Hydrogen Peroxide Pretreatment Mitigates Cadmium-Induced Oxidative Stress in *Brassica napus* L.: An Intrinsic Study on Antioxidant Defense and Glyoxalase Systems

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Cadmium (Cd) is considered as one of the most toxic metals for plant growth and development. In the present study, we investigated the role of externally applied hydrogen peroxide (H_2O_2) in regulating the antioxidant defense and glyoxalase systems in conferring Cd-induced oxidative stress tolerance in rapeseed (*Brassica napus* L.). Seedlings were pretreated with 50 μM H_2O_2 for 24 h. These pretreated seedlings as well as non-pretreated seedlings were grown for another 48 h at two concentrations of $CdCl_2$ (0.5 and 1.0 mM). Both the levels of Cd increased MDA and H_2O_2 levels and lipoxygenase activity while ascorbate (AsA) declined significantly. However, reduced glutathione (GSH) content showed an increase at 0.5 mM $CdCl_2$, but glutathione disulfide (GSSG) increased at any level of Cd with a decrease in GSH/GSSG ratio. The activities of ascorbate peroxidase (APX) and glutathione S-transferase (GST) upregulated due to Cd treatment in dose-dependent manners, while glutathione reductase (GR) and glutathione peroxidase (GPX) increased only at 0.5 mM $CdCl_2$ and decreased at higher dose. The activity of monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), catalase (CAT), glyoxalase I (Gly I), and glyoxalase II (Gly II) decreased under Cd stress. On the other hand, H_2O_2 pretreated seedlings, when exposed to Cd, AsA and GSH contents and GSH/GSSG ratio increased noticeably. H_2O_2 pretreatment increased the activities of APX, MDHAR, DHAR, GR, GST, GPX, and CAT of Cd affected seedlings. Thus enhancement of both the non-enzymatic and enzymatic antioxidants helped to decrease the oxidative damage as indicated by decreased levels of H_2O_2 and MDA. The seedlings which were pretreated with H_2O_2 also showed enhanced glyoxalase system. The activities of Gly I, and Gly II and the content of GSH increased significantly due to H_2O_2 pretreatment in Cd affected seedlings, compared to the Cd-stressed plants without H_2O_2 pretreatment which were

vital for methylglyoxal detoxification. So, the major roles of H_2O_2 were improvement of antioxidant defense system and glyoxalase system which protected plants from the damage effects of ROS and MG. The mechanism of H_2O_2 to induce antioxidant defense and glyoxalase system and improving physiology under stress condition is not known clearly which should be elucidated. The signaling roles of H_2O_2 and its interaction with other signaling molecules, phytohormones or other biomolecules and their roles in stress protection should be explored.

INTRODUCTION

Metal toxicity has been increasing considerably due to increase of toxic metal release as a result of urbanization and industrialization (Hasanuzzaman and Fujita, 2012). Cadmium (Cd) is considered as most toxic considering injurious effects on plant developmental processes and metabolism (Nouairi et al., 2009). Cd has nature to enter through roots readily and easily due to its high solubility in water. Cd content higher than 5–10 µg Cd g^{-1} leaf dry weight is considered toxic for plants, in general (White and Brown, 2010). Cd stress reduces growth and metabolism affecting plants' basic physiological processes including water and nutrient translocation and assimilation, transpiration and photosynthesis (Hasanuzzaman and Fujita, 2012; Khan et al., 2015). At cellular level Cd provokes generation of ROS [may include superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and so forth] which results in oxidative damages to lipids, proteins and fatty acid which disrupt biomembrane, ultrastructural cellular components, DNA, and causes programmed cell death (PCD; Gill and Tuteja, 2010; Nahar et al., 2015, 2016a). Plants' antioxidant system scavenges ROS and keeps a state of balance under non-stress condition. Antioxidant machinery posses non-enzymatic antioxidants [AsA, GSH, flavonoids, phenolic compounds, carotenoids, alkaloids, Pro, non-protein amino acids, and α-tocopherols] and a bunch of antioxidant enzymes [CAT, APX, MDHAR, DHAR, GR, GPX, guaiacol peroxidase, and GST] which works in coordinated manner to scavenge ROS and to minimize oxidative stress (Apel and Hirt, 2004; Hasanuzzaman et al., 2012b). MG generation is an impulsive outcome of the glycolysis. Due to environmental stresses MG is overproduced many times higher than the normal growth condition to create toxic effects (Kaur et al., 2015).

Abbreviations: AO, ascorbate oxidase; APX, ascorbate peroxidase; AsA, ascorbic acid (ascorbate); BSA, bovine serum albumin; CAT, catalase; CDNB, 1-chloro-2, 4-dinitrobenzene; Chl, chlorophyll; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Gly I, glyoxalase I; Gly II, glyoxalase II; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; HSD, honest significant difference; LOX, lipoxygenase; MDA, malondialdehyde; MDHA, monodehydroascorbate; MDHAR, MDHA reductase; MG, methylglyoxal; NADPH, nicotinamide adenine dinucleotide phosphate; NTB, 2-nitro-5-thiobenzoic acid; PEG, polyethylene glycol; Pro, proline; ROS, reactive oxygen species; RWC, relative water content; SLG, S-D-lactoylglutathione; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

In glyoxalase system, utilizing GSH MG is transformed in to SLG by the activity of Gly I, while Gly II transforms SLG to D-lactic acid which is a MG detoxification process. At the end, GSH is regenerated. Tolerance against ROS and MG confers and improves abiotic stress adaptation and tolerance in different plants (Yadav et al., 2008; Hasanuzzaman et al., 2012a; Kaur et al., 2015).

