stress, this might be due to a higher rate of glycolysis, amino acid, and acetone metabolism, or other biochemical processes. BABA treatment increased the Gly I and Gly II level in salt treated rapeseed plants which induces MG detoxification. However, the glyoxylase system composed of Gly I and Gly II regulates the enhanced production of MG under various environmental stresses including salinity [55, 56], drought [20, 57, 58], toxic metals [59, 60], heat exposure [61, 62]. Previously, Reddy and Sopory [63] observed that overexpression of Gly I gene decreased the endogenous MG and enhanced the tolerance against MG and salt stress. Similarly, overexpression of Gly II gene in rice enhances tolerance to MG and salt stress [56]. The glyoxylase system detoxifies MG in two-steps. In the first step, Gly I catalyzed the reaction of MG with GSH, and produces hemithioacetal which is then converted to S-D-lactoylglutathione (SLG). In the second step, SLG is then hydrolysed to D-lactate catalyzed by Gly II and later GSH regenerated. At the end of the reaction, GSH is recycled because the availability of GSH is an important factor for detoxifying MG via the glyoxalase system [55].

#### 4. Materials and methods

##### 4.1 Plant materials, experimental conditions, and treatments

Disease and pest free homogeneous rapeseed (*Brassica napus* L. cv. BARI Sharisha-13) seeds were used in this study. For sterilization, firstly seeds were dipped into 70% ethanol for five minutes and then rinsed with double distilled water thoroughly for several times. 9 cm Petri dishes containing six sheets of filter paper wetted by 10 mL distilled water were used to germinate seeds maintaining equal distance. The Petri dishes containing seeds were transferred in a germinator for uniform and healthy germination (48 hours). Each Petri dish included 60 morphologically identical germinated seedlings. Then the rapeseed seedlings contained Petri dishes were shifted in a cultivation chamber (Iwaki; Asahi Techno Glass, Japan) under proper environment (light: 350  $\mu\text{mol photons m}^{-2}\text{s}^{-2}$ ; temperature: 25 $\pm$ 2°C; relative humidity: 65–70%). For plant nutrition Hyponex solution (Hyponex, Japan; 5,000-fold diluted) was added to the seedlings at regular interval. Finally 6-d-old 50 plants of each Petri dish were pretreated with BABA, 150  $\mu\text{M}$  for 48 h. Both BABA-pretreated and non-pretreated seedlings were then exposed to salt condition (100 and 150 mMNaCl) and further grown for 48 h. A number of trials were performed before deciding the current doses of treatments. Leaves and roots of seedlings were collected for determining different important growth and physiological parameters as per standard techniques. The study was done following completely randomized design (CRD) with six treatments (1. Control; 2. BABA; 3. 100 mMNaCl, S1; 4. 150 mMNaCl, S2; 5. BABA+S1; 6. BABA+ S2) and repeated thrice.

##### 4.2 Measurement of Na<sup>+</sup> and K<sup>+</sup> content

Na<sup>+</sup> and K<sup>+</sup> content of shoots and roots of rapeseed seedlings were estimated by utilizing an atomic absorption spectrophotometer (Z-5000; Hitachi, Japan). Fresh sample of shoots and roots were collected and dehydrated in oven for 72 h at 80°C. Dry samples then transferred for digestion separately into a strong acid mixture (HNO<sub>3</sub>:HClO<sub>4</sub> = 5:1 v/v) at 80°C for 48 h. Finally spectrophotometer was used to read absorbance of samples. A standard curve generated from known concentration was used to estimate the shoots and roots Na<sup>+</sup> and K<sup>+</sup> content.

##### 4.3 Estimation of plant growth parameters

Indiscriminately chosen five plants from every treatment were used to determine the average height of plant in centimeters (cm). Fresh weight (FW) of a plant was measured from the average weight of 10 randomly selected fresh seedlings. Dry weight (DW) of plant was estimated after proper drying in the oven for 48 h at 80°C.

#### 4.4 Determination of leaf relative water content

Whole fresh leaves were used to estimate leaf RWC according to the technique explained by Barrs and Weatherley [64]. The FW was recorded through weighing entire fresh leaves and then dipped them to fresh water for 12 hours and weighed further after take away outside water and documented as turgid weight (TW). Lastly, DW was estimated after dehydrating the turgid leaves for 48 h at 80°C. Following formula was used to determine leaf RWC:

$$\text{RWC (\%)} = [(\text{FW}-\text{DW})/(\text{TW}-\text{DW})]\times 100$$

#### 4.5 Estimation of proline content

For determination of Pro level of rapeseed seedlings, 0.5 g leaves were blended well in 5 mL 3% sulfo-salicylic acid keeping ice-cold condition in mortar and pestle following the method explained by Bates et al. [65]. To get supernatant extracted solution was centrifuged for 12 minutes at 11,500×g. Then 1 mL supernatant was added with 1 mL acid ninhydrin and 1 mL glacial acetic acid and the combination was incubated at 100°C in a hot water bath. After 1 hour incubation, mixture solution was moved to clean test tube and placed in ice containing box for proper cooling. After that, toluene (2 mL) was supplemented to the cooled solution and vortexed well. Finally absorbance of chromophore-containing toluene was recorded spectrophotometrically at 520 nm wavelengths and Pro level was estimated using standard curve generated from known concentration.

