

Article

Physiological and Biochemical Mechanisms of Methylglyoxal-Priming-Mediated Salt Tolerance in Barley Seedlings

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Abstract

Methylglyoxal (MG), a reactive carbonyl species, is now recognized as a novel signaling molecule regulating abiotic stress tolerance and plant growth. Using tolerant (BHL-25 and BHL-27) and susceptible (BARI Barley-6 and BHL-26) genotypes, the study aimed to reveal the detailed mechanisms of MG-priming-induced salt stress tolerance in barley (*Hordeum vulgare* L.). Seeds were primed with MG, and seven-day-old seedlings were transferred to hydroponic solution. After one week of seedling growth in hydroponic solutions, the salinity stress was imposed. The five treatments were as follows: control (0 salt + 0 mM MG), salt (16 dS m⁻¹) + 0 mM MG, salt + 0.25 mM MG, salt + 0.5 mM MG, and salt + 1 mM MG. Significant genotype-dependent changes were observed in response to salt stress based on morphological, physiological and biochemical traits. Salt stress significantly impaired shoot length, shoot dry weight, root volume, root dry weight, relative water content, leaf Na⁺ content and K⁺/Na⁺ ratio. Salinity stress caused a significant increase in oxidative indices in all genotypes; however, the tolerant genotypes showed a lower increase. Importantly, in plants grown from MG-primed seeds, the negative effects of salt stress were reversed by modulating the K⁺/Na⁺ ratio, the activities of antioxidative and glyoxalase pathway enzymes as well as the redox state of ascorbate and glutathione. In conclusion, MG-mediated salt tolerance was linked to a reduction in reactive oxygen species (ROS) and activation of ROS and MG detoxification processes as well as favorable regulation of the K⁺/Na⁺ ratio, glutathione and ascorbate redox state that improved the growth of the barley plants under salinity.

Keywords: antioxidant system; glyoxalase system; methylglyoxal; reactive oxygen species; salt tolerance



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1. Introduction

Soil salinity can cause substantial reductions in arable land area, crop yield, and the quality of harvested products [1]. Soil salinity is currently affecting over one billion hectares worldwide and is continuously increasing [2]. Global agricultural losses due to saline soil

could reach \$27 billion annually [3]. Although plants are susceptible to the negative effects of salinity at every stage of their lives, they are particularly at risk during the germination and seedling stages [4]. There are two main ways that salt stress impacts plants: (i) osmotic stress, which is caused by excess concentrations of soluble salts in the soil, that lower the root water uptake, leading to changes in cell viability, structure, and function [5]; and (ii) ion toxicity, which is particularly related to restricted K^+ uptake relative to Na^+ [6,7]. A decreased intake of mineral nutrients under salt stress, for instance, disrupts cellular metabolism, photosynthesis, and root architecture [4]. In plants, relative leaf water content, transpiration rate, osmotic and leaf water potential and leaf temperature are altered under salinity stress. Additionally, salt has an impact on photosynthetic elements such as carotenoids, chlorophylls, and enzymes [8]. Osmotic and ionic stress due to salinity eventually results in oxidative stress, which is the overabundance of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\bullet$), which can induce oxidative damage to cell organelles and membrane components and, at extreme levels, cause cell and plant death [9]. Under high salinity stress, the activities of the enzymes associated with the antioxidant defense system are also affected adversely [10].

Plants inevitably and spontaneously produce methylglyoxal (MG), a reactive short, oxygenated carbonyl species, when phosphate groups are removed from glycolysis intermediates such as dihydroxyacetone phosphate and glyceraldehyde 3-phosphate [11,12]. Under normal environmental conditions the cellular concentrations of MG are kept at very low levels [13]. However, the cellular concentrations of MG quickly rise to significantly higher levels in response to various abiotic stresses including salinity stress [14–17]. As a hazardous compound, stress-induced accumulation of MG affects various developmental and physiological processes, such as root growth, photosynthesis, and seed germination [15], and also causes deoxyribonucleic acid (DNA) strand breakage and point mutations, deactivates the antioxidant defense system, and accelerates protein breakdown [11,18].

In plants under salinity stress, MG buildup may indirectly lead to increased ROS generation by lowering available reduced glutathione (GSH) levels and compromising the activity of antioxidant enzymes [11,19,20]. While excessive MG accumulation in living cells is harmful, at low concentrations MG functions as a crucial signaling molecule that controls a variety of processes, including cell survival and proliferation, cellular homeostasis, redox status regulation, and numerous other facets of overall metabolism [11,21,22]. Plant stress responses can be influenced by MG through controlling stomatal opening and closure, reducing ROS generation, activating inward rectifying K^+ channels, and expressing many genes that respond to stress [11,15,18,23]. It is now clear that MG plays a part in signal transduction by sending and enhancing cellular signals and processes that help plants adapt to environmental stresses and MG is thought to be a possible biochemical marker for plant stress tolerance [15]. Seed priming with MG was also found to improve salinity stress tolerance [24].

Barley (*Hordeum vulgare* L.) is the fourth most important cereal crop in the world and is regarded as a good model for agronomy, plant physiology and abiotic stress research due to its rapid growth rate and environmental stress tolerance [25,26]. Although much is known about differences among barley genotypes in salinity tolerance, the possible mechanisms of MG-priming-mediated salt tolerance in barley are not reported. We hypothesized that the morpho-physiological and biochemical responses of resistant and sensitive genotypes would be different under salt stress, with or without MG priming. Therefore, the specific objectives of the study were: (i) to study the morphological and physiological responses (root and shoot weight, root dry weight and volume and relative water content) in primed and non-primed barley plants under salt stress; (ii) to examine the biochemical responses (K^+/Na^+ ratio, ROS content, lipid peroxidation, ascorbate and glutathione content and

their redox state, antioxidant enzymes activities) of primed and non-primed barley plants under saline stress; and (iii) to determine the effect of MG priming on endogenous MG levels and MG detoxifying glyoxalase enzymes activities under salinity. To the best of our knowledge, this is the first report on MG-priming-mediated salinity tolerance in barley genotypes having contrasting salinity tolerance.

2. Materials and Methods

2.1. Plant Materials, Growth Conditions

Two saline-susceptible (BARI Barley-6 and BHL-26) and two saline-tolerant (BHL-25 and BHL-27) barley genotypes obtained from the Plant Breeding Division (PBD), Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh were used in the study. Seeds of each genotype were disinfected in 70% ethanol by immersion for three minutes followed by washing. The sterilized seeds were then primed with MG (0, 0.25, 0.5 and 1 mM) [27] for three hours and rinsed thoroughly with double distilled water. After priming, the seeds were sown in coarse sand media for germination at the Green House of PBD, BARI, Gazipur. The uniform 7-day-old seedlings of each treatment were selected and grown in 5 L plastic pots in a hydroponic solution (Hoagland solution) in a 2.8 L pot [28], with continuous aeration using an air pump. A plastic sheet with 6 holes evenly distributed on each sheet (two plants in each hole) was used to cover the container and it was incubated in a greenhouse with sunlight and a temperature of 20 ± 2 °C/day and 15 ± 2 °C/night respectively with relative humidity (60–70%). The photoperiod used was 12 h and photosynthetic active radiation was set to $700 \mu\text{mol m}^{-2} \text{s}^{-1}$ (maximum radiation measured using steady state photometer, LICOR AM 300, Model 1600, Lincoln, NE, USA). The solution pH was maintained at 5.8 during the whole experimental period.

2.2. Imposition of Stress Treatments

The five treatments of the experiment were as follows: control (0 salt + 0 mM MG), salt (16 dS m^{-1}) + 0 mM MG, salt + 0.25 mM MG, salt + 0.5 mM MG, and salt + 1 mM MG. After one week of seedling growth in the hydroponic solution, the imposition of salinity stress was started. On the first day, 4 dS m^{-1} salinity was imposed using sea salt, and it was increased by 4 dS m^{-1} every week to reach the level of 16 dS m^{-1} . This level of salinity was maintained for the rest of the experimental period. The control plants were grown in hydroponic solution only. The salinity level was gradually increased to mimic the real conditions of salinity stress of the farmers' fields of Bangladesh. Similar gradual increase in salinity levels was also followed by Santangeli et al. [29] in rapeseed. A total of sixty hydroponic pots were maintained during the experiment, which was carried out using a completely randomized design (CRD) with three replications. Each pot contained six plants and was treated as an independent biological replicate. In order to make the growing conditions uniform and eliminate any position effect, the plants were randomly rearranged each week through the entire experimental period. Plants were randomly chosen for harvesting at panicle initiation and were either immediately used for analysis or frozen in liquid nitrogen and kept at -80 °C.

2.3. Estimation of Growth Parameters

Three randomly chosen plants from each treatment and replication were measured for shoot length (SL), root volume (RV), shoot fresh weight (SFW), root fresh weight (RFW), shoot dry weight (SDW), and root dry weight (RDW) at 52 days after sowing (DAS). The dry weight of the sample was taken after drying the sample at 75 °C for 48 h and expressed as g plant^{-1} . The Yamasaki and Dillenburg [30] method was used to determine the leaf lamina's relative water content (RWC).