Among the ROS, H_2O_2 has stability, being a versatile molecule shows signaling function (Quan et al., 2008; Saxena et al., 2016). It takes part of oxidative metabolism. It has been proved to involve in signaling cascades and metabolism which are vital for plants growth/developmental processes. Seed germination, initiation of root hair, strengthening of cell wall, cell wall loosening, xylem differentiation and stomatal movement were reported to link with H_2O_2 mediated signaling cascade (Dempsey and Klessig, 1995; Wojtyla et al., 2016). Interacting with other hormones and signaling molecules [abscisic acid (ABA) and ethylene], H_2O_2 regulates plant metabolism (Jubany-Mari et al., 2009; Chen et al., 2012). Recently it has been reported that nitric oxide (NO) and H_2O_2 regulate the salicylic acid (SA)-induced salvianolic acid B production (Guo et al., 2014). Thus, as a signaling molecule H_2O_2 regulates different metabolic pathways to develop stress tolerances (Mittler et al., 2004; Reczek and Chandel, 2015). H_2O_2-induced signal stimulates the expression and activation of stress tolerant genes (Prasad et al., 1994) which mediate stress acclimation and adaptation (Uchida et al., 2002). In different research findings, H_2O_2 mediated chilling (Prasad et al., 1994), salinity (Xu et al., 2008; Li et al., 2011), heat (Gao et al., 2010), osmotic stress (Liu et al., 2010), Cd (Hu et al., 2009), low light (Zhang et al., 2011), and multiple stress (Gong et al., 2001) tolerances were reported. Based on the results of previous studies we hypothesize that application of exogenous H_2O_2 might have a signaling function, influence antioxidant activities which can improve Cd stress tolerance. Very few research works demonstrated the beneficial roles of H_2O_2 on Cd or heavy metal stress (Chao et al., 2009; Hu et al., 2009). In the previous study, only few components of antioxidant defense system have been examined to show the effect of H_2O_2 under Cd stress (Chao et al., 2009; Hu et al., 2009). Moreover, effects of H_2O_2 on MG detoxification system under Cd stress were not reported. Many aspects of H_2O_2-induced Cd stress tolerance are yet to be elucidated. The present study provides a new insight into H_2O_2-induced coordinated effects on antioxidant defense and glyoxalase system to enhance the resistance to

Cd toxicity in rapeseed seedlings. In this study, we will present several components of antioxidant defense and MG detoxification systems which were not mentioned in previous research findings.

MATERIALS AND METHODS

Plant Material, Growth Condition, and Treatments

Healthy and uniform sized rapeseed (*Brassica napus* cv. BINA sharisha 3) seeds were dipped into 70% ethanol for 5 min, then washed with double distilled water (ddH_2O). Seeds had been sown in Petri plates (9 cm) containing six layers of filter paper where filter papers were provided with 10 ml of ddH_2O. The Petridishes containing seeds were kept in a dark germination chamber under controlled conditions, 72 h. Germinated seedlings were removed from the germinator and placed into growth chamber under control environment (providing with light 100 μmol photon m^{-2} s^{-1}, temp 25 ± 2°C, RH 65–70%). Seedlings were supplied with 10,000-fold diluted Hyponex solution (Hyponex, Japan) as nutrient at regular interval. Eleven-day-old seedlings were pretreated with 50 μM H_2O_2 in their root for 24 h. Both H_2O_2-pretreated and non-pretreated seedlings were then exposed to Cd stress (0.5 and 1.0 mM $CdCl_2$) for 48 h. Several trial experiments were conducted before selecting the present doses of treatments. Different doses of Cd were applied in combination with different doses of H_2O_2 and the present combination (0.5 and 1.0 mM $CdCl_2$ with 50 μM H_2O_2; 48 h) showed the better result. We hypothesized that using two concentrations of Cd the trend how the H_2O_2 is affecting the Cd-stressed rapeseed seedlings could be understood better. The same experiment was repeated three times under the same treatment condition. There were 45 seedlings in each Petri dish. In total $6 \times 3 = 18$ dishes were used.

Measurement of Lipid Peroxidation

Lipid peroxidation had been determined by estimating MDA (a product of lipid peroxidation) using TBA (Heath and Packer, 1968; Hasanuzzaman et al., 2011).

Measurement of Hydrogen Peroxide Content

Hydrogen peroxide (H_2O_2) had been determined extracting leaves in potassium phosphate (K-P) buffer (pH 6.5; centrifuging at $11,500 \times g$), then adding it to a mixture of $TiCl_4$ in 20% H_2SO_4 (v/v). The supernatant was read spectrophotometrically at 410 nm (Yu et al., 2003).

Histochemical Detection of Hydrogen Peroxide and Superoxide

The H_2O_2 and $O_2^{\bullet-}$ were determined histochemically (Chen et al., 2010) in the leaves of rapeseed plants by staining leaves with 1% 3,3-diaminobenzidine (DAB; to get brown spots due to the reaction of DAB with H_2O_2) and 0.1% nitroblue tetrazolium chloride (NBT; to get deep blue spots appeared due to the

reaction of NBT with $O_2^{\bullet-}$) solution, respectively. Then, leaves were blanched in boiling ethanol to visualize the spots.

Extraction and Measurement of Ascorbate and Glutathione

The leaves of rapeseed plant (0.5 g) had been homogenized in 5% meta-phosphoric acid containing 1 mM EDTA (centrifuged at $11,500 \times g$; 15 min at 4°C). Supernatant was collected for the assay of AsA and GSH pool. To determine total ascorbate, the oxidized fraction was reduced by adding 0.1 M dithiothreitol for 1 h at room temperature and then read at 265 nm using 1.0 unit AO. Oxidized ascorbate (DHA) content had been assayed by subtracting reduced AsA from total AsA (Hasanuzzaman et al., 2011; Nahar et al., 2016b). The glutathione pool had been determined according to previously described methods (Yu et al., 2003; Hasanuzzaman et al., 2011). Standard curves with known concentrations of GSH and GSSG had been used to calculate the unknown GSH and GSSG pool of plant sample. The content of reduced GSH had been calculated by subtracting GSSG from total GSH.

