

Relative tolerance of different species of Brassica to cadmium toxicity: Coordinated role of antioxidant defense and glyoxalase systems

Jubayer Al Mahmud^{1,2}, Mirza Hasanuzzaman^{3*}, Kamrun Nahar^{1,4}, Anisur Rahman^{1,3}, Masayuki Fujita^{1*}

¹Laboratory of Plant Stress Responses, Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, Miki-cho, Kita-gun, Kagawa 761-0795, Japan

²Department of Agroforestry and Environmental Science, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka 1207, Bangladesh

³Department of Agronomy, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka 1207, Bangladesh

⁴Department of Agricultural Botany, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka 1207, Bangladesh

*Corresponding author: Mirza Hasanuzzaman, e-mail: mhzsauag@yahoo.com; Masayuki Fujita: fujita@ag.kagawa-u.ac.jp

Abstract

The present study was carried out to examine the metal accumulation and tolerance abilities of three *Brassica* species (*B. napus*, *B. campestris*, and *B. juncea*) seedlings exposed to two levels of cadmium (Cd) stress (0.25 and 0.5 mM CdCl₂ for three days). Of the *Brassica* species studied, *B. juncea* accumulated the highest amount of Cd in a dose-dependent manner, and in every case, the Cd content was higher in the roots than the shoots. Cadmium stress reduced seedlings biomass, leaf relative water content (RWC), and chlorophyll (chl) content, whereas proline (Pro), MDA, and H₂O₂ content and lipoxigenase (LOX) activity increased in all species. Under Cd stress, ascorbate (AsA) content reduction was lower and glutathione (GSH) content increase was higher in *B. juncea* compared with other species. Monodehydroascorbate reductase (MDHAR), glutathione reductase (GR), and superoxide dismutase (SOD) activities increased significantly in *B. juncea* under Cd stress compared with the other species. Catalase (CAT) activity did not decrease in *B. juncea* due to Cd stress, compared with the other species. Dehydroascorbate reductase (DHAR) activity decreased with both levels of Cd stress in all species except for *B. juncea* under 0.25 mM CdCl₂ stress. Glyoxalase system components performed better in *B. juncea* than the other species under Cd stress. Methylglyoxal (MG) increased substantially under both levels of Cd stress, but MG content was lower in *B. juncea* compared with the others. Considering the antioxidant defense and glyoxalase systems performance *B. juncea* is relatively tolerant species to Cd toxicity though it accumulated highest Cd.

Key words: Abiotic stress; Heavy metal; Methylglyoxal; Phytoremediation; Reactive oxygen species.

Abbreviations: AO_Ascorbate oxidase, APX_Ascorbate peroxidase, AsA_Ascorbic acid, CAT_Catalase, CDNB_1-Chloro-2,4-dinitrobenzene, DAB_Diaminobenzidine, DHA_Dehydroascorbic acid, DHAR_Dehydroascorbate reductase, DTNB_5,5-Dithio-bis-(2-nitrobenzoic) acid, EDTA_Ethylenediaminetetraacetic acid, Gly_Glyoxalase, GR_Glutathione reductase, GSH_Reduced glutathione, GSSG_Oxidized glutathione, GPX_Glutathione peroxidase, GST_glutathione S-transferase, LOX_Lipoxygenase, MDHAR_Monodehydroascorbate reductase, MG_Methylglyoxal, NBT_Nitroblue tetrazolium chloride, NTB_2-Nitro-5-thiobenzoic acid, Pro_Proline, ROS_Reactive oxygen species, SLG_γ-D-lactoyl-glutathione, SOD_Superoxide dismutase, TBA_Thiobarbituric acid, TCA_Trichloroacetic acid