2.4. Measurement of Sodium (Na^+) and Potassium (K^+) Content

To measure the Na^+ and K^+ content, the sap was obtained from freshly harvested leaves with the help of a tissue sap extractor (Horiba, Kyoto, Japan) using the methods described by Rohman et al. [31]. For determination of Na^+ and K^+ , compact Na^+ (Horiba-731, Japan) and K^+ (Horiba722, Japan) ion meters were used. The collected sap was directly put in the ion meter's probe after calibration of the instrument with the standard solution provided with the meter.

2.5. Ascorbate and Glutathione Assay

The expanded leaves from the top of the shoot were used for the estimation of glutathione (GSH) and ascorbate (AsA). Barley leaves (0.5 g) were mechanically homogenized with three ml of acidic extraction buffer (containing 5% meta phosphoric acid and 1 mM EDTA) on ice. The supernatant was isolated by centrifugation at 4 °C for 15 min at $11,500\times g$. The estimation of AsA level was done based on the method of Huang et al. [32], while that of the glutathione pool (oxidized glutathione, GSSG, and reduced glutathione, GSH) was based on the technique of Yu et al. [33].

2.6. Estimation of MDA and ROS

The amount of malondialdehyde (MDA) was determined to calculate lipid peroxidation levels [34]. Leaf samples (0.5 g) were homogenized with 3 mL of 5% (*w/v*) trichloroacetic acid (TCA) and then centrifuged at $11,500\times g$ for 10 min in 4 °C. Four mL of reagent (0.5% TBA in 20% TCA) was added to 1 mL of supernatant. The mixture was heated for 30 min at 95 °C in a water bath, then rapidly cooled in ice and then centrifuged at $11,500\times g$ for 15 min. The difference between readings of absorbances at 600 nm and 532 nm was measured. The amount of MDA was expressed as nmol of MDA g^{-1} FW and was computed using the extinction coefficient of $155\text{ mM}^{-1}\text{ cm}^{-1}$.

The Yu et al. [33] method was employed for the determination of hydrogen peroxide. In this regard, 3 mL of 50 mM potassium phosphate buffer (K-P) (pH 6.5) was used at 4 °C to homogenize 0.5 g of fresh leaf. Following this, the homogenate was centrifuged for 15 min at $11,500\times g$. Further, 1 mL of 0.1% TiCl_4 solution in 20% H_2SO_4 (*v/v*) was mixed with 3 mL of the supernatant and kept at room temperature for 10 min. Afterwards, this mixture was subjected to centrifugation again for 15 min at $11,500\times g$. The optical absorbance of the supernatant was measured spectrophotometrically at 410 nm to calculate the H_2O_2 content ($\epsilon = 0.28\text{ }\mu\text{M}^{-1}$) and expressed as nmol g^{-1} FW.

A slightly modified version of Elstner and Heupel's [35] approach was used to measure the superoxide radical ($\text{O}_2^{\bullet-}$). Following homogenization of 0.3 g of fresh leaf tissue with 3 mL of 65 mM K-P buffer (pH 6.5) at 4 °C, centrifugation of the samples at 4 °C for 10 min at $5000\times g$ took place. Upon mixing of the supernatant (0.75 mL) with 0.675 mL of 65 mM K-P buffer (pH 7.8) and 0.07 mM hydroxylamine chlorhydrate, incubation of the mixture was done at 25 °C. A total of 0.375 mL of 17 mM sulfanilamide and 0.375 mL of 7 mM α -naphthylamine were then added after 20 min. Incubation was carried out again for another 20 min at 25 °C followed by the addition of 2.25 mL of diethyl ether. The concentration of $\text{O}_2^{\bullet-}$ was determined from the standard NaNO_2 curve measured at 530 nm.

2.7. Determination of Antioxidant Enzyme Activities

The mortar and pestle were precooled before homogenizing freshly harvested leaf samples (0.5 g) in 1 mL of 50 mM ice-cold K-P buffer (pH 7.0), which had 100 mM KCl, 1 mM ascorbate, 5 mM β -mercaptoethanol, and 10% (*w/v*) glycerol. The supernatant from the centrifugation of the homogenate at $11,500\times g$ for 10 min was used to carry out the enzymatic reaction. All the activities were carried out at 0 to 4 °C.

The activity of superoxide dismutase (SOD, EC: 1.15.1.1) was determined based on the work of Beyer and Fridovich [36]. The basis for the assay was the xanthine–xanthine oxidase system. The components of the reaction mixture include 50 mM K-P buffer (pH 7.0), NBT (2.24 mM), catalase (0.1 unit), xanthine oxidase (0.1 unit), xanthine (2.36 mM), and enzyme extract. Catalase was included in the reaction mixture to inhibit the possible inactivation of CuZn-SOD by H_2O_2 . Units of SOD activity were defined as $\text{min}^{-1} \text{mg}^{-1}$ protein, meaning the quantity of enzyme required to inhibit NBT reduction by 50%.

The determination of POD (peroxidase, EC: 1.11.1.7) activity was performed following the protocol described by Hemeda and Klein [37]. All ingredients including enzyme extract, 0.05% guaiacol, 25 mM K-P buffer (pH 7.0), and 10 mM H_2O_2 were added to the reaction mixture. The measurement of activity was based on the change in absorbance at 470 nm due to the oxidation of guaiacol during one minute using an extinction coefficient of $26.6 \text{ mM}^{-1}\text{cm}^{-1}$.

Catalase (CAT, EC: 1.11.1.6) activity was measured through the method described by Iwase et al. [38]. In their method, the reduction in the absorption value at 240 nm during a period of one minute resulting from the degradation of H_2O_2 was measured. The reaction mixture contained 50 mM K-P buffer (pH 7.0), 15 mL of H_2O_2 , and enzyme solution in a total volume of 0.7 mL. The reaction mixture was activated by adding enzyme extract, and the activity was measured using the extinction coefficient of $39.4 \text{ mM}^{-1}\text{cm}^{-1}$.

In order to get the final volume of 0.7 mL for the ascorbate peroxidase (APX, EC: 1.11.1.11) activity test, 50 mM K-P buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2 , 0.1 mM EDTA and enzyme extract were included. H_2O_2 was used to start the reaction. The activity was measured by observing the decrease in the absorbance at 290 nm within a minute, using an extinction coefficient of $2.8 \text{ mM}^{-1}\text{cm}^{-1}$ according to Nakano and Asada [39].

Glutathione peroxidase (GPX, EC: 1.11.1.9) enzyme activity with H_2O_2 as a substrate was determined according to Elia et al. [40]. The composition of the reaction mixture was 100 mM sodium phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM NaN_3 , 0.12 mM NADPH, 2 mM GSH, 1 unit GR, 0.6 mM H_2O_2 , and 5 μL of sample solution. The reaction was started by the addition of H_2O_2 . The NADPH oxidation rate was measured in one minute at 340 nm wavelength. The activity was calculated using the extinction coefficient of $6.62 \text{ mM}^{-1}\text{cm}^{-1}$.

The Hossain and Fujita technique [41] was employed for the determination of activity of glutathione reductase (GR, EC: 1.6.4.2). The total volume of reaction medium was 1 mL with 0.1 M K-P buffer (pH 7.8), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH and enzyme solution. GSSG served as a substrate for initiating reaction, and during the first minute of the reaction process, there was a decrease in absorbance at 340 nm due to the oxidation of NADPH. The extinction coefficient $6.2 \text{ mM}^{-1}\text{cm}^{-1}$ was taken into account when calculating the activity.

The Hossain and Fujita procedure [41] was employed in the determination of MDHAR (monodehydroascorbate reductase) activity (EC: 1.6.5.4). The reaction mixture was made up of 0.7 mL in which 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, and 0.5 units of AO and enzyme were present. The addition of AO served to initiate the reaction. Activity was determined from the rate of decrease in absorbance at 340 nm within one minute using a molar extinction coefficient of $6.2 \text{ mM}^{-1}\text{cm}^{-1}$.

The activity of the enzyme, dehydroascorbate reductase (DHAR, EC: 1.8.5.1), was determined by using the technique described by Nakano and Asada [39]. The buffer solution contained 1 mM dehydroascorbate (DHA), 2.5 mM GSH, and 50 mM K-P buffer (pH 7.0). The reaction was started upon the addition of the sample solution to the buffer solution. Activity was determined based on the absorption difference at 265 nm after one minute with an extinction coefficient of $14 \text{ mM}^{-1}\text{cm}^{-1}$.