#### 4.6 Measurement of chlorophyll content

To measure content of chlorophyll (chl) *a* and chl *b* of rapeseed seedlings, 0.5 g fresh leaves were randomly selected and extracted in 80% (v/v) acetone (10 mL) according to the technique explained by Arnon [66]. Then the extracted homogenate was centrifuged at 5,000×g for 10 min. Finally the absorbance of the solution was read after dilution. The 663 and 645 nm wavelength were used at UV-visible spectrophotometer for determining for chl *a* and chl *b* contents.

#### 4.7 Histochemical detection of superoxide anion

Fresh leaves of rapeseed seedlings were dipped in 0.1% solution of NBT and incubated in dark environment for 24 h according to Nahar et al. [67]. After incubation, NBT treated leaves were bleached by hot/boiled ethanol and photographs were taken where dark blue spots indicate the generation of O<sub>2</sub><sup>•-</sup>.

#### 4.8 Estimation of lipid peroxidation

Malondialdehyde content mostly determines the level of lipid peroxidation in the plant. The technique of Heath and Packer [68] was used to determine MDA with few modification explained by Hasanuzzaman et al. [69]. Fresh leaves (0.5 g) were blended in 5% (w/v) trichloroacetic acid (TCA). Then the extract was centrifuged at 11,500×g for 15 min for getting clear supernatant which (1 mL) was then added with 4 mL of thiobarbituric acid (TBA) reagent (solution of 0.5% of TBA and 20% TCA) and incubated at 95°C in a hot water chamber. Later than 30 minutes, the mixture was transferred in new clear test tube and allowed to ice containing box for proper cooling. UV-visible spectrophotometer was used to measure the absorbance at 532 nm and also at 600 nm (for correction of non-specific absorbance). At last MDA level was estimated utilizing extinction coefficient 155 mM<sup>-1</sup> cm<sup>-1</sup>.

#### 4.9 Determination of hydrogen peroxide content

To determine H<sub>2</sub>O<sub>2</sub> content, protocol of Yu et al. [70] was used in where fresh leaves (0.5 g) were blended in 3 mL of potassium–phosphate (K–P) buffer having concentration of 50 mM with pH 6.5. For getting clear supernatant homogenates were then centrifuged for 15 min at 11,500×g. Two mL of supernatant was added with 666.4 µL mixture of 0.1% TiCl<sub>4</sub> and 20% H<sub>2</sub>SO<sub>4</sub> (v/v). Then the solution was incubated for 10 minutes maintaining 25°C temperature and re-centrifuged again at 11,500×g for 12 min. The resulting final solution was read spectrophotometrically at 410 nm and extinction coefficient 0.28 µM<sup>-1</sup> cm<sup>-1</sup> was used to calculate H<sub>2</sub>O<sub>2</sub> level.

#### 4.10 Extraction and measurement of ascorbate and glutathione

Mixture of meta-phosphoric acid (5%) and EDTA (1 mM) was used as extraction buffer to measure the components of AsA-GSH cycle. Leaves (0.5 g) were blended in 3 ml of extraction buffer and homogenize by ice cooled mortar and pestle. Extracted homogenates were then centrifuged at 11,500×g for 15 min maintaining temperature 4°C. Content of AsA was measured according the procedures explained by Huang et al. [71] and Hasanuzzaman et al. [68]. Then the clear supernatant was neutralized by 0.5 M K-P buffer (pH 7.0). For reduction of oxidized portion 0.1 M of dithiothreitol was added to the neutralized solution. For measuring content of total AsA and reduced AsA changes of absorbance were recorded at UV-visible spectrophotometer (265 nm) after adding final solution in mixture of 100 mM K-P buffer (pH 7.0) and 1.0 U of ascorbate oxidase (AO). To estimate oxidize portion of AsA (DHA content), reduced AsA was deducted from total AsA. For determination of GSH related components enzymatic recycling was used according to Yu et al. [70] with slight amendments as explained by Nahar et al. [69]. Very first supernatants (0.2 mL) were neutralized by 0.3 mL 0.5 M K-P buffer. After that in presence of GR it was oxidized by 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB). Finally the resulted solution was reduced by nicotinamide adenine dinucleotide phosphate (NADPH). Changes of absorbance were recorded spectrophotometrically at 412 nm wavelength for measuring GSH content. To measure oxidized portion of GSH (GSSG), GSH was separated using 2-vinylpyridine derivatization. For measuring the content of total GSH and GSSG standard curves generated from known concentrations were used. To find out reduced GSH content GSSG was subtracted from total GSH.