Introduction

Fast industrialization due to rapid increase of population causes serious environmental problems including the production and release of different toxic metals into the environment (Hasanuzzaman et al., 2013). Among the toxic metals cadmium (Cd) has assumed important environmental contaminant in terms of damage to plant growth and human health (Heyes, 1997; Nouairi et al., 2009; Hasanuzzaman et al., 2013). Plants grown in Cd rich soil show different abnormalities like chlorosis, necrosis, leaf rolling, root growth inhibition and stunted plant growth. Cd also altered stomatal action, decreased water potential, cation efflux, alterations in membrane functions, photosynthesis inhibition, altered metabolism, altered activities of several key enzymes,

and even death (Sharma and Dubey, 2007; Dubey, 2011; Gill et al., 2011; Hasanuzzaman et al., 2013). Normally, Cd stored in the apoplast and the vacuoles of plant cell. Free Cd ions in the cytosol create toxicity to plant cells and as a non-redox metal, Cd is unable to generate reactive oxygen species (ROS) directly through Haber-Weiss reactions. Reactive oxygen species overproduction and occurrence of oxidative stress in plants could be the indirect consequence of Cd toxicity. The mechanisms include interacting with the antioxidant system (Srivastava et al., 2004), upsetting the electron transport chain (Qadir et al., 2004), as well as the metabolism of crucial plant nutrients (Dong et al., 2006). Reactive oxygen species (singlet oxygen, ¹O₂; superoxide, O₂⁻; hydrogen

peroxide, H₂O₂; hydroxyl radical, OH[•]) are able to damage biomolecules including proteins, lipids, and DNA (Hasanuzzaman and Fujita, 2012). Methylglyoxal (MG) is another cytotoxic reactive oxidative compound over produced under abiotic stresses, including metal toxicity (Yadav et al., 2005a; Yadav et al., 2008; Suhartono et al., 2014). Methylglyoxal damages cellular ultra-structural components, and able to damage DNA and cause mutations. To mitigate the deleterious effect of plants have evolved different mechanisms of defense against Cd toxicity. Plants can avoid metal toxicity through metal binding to the cell wall, by reducing transport across the cell membrane and by active efflux (Li et al., 2002; Hall, 2002). Plants can also work against Cd stress through metal chelation, exclusion, active excretion or compartmentalization (Gratão et al., 2005). Moreover, to minimize oxidative stress plant cells are equipped with antioxidant defense system composed of non-enzymatic antioxidants (ascorbic acid, AsA; glutathione, GSH; phenolic compounds; alkaloids; nonprotein amino acids and α -tocopherols) and enzymatic antioxidants (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione peroxidase, GPX; and glutathione S-transferase, GST) (Pang and Wang, 2008; Gill and Tuteja, 2010; Hasanuzzaman et al., 2012a). Plants also have an MG detoxification system including two vital enzymes, glyoxalase I (Gly I) and glyoxalase II (Gly II), where GSH acts as co-factor. However, the efficiency of this system greatly varies with plant genotypes and environmental stimuli. Plant-based remediation system or phytoremediation is well-known all over the globe, and considered one of the low cost, novel, green technology. To find out suitable plants for Cd removal from the contaminated soil, a broader understanding is needed of the physiological and biochemical features of potentially useful species. Members of the Brassicaceae family are promising candidates for phytoextraction of heavy metals such as Cd, Pb, Zn and Ni (Prasad and Freitas, 2003; Robinson et al., 2009), but the degrees of tolerance of different *Brassica* species are not same in terms of metal accumulation.

So far, only a few articles have been published on the growth and physiological differences among *Brassica* species. However, the antioxidant defense and glyoxalase system have not been investigated. Therefore, this study was designed to examine the relative tolerance of *Brassica campestris*, *Brassica napus* and *Brassica juncea* against various Cd concentrations. The antioxidant defense and glyoxalase systems along with Cd accumulation and level of tolerance and detoxification strategy adopted by the plants were investigated.