The enzymatic activity of Glutathione-S-Transferase (GST, EC: 2.5.1.18) was determined by using a spectrophotometric method as described by Rohman et al. [42]. The total

volume of the reaction mixture was 0.7 mL and included 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM 1-Chloro-2,4-Dinitrobenzene (CDNB), and enzyme solution. To initiate the enzymatic reaction, CDNB was added and the increase in absorbance was measured for one minute at 340 nm wavelength. The activity was calculated using the extinction coefficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

2.8. Methylglyoxal Content and Glyoxalase Assay

With slight modifications, methylglyoxal was measured using the Yadav et al. [43] technique. In 3 mL of 0.5 M perchloric acid, fresh barley leaves (0.3 g) were crushed up. After being incubated on ice for 15 min, the homogenate was centrifuged at 4°C for 10 min at $11,500 \times g$. To render the supernatant colorless, 10 g mL^{-1} of charcoal was added. It was centrifuged at 4°C at $11,500 \times g$ for 10 min after being kept at room temperature for 15 min. The supernatant was centrifuged once more for ten minutes at $11,500 \times g$ after being neutralized by a saturated carbonate solution at room temperature. Using N-acetyl-L-cysteine, the supernatant was utilized for MG quantification in accordance with Rohman et al.'s technique [44].

The Glyoxalase I (Gly I, EC: 4.4.1.5) test was carried out based on the guidelines by Yadav et al. [43]. The following is a brief summary of the test mixture. The test mixture consisted of enzyme solution in a total volume of 0.7 mL, 100 mM K-P buffer (pH 7.0), 15 mM magnesium sulphate, 1.7 mM GSH, and 3.5 mM MG. MG was introduced into the test solution to initiate the reaction and the increase in absorbance at 240 nm was measured within a minute. The extinction coefficient of $3.37 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to calculate the activity.

The glyoxalase II (Gly II, EC: 3.1.2.6) enzymatic activity was determined according to the method of Principato et al. [45] based on the generation of GSH at 412 nm for one minute. The reaction medium included 100 mM Tris-HCl buffer (pH 7.2), 0.2 mM 5,5-Dithiobis 2-nitrobenzoic acid (DTNB), 1 mM S-D-lactoylglutathione (SLG) and enzyme solution in a total volume of 1 mL. SLG was used to start the reaction. Extinction coefficient was $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

2.9. Statistical Analysis

The data generated from the experiment was subjected to two-way analysis of variance (cultivar by solution treatment) using STATISTIX-10 software. The data of the three replications were reported as mean $\pm$ SE. The mean values of the treatments were compared using the LSD at 5% level of probability.

3. Results

3.1. MG Induces NaCl Tolerance in Barley

Significant reductions in SL, SDW, RV and RDW due to salt stress were observed in all the genotypes, but the reduction was greater in the sensitive genotypes (BARI Barley-6 and BHL-26) than in the tolerant ones (Table 1). Importantly, the plants developed from MG-primed seeds showed an increase in SL, SDW, RV and RDW under salinity stress relative to the plants treated with salt stress only without MG priming. Under salt stress, tolerant genotypes showed significantly higher recovery as compared to those of the sensitive genotypes when primed with 1 mM MG. The SL and RDW of tolerant genotypes were statistically similar to the control level when primed with 1 mM MG while values of SDW and RV were significantly higher than those with salinity alone. A significant reduction in RWC was noticed in response to salinity stress in all the studied genotypes; however, the primed plants reversed the negative impact of salinity through a significant increase in RWC.

Table 1. Effect of MG priming on root and shoot traits of barley genotypes grown under control and salinity stress conditions.

Genotype	Treatment	SL (cm)	SDW (g)	RV (cc)	RDW (g)	RWC (%)	Leaf Na ⁺ (ppm)	Leaf K ⁺ /Na ⁺ Ratio
BARI Barley-6	Control	31.7 ± 1.3 ^d	3.07 ± 0.17 ^{cd}	3.94 ± 0.17 ^g	0.276 ± 0.009 ^{d-f}	95.6 ± 1.7 ^{ab}	42 ± 4.2 ^{hi}	2.67 ± 0.17 ^c
	Salt + 0 mM MG	20.3 ± 1.6 ^j	1.59 ± 0.16 ^k	2.34 ± 0.20 ^{lm}	0.161 ± 0.012 ^{kl}	78.1 ± 3.0 ^j	286 ± 13 ^a	0.21 ± 0.06 ⁱ
	Salt + 0.25 mM MG	21.4 ± 2.1 ^j	1.77 ± 0.18 ⁱ	2.41 ± 0.17 ^{kl}	0.168 ± 0.010 ^{i-k}	81.7 ± 5.2 ^{hi}	253 ± 10 ^a	0.27 ± 0.05 ^{hi}
	Salt + 0.5 mM MG	24.9 ± 1.4 ^{hi}	2.03 ± 0.15 ^{hi}	2.66 ± 0.15 ^{ij}	0.182 ± 0.009 ^{ij}	82.9 ± 4.4 ^{hi}	198 ± 14 ^b	0.40 ± 0.08 ^{g-i}
	Salt + 1 mM MG	25.1 ± 1.5 ^{hi}	2.15 ± 0.12 ^h	2.71 ± 0.25 ⁱ	0.184 ± 0.007 ⁱ	83.7 ± 3.7 ^{g-i}	173 ± 10 ^{bc}	0.51 ± 0.10 ^{gh}
BHL-26	Control	35.0 ± 2.0 ^{bc}	3.15 ± 0.15 ^c	3.53 ± 0.20 ^h	0.237 ± 0.013 ^h	97.5 ± 4.2 ^{ab}	48 ± 11 ^{hi}	2.50 ± 0.13 ^c
	Salt + 0 mM MG	23.9 ± 1.3 ⁱ	1.91 ± 0.18 ^{ij}	2.23 ± 0.16 ^m	0.149 ± 0.010 ^c	90.0 ± 5.2 ^{ij}	255 ± 15 ^a	0.20 ± 0.04 ⁱ
	Salt + 0.25 mM MG	25.0 ± 1.7 ^{hi}	2.00 ± 0.14 ⁱ	2.48 ± 0.21 ^{kl}	0.165 ± 0.010 ^{j-l}	91.9 ± 4.4 ^{hi}	246 ± 9.5 ^a	0.34 ± 0.05 ^{hi}
	Salt + 0.5 mM MG	26.8 ± 1.4 ^{gh}	2.17 ± 0.19 ^{gh}	2.54 ± 0.19 ^{jk}	0.161 ± 0.008 ^{kl}	93.1 ± 3.5 ^{g-i}	204 ± 11 ^b	0.38 ± 0.04 ^{g-i}
	Salt + 1 mM MG	28.8 ± 0.9 ^{e-g}	2.29 ± 0.15 ^g	2.65 ± 0.23 ^{ij}	0.178 ± 0.012 ^{ij}	93.7 ± 3.2 ^{gh}	141 ± 13 ^{cd}	0.65 ± 0.07 ^{gh}
BHL-25	Control	30.1 ± 1.6 ^{de}	3.42 ± 0.12 ^b	4.58 ± 0.25 ^c	0.299 ± 0.010 ^{bc}	96.0 ± 5.1 ^a	27 ± 4.1 ⁱ	4.74 ± 0.22 ^a
	Salt + 0 mM MG	27.1 ± 1.9 ^g	2.79 ± 0.20 ^f	3.81 ± 0.22 ^g	0.249 ± 0.007 ^{gh}	80.6 ± 2.7 ^{d-f}	132 ± 6.3 ^{de}	0.66 ± 0.09 ^{fg}
	Salt + 0.25 mM MG	28.0 ± 1.3 ^{fg}	2.88 ± 0.16 ^{ef}	4.10 ± 0.19 ^f	0.267 ± 0.008 ^{e-g}	82.9 ± 3.3 ^{c-e}	100 ± 5.4 ^{ef}	0.91 ± 0.07 ^f
	Salt + 0.5 mM MG	28.7 ± 1.5 ^{e-g}	3.04 ± 0.16 ^{c-e}	4.20 ± 0.23 ^{ef}	0.275 ± 0.008 ^{ef}	84.0 ± 4.0 ^{bd}	65 ± 4.7 ^{gh}	1.58 ± 0.11 ^d
	Salt + 1 mM MG	29.2 ± 1.4 ^{ef}	3.11 ± 0.17 ^c	4.35 ± 0.14 ^{de}	0.284 ± 0.010 ^{c-e}	84.9 ± 2.8 ^{bc}	39 ± 10 ^{hi}	2.92 ± 0.15 ^c
BHL-27	Control	38.5 ± 1.6 ^a	3.72 ± 0.21 ^a	5.16 ± 0.15 ^a	0.318 ± 0.011 ^a	96.2 ± 3.6 ^{ab}	31 ± 6.2 ^{hi}	4.03 ± 0.23 ^b
	Salt + 0 mM MG	32.0 ± 1.8 ^d	2.93 ± 0.15 ^{d-f}	4.25 ± 0.24 ^{d-f}	0.262 ± 0.009 ^{fg}	86.7 ± 4.2 ^{fg}	146 ± 7.9 ^{cd}	0.64 ± 0.07 ^{fg}
	Salt + 0.25 mM MG	34.1 ± 1.2 ^c	3.00 ± 0.13 ^{c-e}	4.36 ± 0.18 ^d	0.270 ± 0.010 ^{ef}	89.1 ± 3.0 ^{ef}	122 ± 15 ^{d-f}	0.91 ± 0.11 ^f
	Salt + 0.5 mM MG	36.0 ± 2.1 ^{bc}	3.33 ± 0.15 ^b	4.66 ± 0.21 ^{bc}	0.290 ± 0.012 ^{b-d}	90.6 ± 5.0 ^{c-e}	88 ± 9.5 ^{fg}	1.22 ± 0.10 ^e
	Salt + 1 mM MG	36.8 ± 1.2 ^{ab}	3.45 ± 0.16 ^b	4.78 ± 0.17 ^b	0.306 ± 0.009 ^{ab}	91.2 ± 4.2 ^{c-e}	49 ± 8.3 ^{hi}	2.39 ± 0.14 ^c

Values are expressed as mean ± SE of three replicates ($n = 3$). Different letters in the same column are significant at a 5% level of significance following the LSD test. Here, SL = shoot length, SDW = shoot dry weight, RV = root volume, RDW = root dry weight and RWC = relative water content.