Protein Determination

Following the method of Bradford (1976) the protein content had been measured where we used BSA as a protein standard.

Enzyme Extraction and Assays

Leaves had been homogenized with 50 mM K-P buffer (pH 7.0) containing 100 mM KCl, 1 mM AsA, 5 mM β-mercaptoethanol, and 10% (w/v) glycerol in pre-chilled mortars. Homogenates were centrifuged at $11,500 \times g$. The supernatants were collected and used for the assay of enzyme activity.

Ascorbate peroxidase (EC: 1.11.1.11) activity: The reaction buffer solution contained 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 (final volume 700 μL). The reaction had been initiated adding H_2O_2. Absorbance had been monitored at 290 nm for 1 min and activity has been calculated using an extinction coefficient of 2.8 mM^{-1}cm^{-1} (Nakano and Asada, 1981).

Monodehydroascorbate reductase (EC: 1.6.5.4) activity: The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, 0.5 unit of AO, and enzyme solution (final volume 700 μL). The reaction had been started by adding AO. Absorbance was taken at 340 nm; activity had been calculated from the change in absorbance for 1 min using an extinction coefficient of 6.2 mM^{-1}cm^{-1} (Hossain et al., 1984).

Dehydroascorbate reductase (EC: 1.8.5.1) activity: The reaction buffer contained 50 mM K-P buffer (pH 7.0), 2.5 mM GSH, and 0.1 mM DHA. Activity had been calculated from the change in absorbance at 265 nm for 1 min using an extinction coefficient of 14 mM^{-1}cm^{-1} (Nakano and Asada, 1981).

Glutathione reductase (EC: 1.6.4.2) activity: The reaction mixture contained 0.1 M K-P buffer (pH 7.0), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, and enzyme solution (final volume 1 mL). The reaction had been started with GSSG; the decrease in absorbance at 340 nm was monitored for 1 min and activity had

been calculated using an extinction coefficient of 6.2 mM^{-1}cm^{-1} (Hasanuzzaman et al., 2011).

Glutathione *S*-transferase (EC: 2.5.1.18) activity: The reaction mixture had 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 1 mM CDNB, and enzyme solution (final volume 700 µL). The reaction had been started by CDNB; the raise of absorbance was monitored at 340 nm for 1 min. Activity had been calculated using an extinction coefficient of 9.6 mM^{-1}cm^{-1} (Hossain et al., 2006).

Glutathione peroxidase (EC: 1.11.1.9) activity: The reaction mixture contained of 100 mM K-P buffer (pH 7.0), 1 mM EDTA, 1 mM NaN$_3$, 0.12 mM NADPH, 2 mM GSH, 1 unit GR, 0.6 mM H$_2$O$_2$ (as a substrate), and 20 µL of sample solution. The oxidation of NADPH had been observed at 340 nm for 1 min and the activity was calculated using an extinction coefficient of 6.62 mM^{-1}cm^{-1} (Hasanuzzaman et al., 2011).

Catalase (EC: 1.11.1.6) activity: Decrease of absorbance (by decomposition of H$_2$O$_2$) at 240 nm had been noticed for 1 min. The reaction had been started with enzyme extract; activity has been calculated using an extinction coefficient of 39.4 M^{-1}cm^{-1} (Hasanuzzaman et al., 2011).

Glyoxalase I (EC: 4.4.1.5): The assay mixture contained 100 mM K-P buffer (pH 7.0), 15 mM magnesium sulfate, 1.7 mM GSH, and 3.5 mM MG (final volume 700 µL). Adding MG the reaction had been started; the increase in absorbance was recorded at 240 nm for 1 min. Activity had been calculated using an extinction coefficient of 3.37 mM^{-1}cm^{-1} (Hasanuzzaman et al., 2011).

Glyoxalase II (EC: 3.1.2.6): Formation of GSH was monitored for 1 min at 412 nm. The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.2), 0.2 mM DTNB, and 1 mM SLG (final volume of 1 mL). Reaction had been initiated by adding SLG; activity had been calculated using an extinction coefficient of 13.6 mM^{-1}cm^{-1} (Principato et al., 1987).

Lipoxygenase (EC 1.13.11.12): LOX activity was estimated monitoring the increase of absorbance at 234 nm using linoleic acid as a substrate. Activity had been calculated using an extinction coefficient of 25 mM^{-1}cm^{-1} and expressed as units (1 nmol of substrate oxidized per min) mg^{-1} protein (Doderer et al., 1992).

Statistical Analysis

All data were subjected to analysis of variance (ANOVA). Mean differences had been compared by Tukey's HSD test using XLSTAT v. 2016.04.32525 software (Addinsoft, 2016). Differences at $P \leq 0.05$ were considered significant.

RESULTS

Production of ROS and Oxidative Stress

Cadmium stress imposition in the growing media caused oxidative damage in the seedlings. Membrane lipid peroxidation (increasing MDA levels) has been noticed in Cd-affected rapeseed seedlings (**Figure 1A**). Content of H$_2$O$_2$ increased by 37 and 60%, and activity of LOX increased by 62 and 145% under 0.5 and 1 mM CdCl$_2$ stresses (**Figures 1B,C**), respectively, as

compared with control plants. All these were responsible for peroxidation of membrane lipid. Exogenous H$_2$O$_2$ application reduced H$_2$O$_2$ content and LOX activity which are corroborating with the reduction of MDA contents by 23 and 25% in mild and severe Cd stresses when compared to stress treatments only (**Figures 1A–C**).