Results

Cadmium accumulation in the shoots and roots

Three *Brassica* species accumulated Cd in their shoots and roots as a result of Cd exposure, and the accumulation increased with the increase in stress level. Cadmium accumulation was higher in the roots than in the shoots in all three species. *Brassica juncea* accumulated more Cd in its shoots and roots than *B. campestris* and *B. napus* (Table 1). Under 0.25 mM CdCl₂ stress, the shoots of *B. juncea* accumulated 29 and 27% higher Cd than *B. campestris* and *B. napus*, respectively, whereas under 0.5 mM CdCl₂ stress, *B. juncea* accumulated 13 and 9% higher Cd than *B. campestris* and *B. napus*, respectively. Similarly, under 0.25 mM CdCl₂

stress, the roots of *B. juncea* accumulated 65 and 64% higher Cd than *B. campestris* and *B. napus*, respectively, whereas under 0.5 mM CdCl₂ stress, *B. juncea* accumulated 41 and 19% higher Cd than *B. campestris* and *B. napus*, respectively.

Plant growth and biomass production

Upon exposure to 0.25 mM CdCl₂, no significant change in plant height was observed in any of the species tested, while exposure to 0.5 mM CdCl₂ resulted significant decreasing of plant height in *B. campestris* and *B. napus* but not in *B. juncea* (Table 1). Fresh weight and DW of all three *Brassica* species decreased under Cd stress in a dose-dependent manner (Table 1).

Leaf relative water content and proline content

Leaf RWC of all the *Brassica* seedlings decreased due to Cd stress with the increase of CdCl₂ doses (Table 2). Proline content in all three *Brassica* species increased substantially with the increase of Cd stress, but the increase in Pro was higher in *B. juncea* than the other two species (Table 2).

Photosynthetic pigments

Chlorophyll *a* and *b* contents decreased in all three *Brassica* species with the increase in CdCl₂ dosage, but the decrease in chl was lower in *B. juncea* than the other two species (Table 2).

Oxidative damage

Cadmium resulted in severe oxidative stress in all three *Brassica* species (Fig 1). Histochemical staining indicated accumulation of ROS as dark blue spots of O₂^{•-} and brown spots of H₂O₂ in the leaves of plants (Fig 1A, B). MDA content increased significantly in all three species under both levels of Cd stress, and compared with control, the rate of increase was lower in *B. juncea* (Fig 2A). MDA content increased by 74, 57, and 55% under 0.25 mM CdCl₂, and 114, 94, and 85% under 0.5 mM CdCl₂ in *B. campestris*, *B. napus*, and *B. juncea*, respectively, compared with the control plants. A similar result was also observed for H₂O₂ (Fig 2B). LOX activity markedly increased by 46, 85, and 20% under 0.25 mM Cd stress, and 65, 181, and 39% under 0.5 mM Cd stress in *B. campestris*, *B. napus*, and *B. juncea* respectively, compared with the control seedlings (Fig 2C).

Ascorbate and glutathione pools

Both levels of Cd treatment decreased the AsA content of all three species of *Brassica* (Fig 3A). However, the rate of reduction of AsA content was higher in *B. campestris* and *B. napus* than *B. juncea* under 0.5 mM CdCl₂. Seedlings of all three species treated with Cd had significantly increased dehydroascorbate (DHA) content except *B. campestris* under 0.25 mM Cd (Fig 3B), but in *B. juncea*, the level of DHA increase was lower than the other two species. The AsA/DHA ratio of all *Brassica* species decreased under Cd stress in dose dependant manner (Fig 3C). Cadmium stress significantly increased GSH levels in all three *Brassica* species, compared with the control seedlings (Fig 3D), and the highest increase was observed in *B. juncea*. GSSG content also increased in all three species in the same manner as GSH, but the lowest rate of increase was recorded in *B. juncea* (Fig 3E). Compared with control, the GSH/GSSG ratio decreased by 21 and 32% in *B. campestris*, and 9 and 31%

Table 1. Cd content in shoot and root, plant height, fr wt and dry wt of different *Brassica* species under Cd stress. Cd1 and Cd2 indicate 0.25 mM CdCl₂ and 0.5 mM CdCl₂, respectively.