Significant variations in Na⁺ content and K⁺/Na⁺ ratios in leaf tissues were recorded in the genotypes in response to salinity stress. Under control conditions, Na⁺ contents were similar both in sensitive (BARI Barley-6 and BHL-26) and tolerant (BHL-25 and BHL-27) genotypes (Table 1). The plants grown under salinity stress exhibited a significant increase (5.81-, 4.31-, 2.57- and 2.65-fold increase in BARI Barley-6, BHL-26, BHL-25 and BHL-27, respectively) in Na⁺ contents as compared to the control. The plants developed from MG-primed seeds showed a reduction in Na⁺ accumulation under salt stress and the effect was more prominent in tolerant genotypes than those of the sensitive genotypes. Surprisingly, seed priming with 1 mM MG was able to lower the levels of Na⁺ content close to the control level in tolerant genotypes whereas in sensitive genotypes it showed a sharp increase (4.12- and 2.94-fold in BARI Barley-6 and BHL-26, respectively) as compared to the control. Like Na⁺, significant variation was also recorded in the K⁺/Na⁺ ratio in the studied genotypes under control and salt stress conditions (Table 1). A sharp decrease in the K⁺/Na⁺ ratio was recorded in the plants grown under salt stress without MG priming. Importantly, salt-stressed plants grown from various concentrations of MG priming showed a significant increase in K⁺/Na⁺ ratio as compared to the plants grown under salt stress without MG priming; however, greater effects were recorded in the tolerant genotypes (Table 1). Under salt stress, the plants grown from 1 mM MG priming showed the highest K⁺/Na⁺ ratio in the tolerant genotype BHL-25 (2.92), whereas the lowest K⁺/Na⁺ ratio was observed in the sensitive genotype BARI Barley-6 (0.51) (Table 1).

3.2. Exogenous MG Decreases Lipid Peroxidation and ROS Level Under Salinity Stress

Salinity stress increased the O₂^{•−}, H₂O₂ and MDA content significantly (238%, 226%, 94%, and 101% increase in O₂^{•−} in BARI Barley-6, BHL-26, BHL-25 and BHL-27, respectively; 240%, 258%, 116% and 131% increase in H₂O₂ in BARI Barley-6, BHL-26, BHL-25 and BHL-27, respectively; 95%, 106%, 44% and 48% increase in MDA in BARI Barley-6, BHL-26, BHL-25 and BHL-27, respectively under 16 dS m^{−1}) as compared to respective control in all the genotypes, but a significantly lesser increase was recorded in the tolerant genotypes (Figure 1a–c). Plants grown from MG-primed seeds showed significantly lower ROS and MDA contents than those in the plants grown under salt stress without MG

priming. Moreover, the increments of $O_2^{\bullet-}$, H_2O_2 and MDA due to salinity stress were lower in tolerant genotypes than those of the sensitive genotypes. Among the MG-priming levels, the higher level of MG (1 mM) priming provided the best results in reducing the $O_2^{\bullet-}$ (77%, 80%, 79%, and 73% decrease in BARI Barley-6, BHL-26, BHL-25 and BHL-27, respectively), H_2O_2 (65%, 64%, 101%, and 111% decrease in BARI Barley-6, BHL-26, BHL-25 and BHL-27, respectively) and MDA (23%, 22%, 33% and 37% decrease in BARI Barley-6, BHL-26, BHL-25 and BHL-27, respectively) as compared to the plants treated with salt stress without MG priming under salt stress.

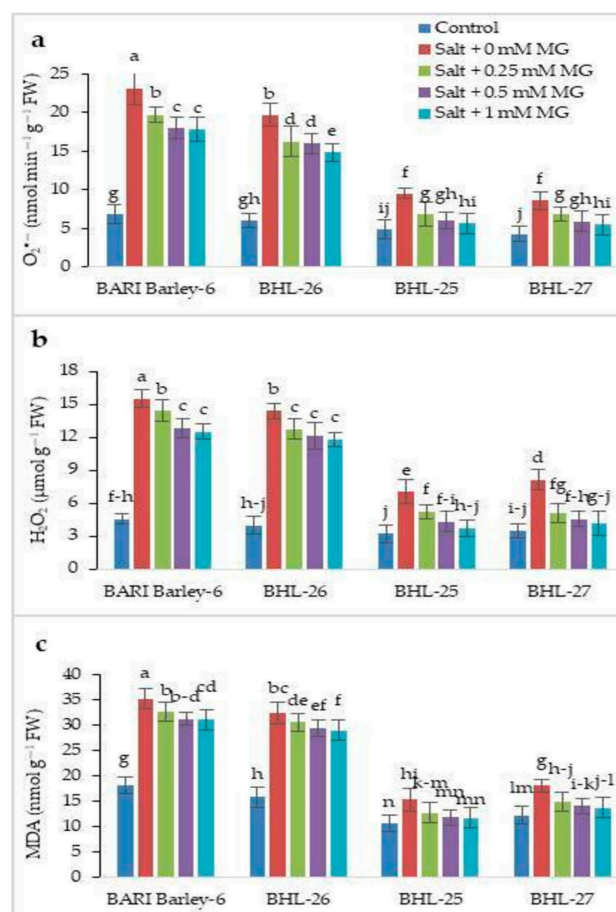


Figure 1. The effect of seed priming with methylglyoxal (MG) on (a) superoxide radical ($O_2^{\bullet-}$), (b) hydrogen peroxide (H_2O_2) and (c) malondialdehyde (MDA) contents of different barley genotypes at the panicle initiation stage under 16 dS m^{-1} salinity stress. The values represent the mean $\pm$ SE ($n = 3$) and different letters on the bars are significantly different at $p \leq 0.05$ following the LSD test.

3.3. Exogenous MG Enhances Ascorbate and Glutathione Content Under Salinity Stress

The AsA content and its redox state decreased significantly in all of the studied genotypes as compared to the control whereas the DHA content increased significantly (Table 2). However, the plants grown after MG priming showed a significant increase in ascorbate content and its redox state as compared to the plants treated with salt stress only. Similarly, GSH content and its redox state showed a decrease in response to salt stress in the studied genotypes whereas the GSSG content increased significantly as compared to the control. The plants developed from either 0.5 and/or 1 mM MG priming showed a significant increase in GSH content and its redox state under salt stress as compared to the control. Both of the tolerant genotypes showed a constitutively higher amount of AsA and GSH as compared to the salt-susceptible genotypes (Table 2).

Table 2. Effect of seed priming with MG on AsA and glutathione pool of barley genotypes under salinity and control condition at panicle initiation stage.