Histochemical Detection of ROS in Rapeseed Leaves

Leaves were dipped into DAB and NBT solution to visualize the generation and spots of H$_2$O$_2$ and O$_2^{\bullet-}$, respectively. The leaves under Cd stress showed a high frequency of dark brown patches of H$_2$O$_2$ and deep blue spots of O$_2^{\bullet-}$ anions (**Figures 2A,B**). The spots were darker and larger in severe Cd stress, compared to the mild Cd stress. However, these spots of H$_2$O$_2$ and O$_2^{\bullet-}$ were somewhat reduced, compared to Cd stress alone when exogenous H$_2$O$_2$ was added with Cd stresses which are indicators for oxidative stress reduction (**Figures 2A,B**).

ASA-GSH Cycle

Ascorbate content decreased by 20 and 32%; in contrast, DHA content increased by 7 and 43% which resulted in 25 and 52% decrease of AsA/DHA ratio under mild and severe Cd stresses when compared to Cd untreated control. Increase of GSH pool and also with the high increase of GSSG resulted in decreased ratio of GSH/GSSG by 15 and 44%, respectively, under 0.5 and 1 mM CdCl$_2$ stress, respectively, compared to control. Exogenous H$_2$O$_2$ addition inverted the AsA-GSH pool by increasing AsA content by 32 and 30% (**Figure 3A**), increasing GSH content by 38 and 25% (**Figure 3D**), decreasing DHA content by 12 and 21% (**Figure 3B**), and decreasing GSSG content by 17 and 8% (**Figure 3E**), under mild and severe Cd stresses, respectively. Alteration of AsA and GSH contents by H$_2$O$_2$ pretreatment were vital in improving AsA/DHA (**Figure 3C**) and GSH/GSSG (**Figure 3F**) ratios, compared to Cd stress alone.

The enzymes [APX, MDHAR, DHAR, and GR] (**Figures 4A–D**) of AsA-GSH cycle responded differentially in Cd-exposed seedlings. APX activity increased, MDHAR and DHAR activities reduced with the increase of Cd dose, compared to control, whereas, GR activity increased under mild Cd stress but reduced under severe Cd stress in comparison to their respective control (**Figures 4A–D**). External application of H$_2$O$_2$ under Cd stress increased activities of APX (40 and 39%), DHAR (77 and 67%), and GR (36 and 79%), respectively, under mild and severe Cd stresses, respectively, in contrast to Cd stress alone (**Figures 4A–D**).

CAT, GPX, and GST Activities

Both levels of Cd stress-affected seedlings showed higher GST activity whereas GPX activity increased only under mild Cd stress level, but CAT activity decreased at both levels of Cd stresses when compared to control. Activity of GST upregulated by 115 and 145%, the activities of CAT reduced by 28 and 44% under mild and severe Cd stress, respectively; activity of GPX amplified by 23% under mild Cd stress but it decreased by 23% under severe
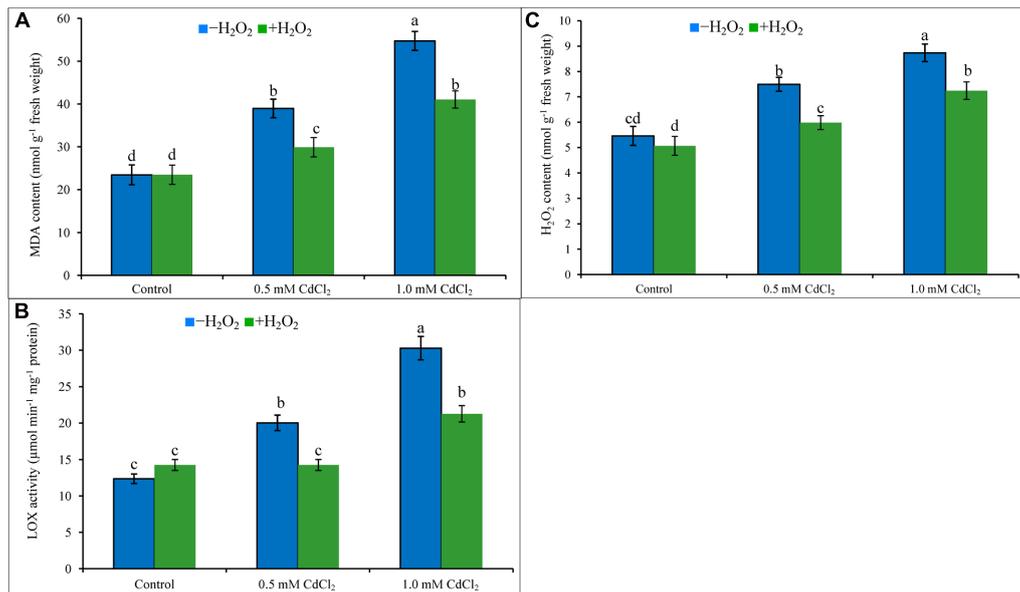
FIGURE 1 | Malondialdehyde content (A), H₂O₂ content (B), and LOX activity (C) in rapeseed leaves induced by exogenous H₂O₂ under Cd stress. Mean (±SD) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.
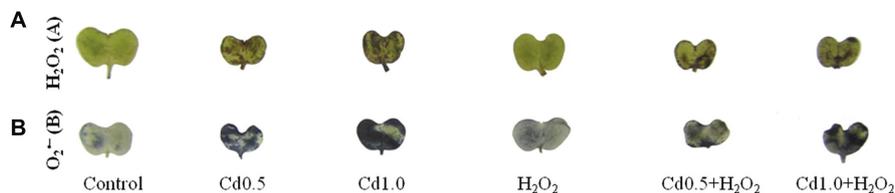
FIGURE 2 | 3,3-Diaminobenzidine staining (A) of H₂O₂ and NBT staining (B) of superoxide $O_2^{\bullet -}$ in rapeseed leaves induced by exogenous H₂O₂ under Cd stress.