Treatments		Cd content in shoot (mg g ⁻¹ DW)	Cd content in root (mg g ⁻¹ DW)	Plant height (cm)	Fresh weight (mg seedling ⁻¹)	Dry weight (mg seedling ⁻¹)
<i>B. campestris</i>	Control	ND	ND	3.63±0.18 e	54.3 ±0.9 d	5.6± 0.18 d
	Cd1	0.70±0.03 d	6.39±0.23 e	3.48±0.18 ef	49.3 ±0.6 f	4.8± 0.04 e
	Cd2	1.09±0.04 b	11.26±0.07 c	3.37±0.15 f	47.3 ±0.6 g	4.6± 0.04 f
<i>B. napus</i>	Control	ND	ND	4.77±0.10 a	66.4 ±1.0 a	6.5± 0.08 a
	Cd1	0.71±0.07 d	6.45±0.05 e	4.58±0.08 ab	61.8 ±1.2 b	5.9± 0.04 b
	Cd2	1.13±0.01 b	13.33±0.42 b	4.48±0.08 bc	59.8 ±0.4 c	5.7± 0.14 c
<i>B. juncea</i>	Control	ND	ND	4.30±0.05 cd	59.4 ±0.7 c	6.4± 0.06 a
	Cd1	0.90±0.01 c	10.55±0.09 d	4.23±0.03 d	55.0 ±1.3 d	5.8± 0.04 bc
	Cd2	1.23±0.01 a	15.91±0.01 a	4.18±0.03 d	52.4 ±0.8 e	5.5± 0.08 d

Means (±SD) were calculated from three replications for each treatment. Values with different letters are significantly different at P ≤ 0.05 applying the Fisher's LSD test.

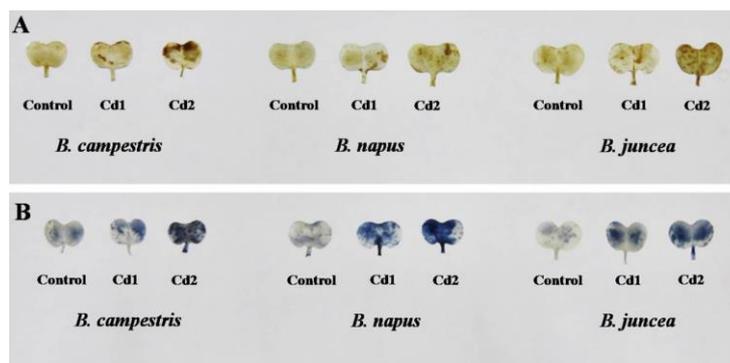


Fig 1. Histochemical detection of H₂O₂ (A) and O₂⁻ (B) of different *Brassica* species under Cd stress. Cd1 and Cd2 indicate 0.25 mM CdCl₂ and 0.5 mM CdCl₂, respectively.

Table 2. Leaf RWC, Pro content, chl *a* and chl *b* content of different *Brassica* species under Cd stress. Cd1 and Cd2 indicate 0.25 mM CdCl₂ and 0.5 mM CdCl₂, respectively

Treatments		Leaf RWC (%)	Pro content (μmol g ⁻¹ FW)	chl <i>a</i> content (mg g ⁻¹ FW)	chl <i>b</i> content (mg g ⁻¹ FW)
<i>B. campestris</i>	Control	89.43±0.9 a	1.23±0.13 e	0.97±0.027 b	0.34±0.009 a
	Cd1	87.72±1.6 bc	1.71±0.11 d	0.49±0.004 g	0.19±0.026 c
	Cd2	84.92±1.6 d	2.14±0.05 c	0.27±0.014 i	0.12±0.009 d
<i>B. napus</i>	Control	90.99±0.9 a	1.20±0.11 e	1.03±0.014 a	0.34±0.019 a
	Cd1	86.87±0.3 b	1.82± 0.14 d	0.59±0.016 e	0.22±0.009 b
	Cd2	82.54±1.0 d	2.55±0.05 b	0.35±0.004 h	0.14±0.008 d
<i>B. juncea</i>	Control	88.34±1.6 b	1.27±0.13 e	0.92±0.015 c	0.33±0.012 a
	Cd1	85.21±1.4 cd	2.22±0.06 c	0.67±0.029 d	0.23±0.013 b
	Cd2	83.97±1.5 d	3.09±0.04 a	0.53±0.019 f	0.19±0.011 c

Means (±SD) were calculated from three replications for each treatment. Values with different letters are significantly different at P ≤ 0.05 applying the Fisher's LSD test.