Genotype	Treatment	AsA (n mol g ⁻¹ FW)	DHA (n mol g ⁻¹ FW)	AsA/ (AsA + DHA)	GSH (nmol g ⁻¹ FW)	GSSG (n mol g ⁻¹ FW)	GSH/ (GSH + GSSG)
BARI Barley-6	Control	180 ± 6 ^{de}	183 ± 6 ^g	0.47 ± 0.02 ^{ef}	1192 ± 39 ^{fg}	401 ± 19 ^{ij}	0.727 ± 0.029 ^e
	Salt + 0 mM MG	100 ± 8 ^c	296 ± 10 ^a	0.25 ± 0.02 ⁱ	487 ± 54 ^m	642 ± 30 ^{ab}	0.478 ± 0.023 ^l
	Salt + 0.25 mM MG	120 ± 8 ^h	283 ± 8 ^{ab}	0.30 ± 0.02 ^{g-i}	636 ± 67 ^l	628 ± 17 ^{bc}	0.540 ± 0.042 ^k
	Salt + 0.5 mM MG	124 ± 5 ^{gh}	279 ± 9 ^b	0.31 ± 0.017 ^{g-i}	725 ± 49 ^{jk}	602 ± 19 ^{de}	0.630 ± 0.039 ⁱ
	Salt + 1 mM MG	132 ± 7 ^g	272 ± 9 ^{bc}	0.33 ± 0.017 ^{gh}	752 ± 61 ^j	584 ± 21 ^e	0.656 ± 0.024 ^h
BHL-26	Control	176 ± 6 ^{d-f}	172 ± 7 ^{gh}	0.51 ± 0.021 ^{c-e}	1258 ± 70 ^f	386 ± 32 ^{jk}	0.776 ± 0.034 ^c
	Salt + 0 mM MG	94 ± 7 ⁱ	282 ± 8 ^{ab}	0.25 ± 0.018 ⁱ	662 ± 60 ^{kl}	661 ± 24 ^a	0.586 ± 0.032 ^j
	Salt + 0.25 mM MG	101 ± 8 ⁱ	264 ± 11 ^{cd}	0.28 ± 0.016 ^{hi}	729 ± 74 ^{jk}	637 ± 24 ^{bc}	0.627 ± 0.027 ⁱ
	Salt + 0.5 mM MG	120 ± 7 ^h	250 ± 7 ^{de}	0.32 ± 0.019 ^{gh}	875 ± 67 ⁱ	618 ± 17 ^{cd}	0.705 ± 0.028 ^f
	Salt + 1 mM MG	122 ± 6 ^{gh}	244 ± 8 ^e	0.33 ± 0.020 ^g	916 ± 56 ^{hi}	592 ± 20 ^e	0.737 ± 0.035 ^{de}
BHL-25	Control	204 ± 8 ^a	141 ± 8 ^k	0.59 ± 0.018 ^a	1882 ± 63 ^a	334 ± 14 ^{no}	0.830 ± 0.029 ^a
	Salt + 0 mM MG	175 ± 6 ^{d-f}	203 ± 8 ^f	0.46 ± 0.017 ^{ef}	1134 ± 45 ^g	468 ± 19 ^f	0.632 ± 0.036 ⁱ
	Salt + 0.25 mM MG	184 ± 9 ^{c-e}	178 ± 7 ^g	0.51 ± 0.021 ^{c-e}	1448 ± 59 ^{de}	434 ± 32 ^g	0.69 ± 0.022 ^f
	Salt + 0.5 mM MG	193 ± 5 ^{a-c}	163 ± 10 ^{hi}	0.54 ± 0.02 ^{a-c}	1653 ± 65 ^c	366 ± 15 ^{lm}	0.728 ± 0.022 ^e
	Salt + 1 mM MG	195 ± 7 ^{a-c}	152 ± 9 ^{i-k}	0.56 ± 0.015 ^{ab}	1735 ± 48 ^b	352 ± 23 ^{mn}	0.746 ± 0.025 ^d
BHL-27	Control	196 ± 6 ^{ab}	147 ± 9 ^{jk}	0.57 ± 0.018 ^{ab}	1646 ± 54 ^c	322 ± 18 ^o	0.836 ± 0.023 ^a
	Salt + 0 mM MG	167 ± 7 ^f	215 ± 7 ^f	0.44 ± 0.016 ^f	995 ± 32 ^h	429 ± 21 ^{gh}	0.676 ± 0.036 ^g
	Salt + 0.25 mM MG	173 ± 6 ^{ef}	182 ± 8 ^g	0.49 ± 0.024 ^{d-f}	1139 ± 43 ^g	410 ± 28 ^{hi}	0.735 ± 0.033 ^{de}
	Salt + 0.5 mM MG	181 ± 7 ^{de}	173 ± 9 ^{gh}	0.51 ± 0.023 ^{c-e}	1409 ± 57 ^e	377 ± 31 ^{kl}	0.789 ± 0.030 ^c
	Salt + 1 mM MG	185 ± 6 ^{b-d}	157 ± 9 ^{ij}	0.54 ± 0.019 ^{b-d}	1527 ± 63 ^d	351 ± 26 ^{mn}	0.813 ± 0.042 ^b

Values are expressed as mean ± SE of three replicates ($n = 3$). Different letters in the same column are significant at 5% level of significance following LSD test. Here, AsA = ascorbic acid, DHA = dehydroascorbate, GSH = glutathione, GSSG = oxidized glutathione.

3.4. Exogenous MG Increases the Activity of Antioxidant Enzymes Under Salinity Stress

The SOD activity showed a significant increase in all the genotypes studied (42, 35, 22 and 37% increase in BARI Barley-6, BHL-26, BHL-25 and BHL-27, respectively) in response to salt stress (Figure 2a). Plants developed from 0.5 or 1 mM MG priming also showed further increase in SOD activity in all the studied genotypes under salt stress; however, the highest increase (67%) was recorded in BARI Barley-6 when primed with 0.5 mM MG. A non-significant increase in POD activity was noted under salt stress in all the studied genotypes (Figure 2b). The plants developed from 0.25 to 1 mM MG priming also failed to increase POD activity significantly under salt stress in susceptible genotypes; however, the activity was increased in the tolerant genotypes when primed with various concentrations (0.25, 0.5 and 1 mM) of MG. Under salt stress, CAT activity showed a significant increase in the tolerant genotypes (13.8 and 15.6% in BHL-25 and BHL-27, respectively, as compared to the control); whereas the susceptible genotypes showed a non-significant change (Figure 2c). CAT activity in the MG-primed plants also showed a non-significant change in the susceptible genotypes whereas the activity increased significantly in the tolerant genotypes when primed with either 0.5 or 1 mM MG. The plants developed from 1 mM MG priming showed the highest increase (36%) in CAT activity in the tolerant genotype BHL-25 as compared to the control. GST activity showed a non-significant change in response to salt stress in the susceptible genotype whereas it showed significant increase in the tolerant genotypes (Figure 2d). The plants developed from MG-primed seeds also showed a significant increase both in tolerant and susceptible genotypes; however, a sharp increase was noted in the tolerant genotypes when the seeds were primed with 1 mM MG. There was no significant variation in GPX activity in both the tolerant and susceptible genotypes due to salt stress (Figure 2e). The plants developed from 1 mM MG priming showed a significant increase in GPX activity in both the tolerant genotypes (33 and 19% in BHL-25 and BHL-27, respectively) as compared to the plants subjected to salt stress without priming.

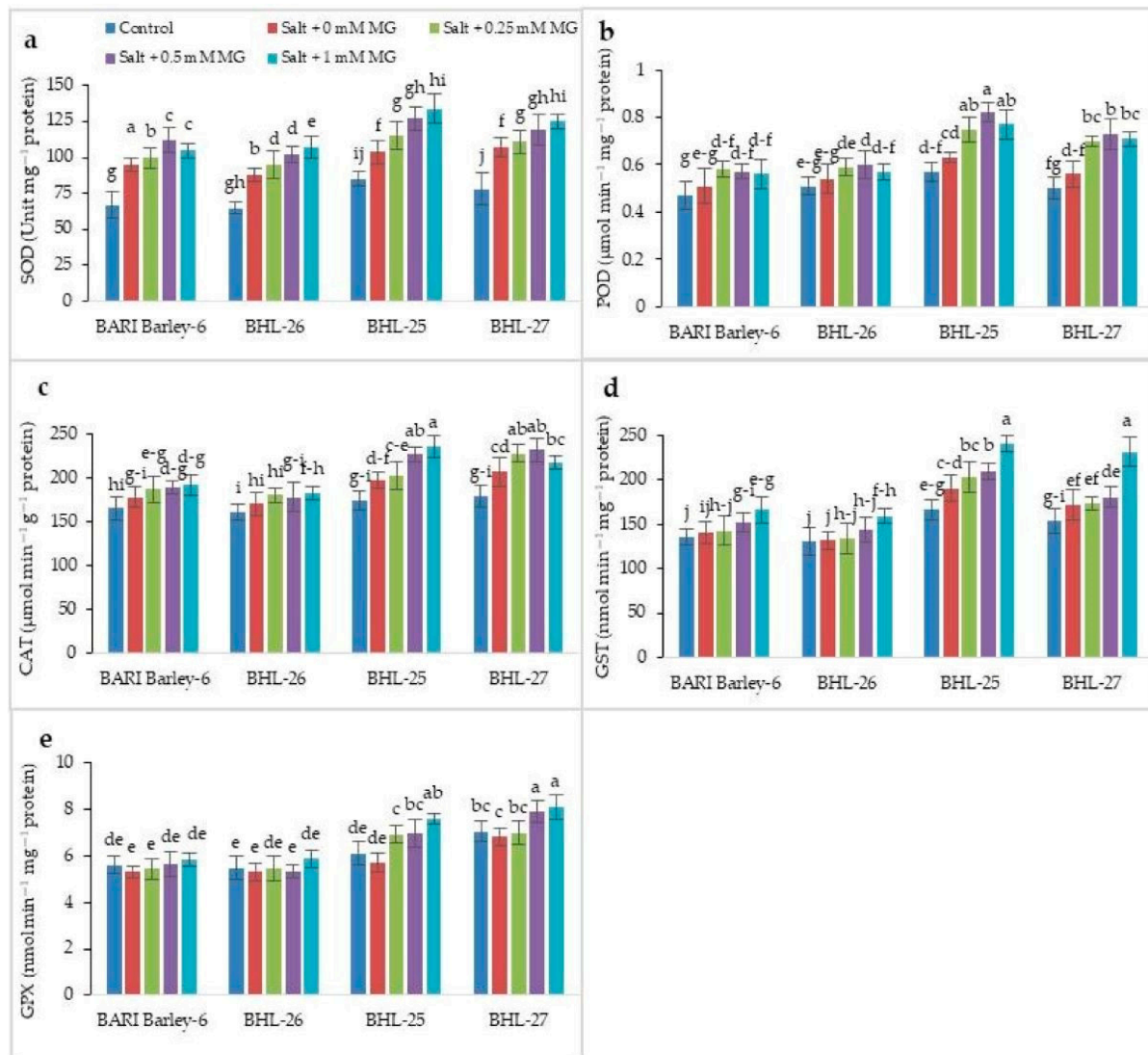


Figure 2. The effect of seed priming with methylglyoxal (MG) on (a) superoxide dismutase (SOD), (b) peroxidase (POD), (c) catalase (CAT), (d) glutathione S-transferases (GST) and (e) glutathione peroxidase (GPX) of different barley genotypes at the panicle initiation stage under 16 dS m⁻¹ salinity stress. The values represent the mean ± SE ($n = 3$) and different letters on the bars are significantly different at $p \leq 0.05$ following the LSD test.