Cd stress, compared to control (**Figures 5A–C**). Supplementation of H₂O₂ with Cd improved GST activities by 44 and 43%, and CAT activities by 79 and 47%, under mild and severe Cd stresses whereas augmented GPX activity by 40% under severe stress (**Figures 5A–C**), respectively, compared to Cd stress alone.
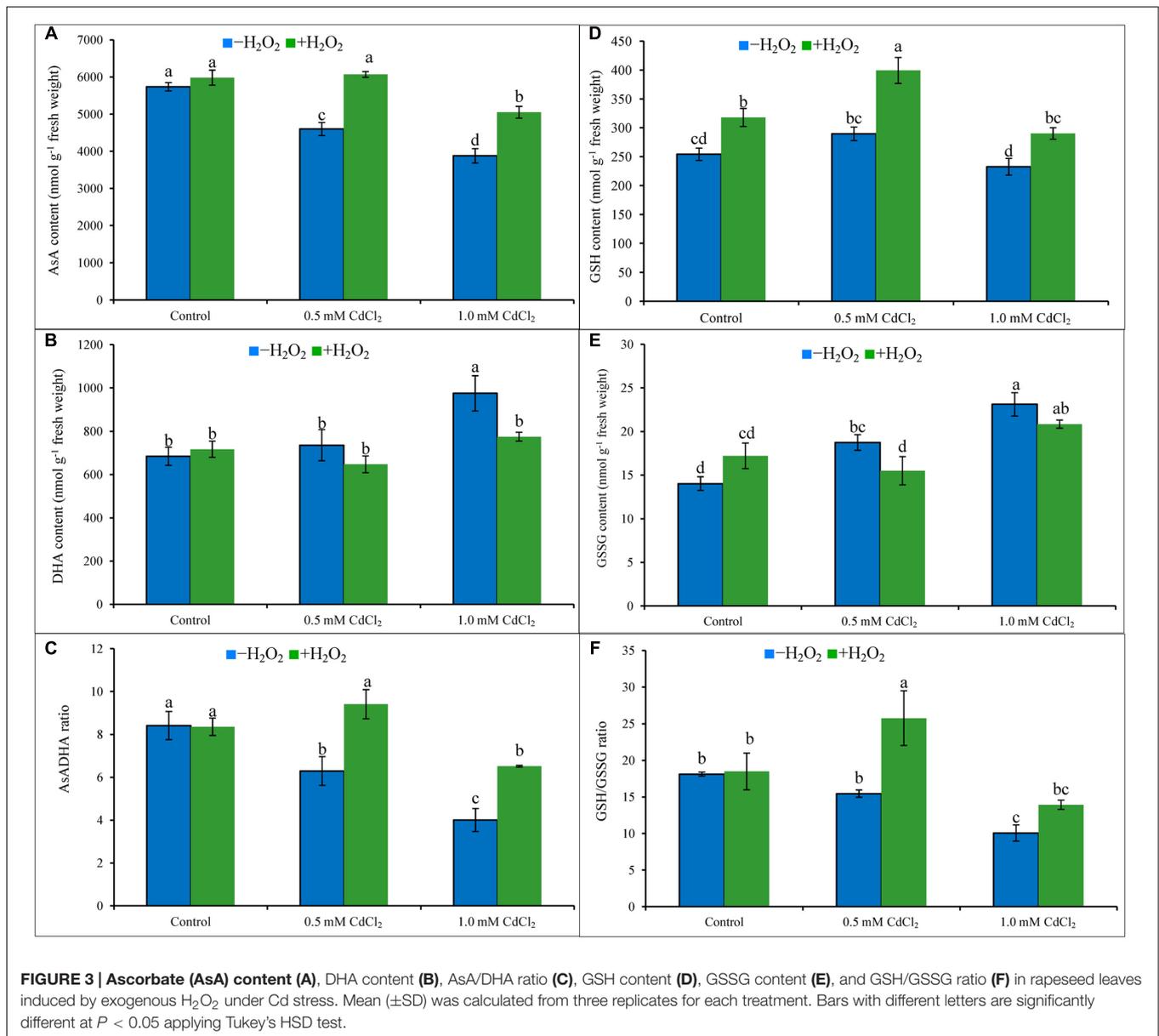
Glyoxalase System

Activities of Gly I and Gly II decreased due to exposure of Cd (**Figures 6A,B**). Their activities increased in both doses of Cd stress treatments supplemented with H₂O₂ except for Gly I activity at severe stress. The increase of Gly I activity under mild Cd stress was 35% after H₂O₂ supplementation, compared to Cd stress alone. Gly II activity increased by 47 and 55% in H₂O₂ added mild and severe Cd stresses, compared to Cd stress alone (**Figures 6A,B**).

DISCUSSION

Showing toxicity at higher concentration and acting as signaling molecule initiate, H₂O₂ plays a dual role and is considered as rival and comrade of stress tolerance