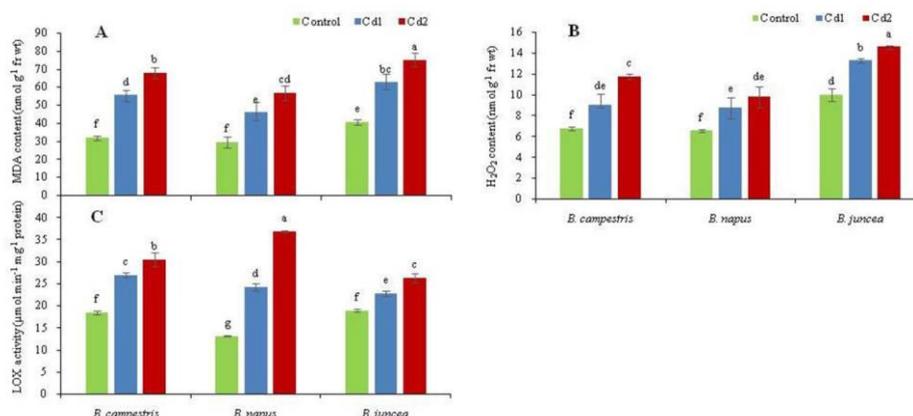


Fig 2. MDA content (A), H₂O₂ level (B), and LOX activity (C) of different *Brassica* species under Cd stress. Cd1 and Cd2 indicate 0.25 mM CdCl₂ and 0.5 mM CdCl₂, respectively. Means (±SD) were calculated from three replications for each treatment. Bars with different letters are significantly different at P ≤ 0.05 applying Fisher's LSD test.