Among the AsA-GSH pathway enzymes, APX activity showed a non-significant change in the salt-susceptible genotypes whereas it showed a significant increase in the tolerant genotypes under salt stress. Importantly, the activity of this enzyme also showed meaningful increase under salt stress in the tolerant plants primed with 0.5 or 1 mM MG (Figure 3a). In contrast, a significant decrease in MDHAR and DHAR activities were recorded in all the genotypes studied as compared to the control under salt stress. Importantly, MDHAR activity showed a significant increase in the susceptible genotypes under salt stress when primed with 1 mM MG whereas it showed significant increase in the tolerant genotypes when primed with both 0.5 and/or 1 mM MG (Figure 3b,c). GR activity showed a non-significant change in the tolerant genotypes whereas it showed a non-significant increase in the tolerant genotypes under salt stress. Importantly, a sharp increase in GR activity of this enzyme was recorded in the tolerant plants when primed with 0.5 and/or 1 mM MG (Figure 3d).

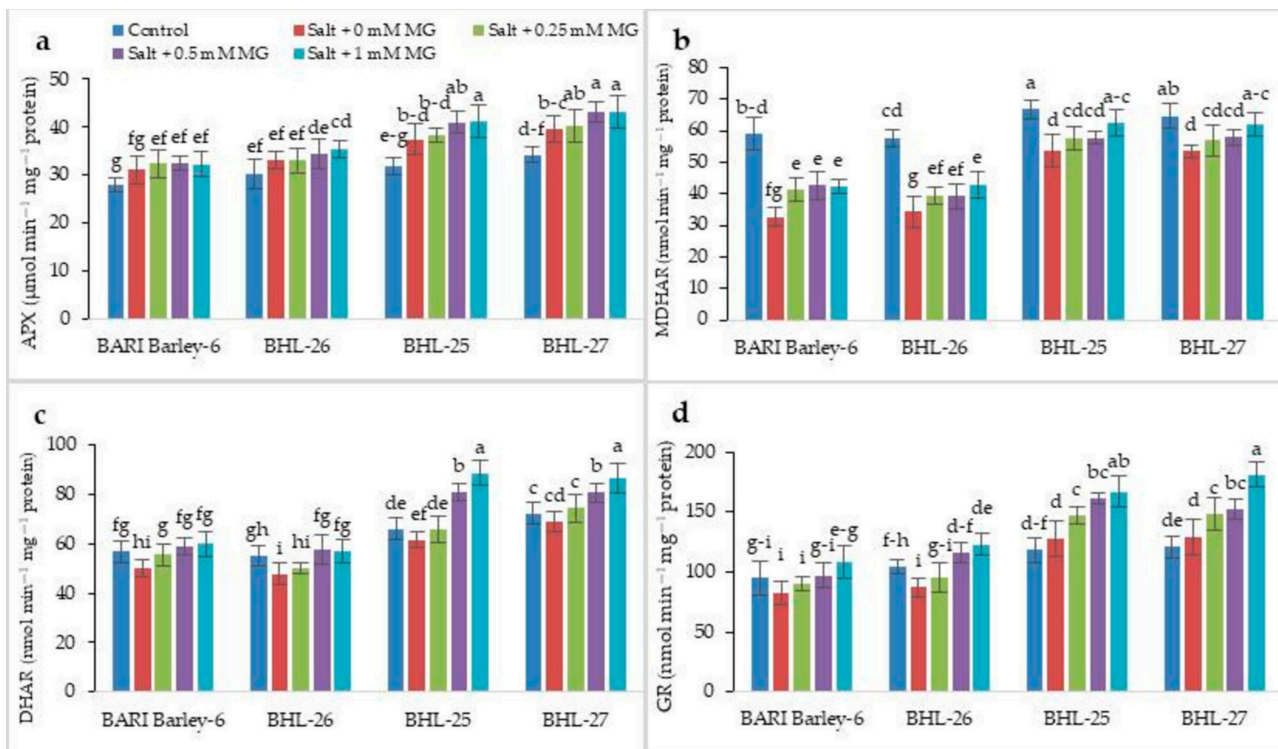


Figure 3. The effect of seed priming with methylglyoxal (MG) on (a) ascorbate peroxidase (APX), (b) monodehydroascorbate reductase (MDHAR), (c) dehydroascorbate reductase (DHAR) and (d) glutathione reductase (GR) of different barley genotypes at the panicle initiation stage under 16 dS m^{-1} salinity stress. The values represent the mean $\pm$ SE ($n = 3$) and different letters on the bars are significantly different at $p \leq 0.05$ following the LSD test.

3.5. Exogenous MG Increased MG Content and Increased Activities of Gly I and Gly II Activity Under Salinity Stress

Under both control and stress conditions, endogenous MG content was higher in sensitive genotypes than that in the tolerant ones, but the content increased enormously under salt stress conditions particularly in the sensitive genotypes (Figure 4a). Sixteen dS m^{-1} salinity stress increased MG by 126% and 112% in susceptible genotypes (BARI Barley-6 and BHL-26, respectively) whereas in the tolerant genotypes (BHL-25 and BHL-27) the increase was 57% and 78%, respectively. The plants grown from MG-primed seeds showed a significant decrease in MG content relative to those in the plants grown under salt stress without MG priming. Surprisingly, seed priming with 1 mM MG was able to bring down the endogenous MG content to the control level in tolerant genotypes under salt stress. In contrast to MG levels, a significant decrease in Gly I (34%, 30%, 10%, and 15% in BARI Barley-6, BHL-26, BHL-25 and BHL-27, respectively) and Gly II (20%, 21%, 8%, and 14% in BARI Barley-6, BHL-26, BHL-25 and BHL-27, respectively) activity was recorded in response to salinity stress (Figure 4b,c). Plants grown from MG priming were found to increase the Gly I and Gly II activities under salt stress in all the studied genotypes; however, a higher increase was found in the tolerant genotypes. The plants grown from seed priming with 1 mM MG showed the highest increase in glyoxalase activities as compared to the other concentrations of MG priming.

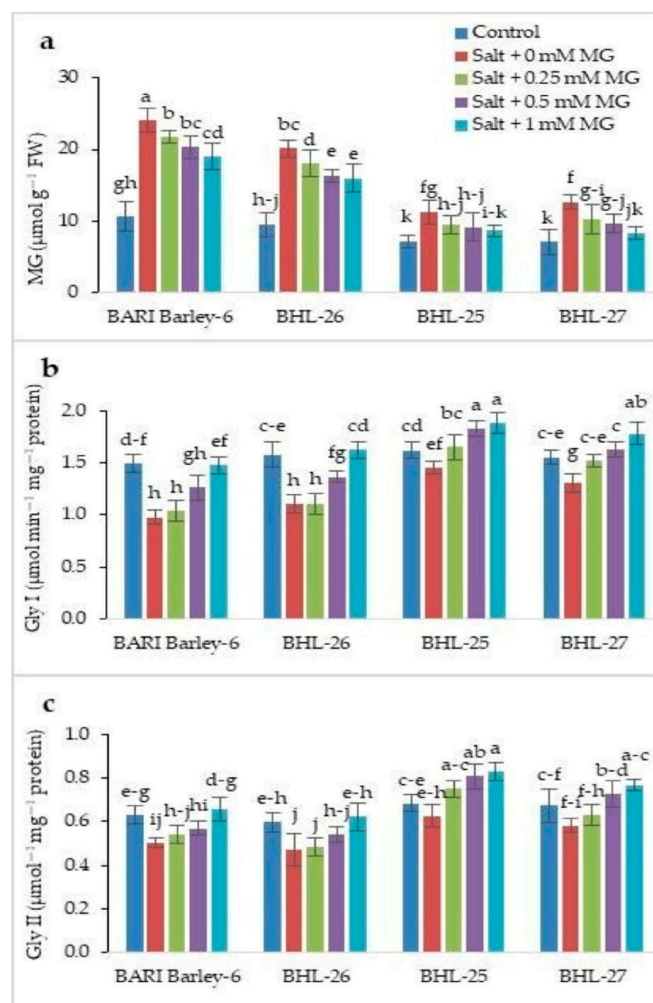


Figure 4. The effect of seed priming with methylglyoxal (MG) on (a) MG levels, (b) glyoxalase I (Gly I) and (c) glyoxalase II (Gly II) activities of different barley genotypes under 16 dS m⁻¹ salinity stress. The values represent the mean $\pm$ SE ($n = 3$) and different letters on the bars are significantly different at $p \leq 0.05$ following the LSD test.