#### 4.11 Enzyme extraction and assays

Ice-cold 50 mM K-P buffer (pH 7.0), 100 mM KCl, 1 mM AsA, 5 mM β-mercaptoethanol, and 10% (w/v) glycerol were mixed together to prepare extraction buffer. Leaves (0.5 g) were blended in 1 mL of extraction buffer using pre-cooled mortar and pestle. Then the resulted homogenates were centrifuged for 12 min at 11,500×g. For executing the protein and enzyme assay procedure supernatant should be stored at 0–4°C temperature.

Well established protocol described by Bradford [72] was followed to estimate protein content of plant sample utilizing Bovine serum albumin as a protein standard.

Activity of Lipoxygenase (LOX, EC 1.13.11.12) was estimated according to the procedure explained by Doderer et al. [73]. The changes of sample absorbance were monitored at 234 nm wavelength where as a substrate linoleic acid was used. The extinction coefficient 25 mM<sup>-1</sup>cm<sup>-1</sup> was employed for calculated the activity of LOX.

To determine the action of APX (EC: 1.11.1.11) protocol explained by Nakano and Asada [74] was followed where a reaction buffer was prepared using 50 mM K-P buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H<sub>2</sub>O<sub>2</sub>, 0.1 mM EDTA. Then the extracted solution was added to the reaction buffer and the absorbance was recorded at 290 nm. Finally action of APX was determined utilizing extinction coefficient 2.8 mM<sup>-1</sup> cm<sup>-1</sup>. The unit of APX activity was µmol min<sup>-1</sup>mg<sup>-1</sup>.

Hossain et al. [75] developed the method of determining the action of MDHAR (EC: 1.6.5.4) which was followed in this study. A solution buffer was arranged by mixing 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA and 0.5 units of AO (reaction initiator) where enzyme extract was added and the changes in absorbance was recorded at 340 nm for 60 sec. The extinction coefficient 6.2 mM<sup>-1</sup> cm<sup>-1</sup> was employed for calculation of MDHAR. The unit of MDHAR activity was nmol min<sup>-1</sup> mg<sup>-1</sup>.

Here the methodology of Nakano and Asada [74] was used to quantify action of DHAR (EC: 1.8.5.1). The reaction buffer was assayed to determine the activity DHAR where 50 mM K-P buffer (pH 7.0), 2.5 mM GSH, 0.1 mM EDTA, and 0.1 mM dehydroascorbic acid (DHA) was used. Finally, extracted sample solution was added to the mixture of distilled water, buffer solution and DHA and the changes in absorbance were recorded at 265 nm for 60 sec. The extinction coefficient  $14 \text{ mM}^{-1} \text{ cm}^{-1}$  was utilized for estimation of DHAR. The action of DHAR was expressed as  $\mu\text{mol min}^{-1} \text{ mg}^{-1}$ .

Hasanuzzaman et al. [69] developed the method of determining the action of GR (EC: 1.6.4.2) which was followed in this study. A reaction buffer was prepared by 0.1 M K-P buffer (pH 7.0), 1 mM EDTA, 1 mM oxidized GSG, and 0.2 mM NADPH where enzyme extract was added and the reduction in absorbance was recorded at 340 nm for 60 sec. The extinction coefficient  $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$  was utilized for estimation of GR activity where the unit of GR activity was  $\text{nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ .

Xanthine-xanthine oxidase system was utilized to estimate the activity of SOD (EC 1.15.1.1). Following the protocol described by El-Shabrawi et al. [76] was used to assay SOD. 50 mM K-P buffer (pH 7.0), 2.24 mM NBT, xanthine oxidase (0.1 U), xanthine (2.36 mM) and catalase (0.1 U) were mixed together for preparing reaction mixture for SOD. After that, enzyme extract was added to reaction mixture and the change in absorbance was recorded spectrophotometrically at 560 nm wavelength. The unit of SOD activity was  $\text{min}^{-1} \text{ mg}^{-1} \text{ protein}$ .

To determine action of CAT (EC: 1.11.1.6), protocol developed by Hasanuzzaman et al. [69] was used. Following the procedure a reaction mixer was developed utilizing 50 mM K-P buffer (pH 7.0) and  $\text{H}_2\text{O}_2$  (15 mM). After that extracted plant sample was added with reaction mixer and monitored the changes in absorbance at 240 nm for 60 sec. The extinction coefficient  $39.4 \text{ mM}^{-1} \text{ cm}^{-1}$  was employed for calculation. Unit of CAT activity was  $\mu\text{mol min}^{-1} \text{ mg}^{-1}$ .