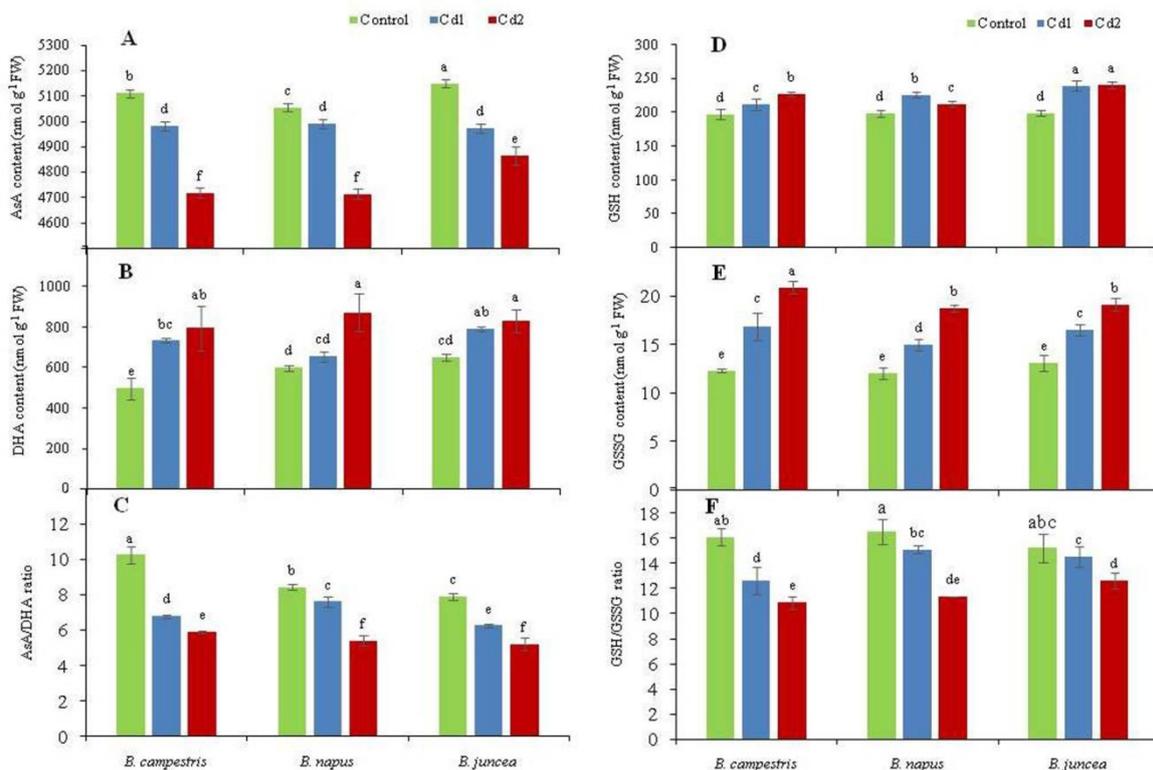


Fig 3. AsA content (A), DHA content (B), AsA/DHA ratio (C), GSH content (D), GSSG content (E), and GSH/GSSG ratio (F) of different *Brassica* species under Cd stress. Cd1 and Cd2 indicate 0.25 mM CdCl₂ and 0.5 mM CdCl₂, respectively. Means (\pm SD) were calculated from three replications for each treatment. Bars with different letters are significantly different at P \leq 0.05 applying Fisher's LSD test.

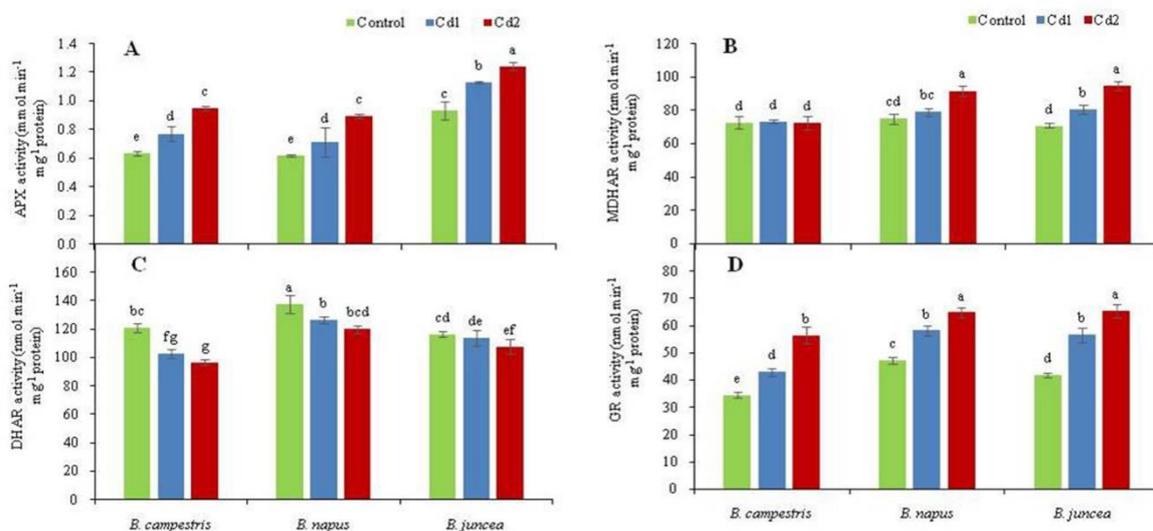


Fig 4. APX (A), MDHAR (B), DHAR (C), and GR (D) activities of different *Brassica* species under Cd stress. Cd1 and Cd2 indicate 0.25 mM CdCl₂ and 0.5 mM CdCl₂, respectively. Means (\pm SD) were calculated from three replications for each treatment. Bars with different letters are significantly different at P \leq 0.05 applying Fisher's LSD test.