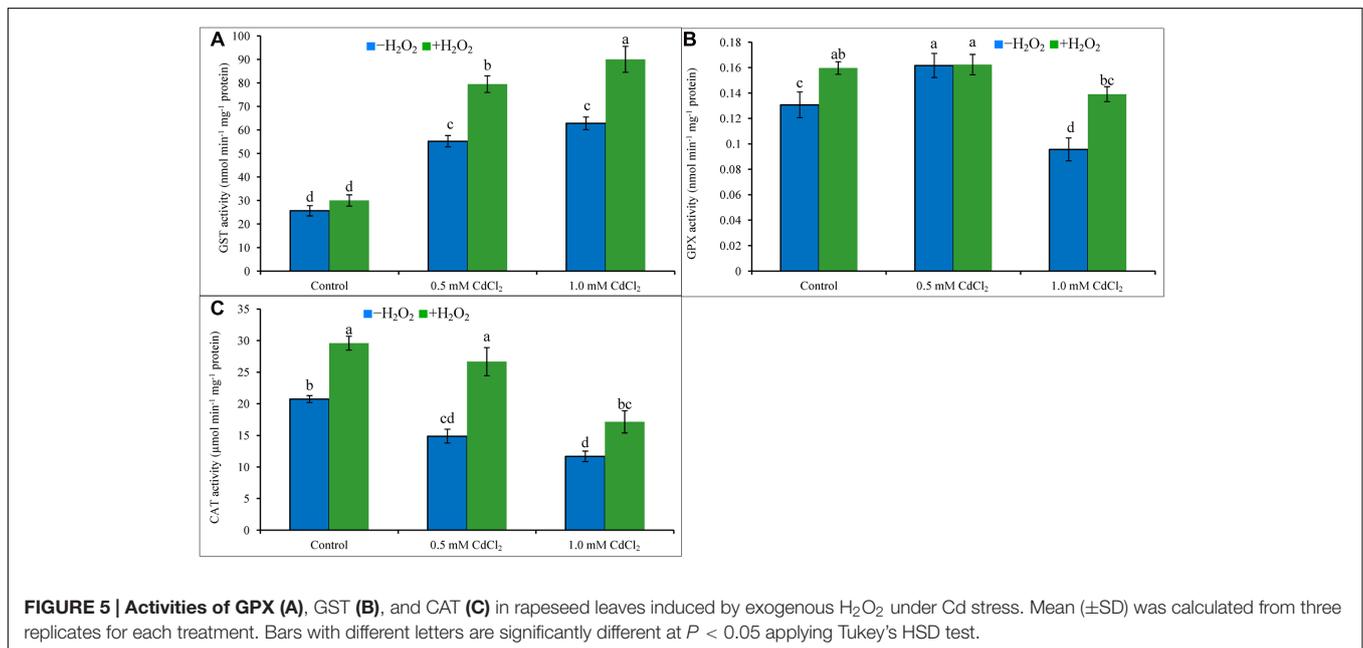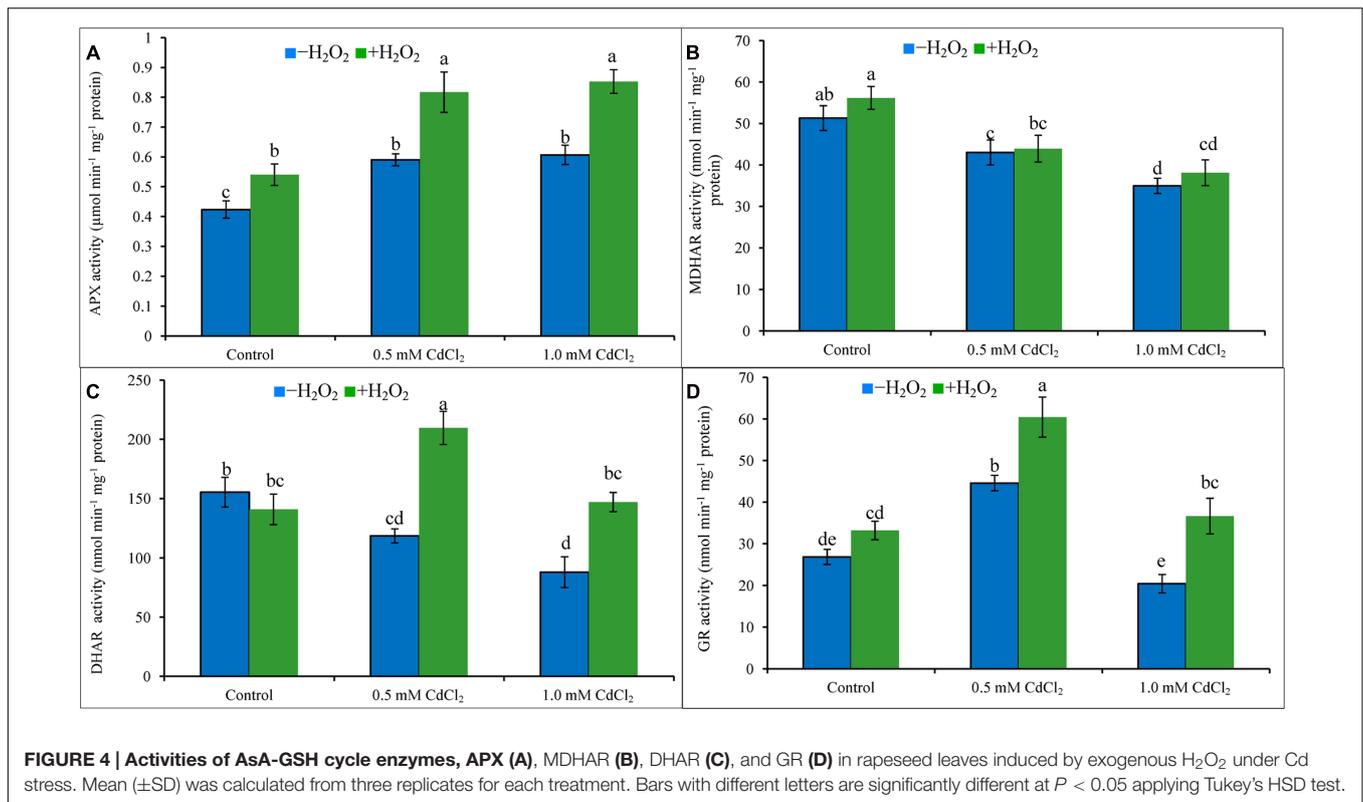development in plants. Due to dual roles of H₂O₂ and due to various unidentified roles of H₂O₂, recent research with H₂O₂ concentrate on diversified plausible mechanisms through which H₂O₂ is related to plant stress tolerance development. Present study has been executed to reveal the pivotal roles of H₂O₂ in relation to Cd stress tolerance in rapeseed.