The activity of GPX (EC: 1.11.1.9) was estimated according to the protocol described by Elia et al. [77] after minor change as explained by Hasanuzzaman et al. [69]. 100 mM K-P buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide ( $\text{NaN}_3$ ), 0.12 mM NADPH, 2 mM GSH, 1 U GR and 0.6 mM  $\text{H}_2\text{O}_2$  (as a substrate) were mixed together for preparing the reaction mixture. Then 20 mL extracted plant sample was mixed with assay buffer and oxidation of NADPH was recorded at 340 nm wavelength for 60 sec. Extinction coefficient  $6.62 \text{ mM}^{-1} \text{ cm}^{-1}$  was utilized to calculate GPX activity and the unit of activity was  $\text{nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ .

To measure Gly I (EC: 4.4.1.5) activity a reaction buffer was prepared following the method explained by Hasanuzzaman et al. [69]. Reaction buffer contained 100 mM K-P buffer (pH 7.0), 15 mM  $\text{MgSO}_4$ , 3.5 mM MG, 1.7 mM GSH and extracted sample. Finally the change in absorbance was recorded for 60 sec at 240 nm wavelength. Action of Gly I was estimated using extinction coefficient  $3.37 \text{ mM}^{-1} \text{ cm}^{-1}$ . The unit of Gly I activity was  $\mu\text{mol min}^{-1} \text{ mg}^{-1} \text{ protein}$ .

Protocol developed by Principato et al. [78] was used to determine action of Gly II (EC: 3.1.2.6). An assay mixture including 100 mM Tris-HCl buffer (pH 7.2), 0.2 mM DTNB, and 1 mM S-D-lactoylglutathione (SLG) was mixed with sample and the change of absorbance was read spectrophotometrically at 412 nm. Extinction coefficient  $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$  was employed to estimate action of Gly II which expressed as  $\mu\text{mol min}^{-1} \text{ mg}^{-1} \text{ protein}$ .

#### 4.12 Determination of methylglyoxal content

To determine MG level, protocol explained by Wild et al. [79] was used where leaves (0.25 g) were blended well in 2.5 mL 5% perchloric acid maintaining ice-cold condition. Then charcoal was used to decolorize the supernatant followed by neutralization with sodium carbonate. After that neutralize solution was assorted with sodium dihydrogen phosphate and N-acetyl-L-cysteine. Ten minutes later development of N- $\alpha$ -acetyl-S-(1-hydroxy-2-oxo-prop-1-yl) cysteine was documented by UV-visible spectrophotometer at 288 nm wavelengths. Level of MG was determined using standard curve generated from known concentration. The unit of MG content was  $\mu\text{mol g}^{-1} \text{ FW}$ .

#### 4.13 Statistical Analysis

Mean values of each parameter was calculated from three replications and they were subjected to analysis of variance (ANOVA) applying XLSTAT v. 2018 software [80]. Average from three replications (n = 3) was used to determine mean ( $\pm$ SD) for each treatment and mean differences was compared using Fisher's LSD test where variation at  $p \leq 0.05$  due to Fisher's LSD test was considered significant.

## 5. Conclusion

Our results suggest that salt stress damages rapeseed seedlings due to excessive accumulation of Na<sup>+</sup> in dose dependant manner. It creates severe oxidative stress through over generation of ROS and enhancing production of MDA and MG together with increased activity of LOX. Moreover it disrupted the antioxidant defense and glyoxalase system with the reduction of photosynthetic pigments and destroying water balance of seedlings. However rapeseed seedling pretreated with BABA showed tolerance against salt toxicity as BABA up-regulated most of the antioxidants to scavenge toxic ROS and MG. We hope that the results of the study will help to elucidate molecular and genetical basis of rapeseed seedlings under salt toxicity.

**Author Contributions:** J.A.M., M.H. and M.F. designed the experiment. J.A.M. performed the experiment. K.N. helped in the executing the experiment. M.H. and M.I.R.K. did the statistical analysis. J.A.M.; M.H. and M.I.R.K. have written the manuscript. M.H.; M.I.R.K. critically reviewed and edited the manuscript. M.F. supported the laboratory and other technical facilities. All the authors read and approved the final manuscript.

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**Conflicts of Interest:** The authors confirm no conflict of interest.

**Ethical statement:** The plant species and variety used in this experiment is a widely cultivated variety and we have followed all proper ethical standard. All of the reagent and fertilizers used are properly recommended by the authority.

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