4. Discussion

The physiological process via which a plant's biochemical defenses are triggered in response to impending abiotic stress is known as priming [46]. Priming is linked to a greater buildup of dormant cellular signaling molecules, which are crucial for stress resistance signal amplification [47]. Aside from activating signaling proteins and transcription factors for quick and effective stress tolerance, priming can also increase the effectiveness of the DNA repair system [48,49]. Since MG functions as a signaling molecule at low concentrations to improve plant performance in the face of abiotic stresses [11,15,24], here we tested different MG concentrations for seed priming in order to reveal the in-depth physiological and biochemical mechanisms of MG-mediated salinity stress tolerance in barley plants. From the results of the current study, it is evident that significant reductions in root and shoot weight, dry weight and relative water content were observed in all the genotypes studied under salt stress. High concentrations of Na⁺ and Cl⁻ are built up within leaf cells in a saline environment due to the continuous uptake of these elements through the root system and their delivery to the shoot via the transpiration stream. This nutrient imbalance eventually inhibits leaf biochemical processes, especially photosynthesis, which suppresses the dry weight production [50]. In addition, at low salinity, the plant may not exhibit any change in water intake, whereas high levels of salt stress cause significant damage

to the plant, particularly its shoot, with a decreased RWC and a dehydrated state at the cellular level [51]. Importantly, seed priming with MG induces the salt tolerance of barley by improving various morphological traits like, SL, SDW, RV, and RDW (Table 1). The protective role of MG (1 mM) under salinity stress (150 mM) in improving wheat (*Triticum aestivum* L. cv. Yunmai 41) seedling growth and development was also reported [24].

The ionic toxicity resulting from the excess accumulation of Na^+ ions under salt stress is the main effect which also triggers secondary stresses including disturbance of nutrient homeostasis by lowering nutrient uptake [52]. The ability to control the accumulation of Na^+ ions under salt stress is critically important for salt tolerance [53]. This experiment demonstrated that salt stress increased Na^+ accumulation and K^+ exclusion from leaf tissues leading to a low K^+/Na^+ ratio, indicating ionic toxicity and disruption of nutrient homeostasis. However, plants grown from seed priming with MG had a lower accumulation of Na^+ and a higher K^+/Na^+ ratio, resulting in better ion balance and hence improved salt tolerance. Similar results were obtained by Sadiq et al. [54] who also observed higher K^+/Na^+ is correlated with salt tolerance in barley.

MDA is regarded as an indicator of oxidative stress since its buildup suggests elevated lipid peroxidation and the generation of ROS in response to environmental stressors [55]. Salt stress leads to lipid peroxidation (higher MDA) that can disrupt the cell membrane, leading to higher solute leakage and decreased photosynthetic function (lower chlorophyll, net photosynthetic rate and stomatal conductance) [56]. Higher levels of MDA may subsequently lead to cellular dysfunction and apoptosis. Our study revealed that salinity stress increased MDA content in all genotypes, but tolerant genotypes showed a lower accumulation of MDA (Figure 1c). H_2O_2 and $\text{O}_2^{\bullet-}$ accumulation also showed similar patterns in response to salinity stress. Importantly, the plants developed from MG-primed seeds maintained significantly lower levels of ROS and MDA. The results were aligned with the findings of Li et al. [27]. The lower levels of ROS and MDA in the MG-treated plants can be attributed to a more efficient ROS and MG detoxification system, as discussed below.

Barley plants grown from MG seed-priming were able to activate enzymatic and non-enzymatic defenses against salinity stress, leading to decreases in ROS and MDA levels; however, a greater effect was measured when the seeds were primed with 1 mM MG (Table 2; Figures 1–3). Importantly, salt-tolerant barley cultivars appeared to have inherently greater defense against salinity, and plants grown from MG seed-priming were able to almost fully reverse the negative effects of salinity on ROS, enzymatic and non-enzymatic defenses along with the glyoxalase system (Figures 2–4). Consequently, the growth of the tolerant barley cultivars was comparable to those of the control plants. Similar to our results, Li et al. [24] also observed that the addition of low concentrations (≤ 3 mM) of MG in hydroponic solution significantly boosted the seedling growth of wheat, as evidenced by plant height and root length under 15 dS m^{-1} NaCl stress.

The antioxidants AsA and GSH are now treated as indicators of stress tolerance due to their scavenging role against ROS and maintaining cellular redox potential under abiotic stress [19]. In comparison to the untreated control, salt stress reduced the AsA content, raised the DHA level, and resulted in a reduction in AsA/GSH ratio in barley plants (Table 2). Under salt stress, AsA is transformed into DHA during the ROS-scavenging process, which lowers the AsA/DHA ratio. This is a typical occurrence under salt stress. The MG-primed barley plants showed an increase in AsA level and AsA redox under salinity stress as compared to the plants treated with salinity stress only, whereas an inverse response was observed in DHA content. Favorable modulation of the ascorbate (AsA) redox state—characterized by maintaining a high ratio of reduced ascorbate (AsA) to oxidized dehydroascorbate (DHA)—is a critical mechanism for salt stress tolerance in crop plants [57]. Similar to AsA, GSH is essential for balancing redox state and protect-

ing cells from oxidative damage [44]. Higher GSH and lower GSSG levels sustain the GSH/GSSG ratio, which is essential for ROS detoxification and redox signaling under stress conditions [58]. In addition to regulating several metabolic processes, GSH also protects membranes by keeping zeaxanthin and α -tocopherol in their reduced states, protects proteins' thiol groups from oxidative denaturation under stress, and acts as a substrate for both GPX and GST [58]. Plants with a higher GSH pool are better able to withstand a variety of abiotic challenges [59]. In addition to scavenging H_2O_2 via the AsA-GSH cycle, GSH can also directly interact with other ROS [60]. In addition to ROS detoxification, MG detoxification is linked to GSH (which will be described in more detail later). In this present study, GSH content was found to decrease in all the genotypes under salinity stress resulting in the alteration of GSH redox state $[(GSH)/(GSH + GSSG)]$; however, more acute decreases in GSH and its redox state were recorded in the susceptible genotypes (Table 2). Both GSH and GSH redox state increased markedly in response to seed priming with MG as compared to salinity stress only. Induced GSSG by salinity stress was reduced to some extent by seed priming with MG. Increased GSH helped to lower the hazardous level of MG through the modulation of the glyoxalase system [61,62]. Increased GSH level and GSH redox state due to seed-priming with MG, associated with salinity stress tolerance, was confirmed by Li et al. [24] by using the MG scavenger NAC in wheat.

SOD, POD, CAT, GPX, GST, APX, GR, DHAR, MDHAR, and GR are the major components of the plant antioxidant defense system that play crucial roles in a tissue defense system against the toxic effects of ROS (mainly $O_2^{\bullet-}$, H_2O_2) generated during various environmental stresses [19,63]. SOD catalyzes the initial step of the detoxification process by converting the highly reactive $O_2^{\bullet-}$ into oxygen (O_2) and H_2O_2 . Then CAT efficiently converts H_2O_2 into H_2O and O_2 without the need for an additional electron donor. POD, a non-specific enzyme, often found in the extracellular space and other compartments, scavenges H_2O_2 by using various co-substrates as electron donors. GPX catalyzes the reduction of H_2O_2 and organic hydroperoxides (like lipid peroxides) to water and their corresponding alcohols, using GSH as a substrate. With the presence of GSH, GSTs are involved in the detoxification of a broad range of harmful xenobiotic compounds and safeguard the cell from oxidative stress. The activities of SOD, POD, CAT, GPX, and GST increased under salt stress in barley plants, and further they showed a concentration-dependent increment in those plants that developed from the MG-priming of seeds, an indicator of efficient ROS detoxification. In addition, the activities of the enzymes of the AsA-GSH pathway (i.e., APX, MDHAR, DHAR, and GR) are the major parts of the AsA-GSH cycle. APX is the central enzyme in this cycle that uses AsA as a specific electron donor to reduce H_2O_2 to water [64]. This reaction produces monodehydroascorbate (MDHA). MDHAR recycles MDHA back to AsA, using NAD(P)H as an electron donor, thus maintaining the pool of reduced ascorbate [60]. If MDHA is spontaneously disproportionate to dehydroascorbate (DHA), DHAR reduces DHA back to AsA, using GSH as an electron donor, which in turn produces GSSG [65]. GR is crucial for maintaining a high ratio of GSH/GSSG; it catalyzes the NADPH-dependent reduction of GSSG back to GSH, regenerating the glutathione pool [66]. In the present study, under salt stress, a decrease in APX, GR, DHAR and MDHAR activities were noticed in all the studied genotypes as compared to the control. In contrast, the plants developed from MG-primed seeds modulated the activities of these enzymes, although priming with 1 mM MG was most effective. Li et al. [24] noticed the enhancement of salinity tolerance in wheat due to the exogenous application of MG by enhancing antioxidant enzymes like APX, GPX, SOD, CAT and GR to ensure better protection of ROS and ROS-induced damage conferring better growth and development. Salinity tolerance is a polygenetic characteristic that is controlled by several genes and stress-responsive pathways. Crucially, integrated genetic processes that control ion transport, water balance,