The mechanism of Cd-induced oxidative stress is different from other stresses; Cd^{2+} cannot produce ROS directly as it through Fenton reaction or Haber Weiss reaction. Showing affinity to thiol Cd run downs GSH (Lopez et al., 2006). Cd enhances ROS production by weakening antioxidant defense mechanism (Srivastava et al., 2004; Gill and Tuteja, 2010), distressing photosystem II activity (Sigfridsson et al., 2004), disturbing functioning of vital enzymes (Dong et al., 2006). Cd displaces iron (Fe) from proteins and increases free Fe that is responsible for ROS generation. Cd also increases ROS production distorting mitochondrial function (Dorta et al., 2003). Spots of H₂O₂ and $O_2^{\bullet -}$ in leaves of rapeseed and the contents of H₂O₂ and MDA have been increased considerably in Cd affected rapeseed plants clearly indicating oxidative damage corroborating the results of previous studies (Dong et al.,

FIGURE 3 | Ascorbate (AsA) content (A), DHA content **(B)**, AsA/DHA ratio **(C)**, GSH content **(D)**, GSSG content **(E)**, and GSH/GSSG ratio **(F)** in rapeseed leaves induced by exogenous H$_2$O$_2$ under Cd stress. Mean (±SD) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

2006; Hu et al., 2009; Hasanuzzaman et al., 2012a). H$_2$O$_2$ pretreatment reduced oxidative damage by decreasing the spots of O$_2^{\bullet-}$ and H$_2$O$_2$ and reducing the amount of H$_2$O$_2$ and MDA contents against Cd toxicity (Hu et al., 2009), reducing contents of MDA and O$_2^{\bullet-}$ in salinity affected wheat plants (Li et al., 2011), decreasing O$_2^{\bullet-}$, H$_2$O$_2$ and MDA in chill affected cucumber seedlings (Zhang et al., 2011). The results of these previous reports indicate the decisive functions of H$_2$O$_2$ in reducing oxidative stress. At low concentration, H$_2$O$_2$ can as signaling molecule which modulates various genes related to stress defense mechanism. H$_2$O$_2$ implicated NO-mediated ABA-induced activation of mitogen-activated protein (MAP) kinase cascade which modulated antioxidant defense mechanism maize leaves. H$_2$O$_2$ can modulate NO and NO itself is an ROS scavenger (Zhang et al., 2007). In present study, the advantageous roles of
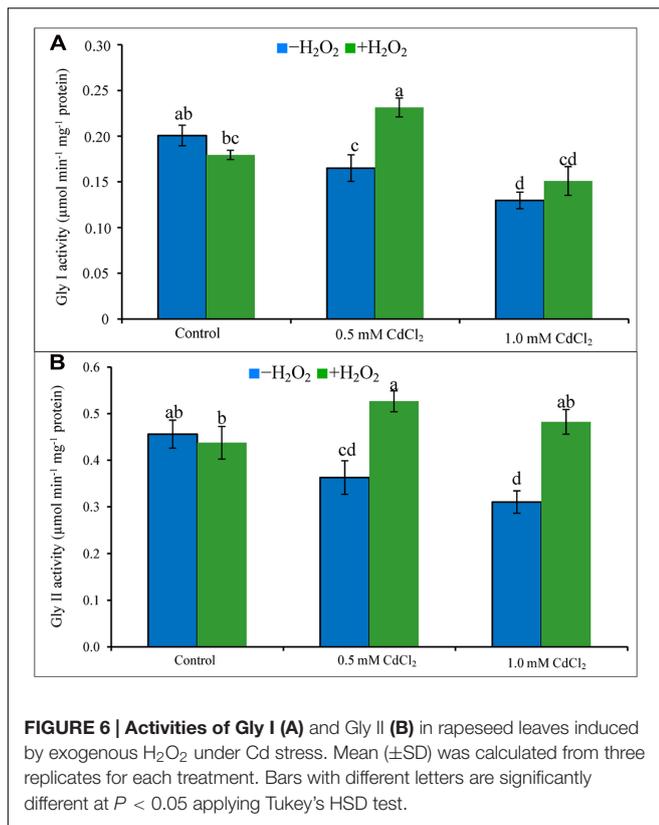
H$_2$O$_2$ have been presented in later section where application of very low concentration exogenous H$_2$O$_2$ pretreatment induced and enhanced the antioxidant defense system components which in turn helped in decreasing the endogenous contents of ROS including H$_2$O$_2$ and O$_2^{\bullet-}$ and in decreasing the oxidative damage which is parallel with the results of the previous findings (Zhang et al., 2007, 2011). Both AsA and GSH presenting in chloroplast, cytoplasm, apoplast, mitochondria, peroxisome effectively scavenge H$_2$O$_2$. CAT, APX, GPX, and GST directly catalyze the reactions of H$_2$O$_2$ scavenging. Exogenous low dose of H$_2$O$_2$ in the present study enhanced the activities of these enzymes and increased the contents of AsA and GSH of Cd affected rapeseed seedlings which are directly related to H$_2$O$_2$ scavenging process and that is why H$_2$O$_2$ pretreatment decreased the endogenous H$_2$O$_2$ levels and subsequent oxidative damage of

FIGURE 4 | Activities of AsA-GSH cycle enzymes, APX (A), MDHAR **(B)**, DHAR **(C)**, and GR **(D)** in rapeseed leaves induced by exogenous H$_2$O$_2$ under Cd stress. Mean (±SD) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

FIGURE 5 | Activities of GPX (A), GST **(B)**, and CAT **(C)** in rapeseed leaves induced by exogenous H$_2$O$_2$ under Cd stress. Mean (±SD) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

Cd affected seedlings (Mittler, 2002; Blokhina et al., 2003; Ashraf, 2009; Gill and Tuteja, 2010).