redox regulation, and tissue specialization allow tolerant plants to survive growth and reproduction in deadly conditions while maintaining cellular homeostasis under high salinity [67]. In the present study, one striking observation is that the tolerant genotypes have higher AsA and GSH content and their redox state whereas the sensitive genotypes have lower levels of antioxidants and their redox state. This difference might be due to the inherent genetic differences between tolerant and susceptible genotypes. Like our results, El-Shabrawi et al. [68] also showed that salt-tolerant rice species have higher antioxidant contents, antioxidant enzyme activities under saline stress that ensure lower MG levels, and lower ROS and ROS-induced damage as compared to the sensitive genotypes. This phenomenon also indicates that upregulation of both the antioxidant defense system and glyoxalase defense system is essential for better survival and growth of barley plants under salinity stress. In accordance with our results, the enhanced level of antioxidant enzymes due to the application of exogenous priming agents also showed better scavenging of overproduced ROS and augmenting salinity tolerance in barley [69,70], wheat [71], and rice [72].

MG detoxification is accomplished through the glyoxalase pathway which is the only method known in living organisms like bacteria, yeast, plants, and animals. This is done using two enzymes, Gly I and Gly II, which are responsible for two reaction steps respectively [73]. MG is changed into S-D lactoylglutathione (SLG) by Gly I, while SLG is changed into D-lactate by Gly II [14]. Recently, the removal of MG has also been associated with an enzyme called D-lactate dehydrogenase (D-LDH). This enzyme helps convert D lactate, which is produced during the end phase of the glyoxalase pathway, to D-pyruvate, which forms ATP within the TCA cycle [74]. GSH contributes to this system as a co-factor. So, in addition to detoxifying MG, the glyoxalase system also helps to keep GSH homeostasis, which leads to ROS detoxification [75,76]. The prominent role of the glyoxalase system has been well documented in plants in modulating abiotic stress tolerance through the regulation of MG and ROS detoxification systems as well as stress-responsive gene expression. Transgenic plants over-expressing glyoxalase pathway genes were found to improve abiotic and biotic stress tolerance through the regulation of MG levels as well as favorable modulation of the AsA and GSH redox states [76]. In the current investigation, salt stress dramatically reduced Gly I and Gly II activities as well as MG levels as compared to the control plants. In contrast, the plants grown from MG seed-priming significantly increase Gly I and Gly II activities and decrease endogenous MG levels under salt stress, but a greater effect was evident in the tolerant cultivars primed with 1 mM MG (Figure 4a–c). In plant cells, at low concentration, MG functions as a stress-signaling molecule. Protective defense genes are expressed when MG is administered exogenously to plant cells. The glyoxalase system is overstimulated by this defense activity, which removes internal MG and eventually lowers the endogenous MG levels in the plant cell [15,21]. Like our study, Li et al. [24] also found that the seed-priming of plants with MG can lower the endogenous MG levels by enhancing the activity of Gly I and Gly II in NaCl-stressed wheat plants. In this study, barley genotypes showed better adaption to high salinity when the seeds were primed with 1 mM MG for a short time. Although 1 mM concentration is higher, however it might be a non-toxic level to modulate glyoxalase system as well as antioxidant defense system to maintain optimum levels of ROS and MG to confer their signaling function. Importantly, priming with a low concentration of MG (0.25 or 0.50 mM) failed to activate the signaling system to orchestrate resistance reactions in both tolerant and susceptible genotypes under high salinity stress.

The schematic diagram (Figure 5) depicts how salinity negatively impacts the growth and development of barley seedlings and how the MG-priming of seeds reversed the negative effects of salinity stress.

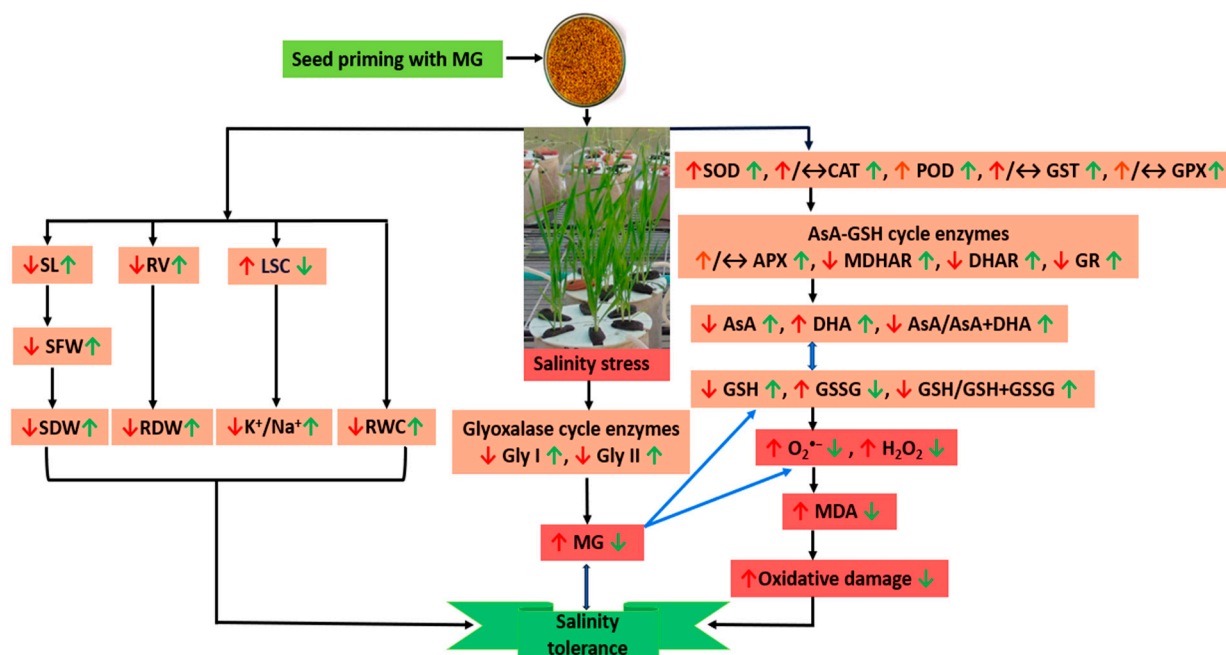


Figure 5. Diagram illustrates how salt inhibits barley seedlings' growth and how priming seeds with MG can restore it. Here, SL = shoot length; SFW = shoot fresh weight; SDW = shoot dry weight; RV = root volume; RDW = root dry weight; LSC = leaf sodium content; K^+/Na^+ = potassium–sodium ratio; RWC = relative water content; ROS = reactive oxygen species; $O_2^{\bullet-}$ = superoxide; H_2O_2 = hydrogen peroxide; MG = methylglyoxal; MDA = malondialdehyde; AsA = ascorbate; DHA = dehydroascorbate; GSH = reduced glutathione; GSSG = oxidized glutathione; SOD = superoxide dismutase; CAT = catalase; POD = peroxidase; GST = glutathione S-transferase; GPX = glutathione peroxidase; APX = ascorbate peroxidase, MDHAR = monodehydroascorbate reductase; DHAR = dehydroascorbate reductase; GR = glutathione reductase; Gly I = glyoxalase I; Gly II = glyoxalase II. ↓ and ↑, indicates the decreasing and increasing effect of salinity on morpho-physiological and biochemical traits of barley seedling without MG priming, whereas ↑ and ↓ indicates the increasing and decreasing effects of salinity after MG priming, respectively. ↔ indicates unchanged enzyme activity under salinity stress without MG priming.

5. Conclusions

Barley plants exposed to salinity stress under hydroponic conditions had significant inhibition in growth and RWC due to higher accumulation of Na^+ , especially in salt-sensitive cultivars. Additionally, salinity stress increased oxidative damage by interfering with the antioxidant defense system by causing an excess of ROS and lipid peroxidation. Salinity stress also affected the glyoxalase system, which led to increased MG levels. However, salt-tolerant cultivars primed with 1 mM MG under salt stress enabled recovery from growth inhibition while salt-sensitive cultivars experienced partial recovery. The MG priming conferred salinity stress tolerance by reducing lipid peroxidation as well as ROS and MG production through the activation of antioxidant defense and glyoxalase systems as well as favorable modulation of the K^+/Na^+ ratio and AsA and GSH redox state (summarized in Figure 5). Further studies need to be conducted at the proteomic and transcriptomic levels to reveal more insights into the possible mechanisms of MG-priming-induced salinity tolerance in plants.

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