Ascorbate is water-soluble non-enzymatic antioxidant in cell decreasing oxidative stress scavenging O$_2^{\bullet-}$ and OH$^\bullet$ (Gill and Tuteja, 2010). In rapeseed seedlings of present study, AsA level reduced and DHA level increased (because AsA is oxidized to

DHA after scavenging ROS) due to reduced MDHAR and DHAR activities (which are AsA recycling enzymes) which decreased the AsA/DHA ratio and increased oxidative stress (Chao et al., 2010). APX activity upregulated due to Cd exposure, which is correlated to the reduced AsA content. H$_2$O$_2$ pretreatment followed by Cd exposure upregulated APX, MDHAR and DHAR

FIGURE 6 | Activities of Gly I (A) and Gly II **(B)** in rapeseed leaves induced by exogenous H_2O_2 under Cd stress. Mean (±SD) was calculated from three replicates for each treatment. Bars with different letters are significantly different at $P < 0.05$ applying Tukey's HSD test.

activities in the seedlings which restored AsA and decreased oxidative stress which is supported by previous findings (Hu et al., 2009; Li et al., 2011; Zhang et al., 2011). In rapeseed seedlings, increased levels of GSH and GSSG but decreased GSH/GSSG ratio have been noticed in exposure to Cd which are supported by previous studies (Molina et al., 2008; Hu et al., 2009; Hasanuzzaman et al., 2012a). GSH is the thiol group of non-enzymatic antioxidant showing an imperative role in the stress signal, adaptation and defense mechanism of plants (Noctor et al., 2012). GSSG is recycled to GSH involving the GR activity (Gill and Tuteja, 2010). Increased GSH content after H_2O_2 application was found beneficial under Cd stress (Chao et al., 2009). The application of H_2O_2 upregulated AsA and GSH levels and improved CAT, POD, SOD, GPX, GR, MDHAR, and DHAR metabolism as reported in Al affected wheat seedlings (Xu et al., 2010). Seedlings pretreated with H_2O_2 increased GR activity (which recycles GSSG to GSH) which resulted in decreased GSSG level and increased GSH content which increased GSH/GSSG ratio in Cd affected rapeseed seedlings which is supported by previous findings (Hu et al., 2009; Li et al., 2011; Zhang et al., 2011).

The multifunctional isoenzymes GSTs are vital antioxidant enzymes, involved xenobiotic and toxic compound detoxification process (Polidoros and Scandalios, 1999). In the present investigation, GST activity and GSH amplified due to Cd exposure which were also observed in other studies (Hu et al., 2009; Hasanuzzaman et al., 2012a). Nonetheless, a further increase of GST activity and GSH content was noticed in

H_2O_2 pretreated rapeseed seedlings under Cd stress which reduced adverse effects of Cd on physiology and growth which is similar to the findings of Hu et al. (2009) in rice. Increased Cd sequestration by H_2O_2 pretreatment in rice roots is an indication of crucial roles of H_2O_2 to further decline of Cd translocation to shoot (Hu et al., 2009). Cd stress reduced CAT and GPX activities in rapeseed seedlings that are correlated to a generation of high H_2O_2 which is comparable with previous findings (Hu et al., 2009; Hasanuzzaman et al., 2012a). The Cd has an affinity to proteins or –SH compounds and other side chains, Cd disturbs protein and enzymes synthesis which impair enzymatic activity (Sanitá di Toppi and Gabbrielli, 1999). CAT and GPX activities were restored and increased in H_2O_2 pretreated Cd-stressed seedlings which played vital roles in reducing the H_2O_2 level in Cd affected seedlings. Similar roles of H_2O_2 were observed in Cd affected rice seedlings (Hu et al., 2009), cucumber plants subjected to low light stress (Zhang et al., 2011) and in salt affected rice seedlings (Li et al., 2011).

Like other abiotic stresses, MG is overproduced within the plants under Cd stress (Hasanuzzaman et al., 2012a; Kaur et al., 2015; Nahar et al., 2015). Cd decreased both the activities of Gly I and Gly II that indicated the reduced MG detoxification via the glyoxalase system (Nahar et al., 2015). In this study, rapeseed seedlings exposed to Cd decreased Gly I and Gly II activities but pretreatment with H_2O_2 increased Gly I and Gly II activities and GSH content indicating the imperative roles of H_2O_2 in MG detoxification which are in the same line with the previous findings (Hasanuzzaman et al., 2012a; Nahar et al., 2016a,b).

CONCLUSION

In this study, we provided evidence for a specific pattern of ROS generation (H_2O_2 and $O_2^{\bullet-}$) and oxidative damage (MDA content) with the raise of Cd dose. Exogenous H_2O_2 treatment increased the amount of the most important ROS scavenging molecules AsA and GSH and increasing the antioxidant enzyme activities which enhanced ROS scavenging process. The Gly I and Gly II activities and content of GSH increased after H_2O_2 pretreatment indicating the roles of exogenous H_2O_2 in MG detoxification process. In contrast to the evidence of exogenous H_2O_2-induced ROS and MG detoxification in the present study, a number of unanswered questions still remain unclear. Why and how does H_2O_2 induce production of antioxidant molecules (AsA and GSH) and activities antioxidant enzymes? Previous reports support the notion that H_2O_2 induced signaling is involved with phytohormones and signaling molecules ABA, SA, JA (jasmonic acid), GA (gibberallic acid), ethylene, NO, Ca^{2+}-mediated development of abiotic stress tolerances in plant (Mittler et al., 2004; Jubany-Mari et al., 2009; Chen et al., 2012; Guo et al., 2014; Reczek and Chandel, 2015; Saxena et al., 2016). In relation to the findings of present study, new questions arise: is there any signaling function of H_2O_2 in regulating the biosynthesis or degradation/metabolism of antioxidants

components or, other hormones or signaling molecules affecting these processes? The possible mechanisms and signaling action of H_2O_2 in these aspects should be further elucidated.

AUTHOR CONTRIBUTIONS

MH, MF, and KN conceived and designed the experiments; MH and KN performed the experiments; HA and BR analyzed the data; MF contributed reagents/materials/analysis tools; MH, KN, SG, HA, and BR wrote the manuscript. BR edited the manuscript. All authors read and approved the final manuscript.

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