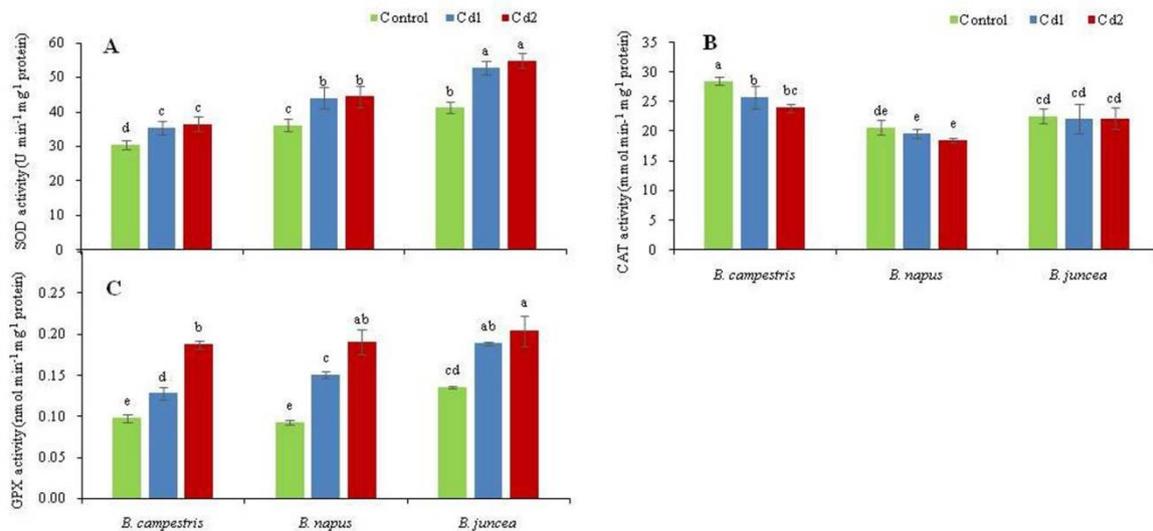


Fig 5. SOD (A), CAT (B), and GPX (C) activities of different *Brassica* species under Cd stress. Cd1 and Cd2 indicate 0.25 mM CdCl₂ and 0.5 mM CdCl₂, respectively. Means (\pm SD) were calculated from three replications for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying Fisher's LSD test.

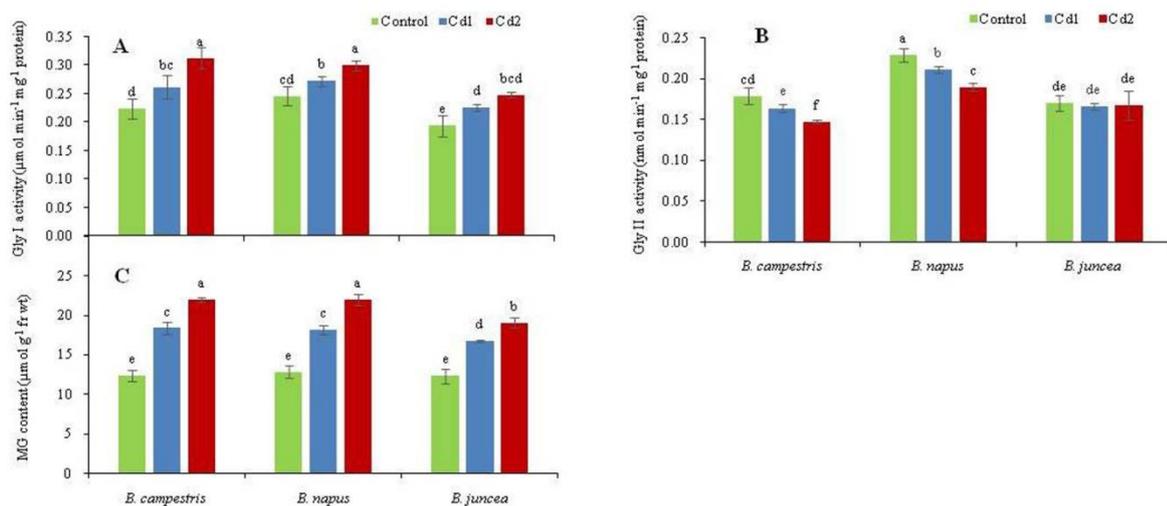


Fig 6. Gly I (A) activity, Gly II (B) activity, and MG content (C) of different *Brassica* species under Cd stress. Means (\pm SD) were calculated from three replications for each treatment. Bars with different letters are significantly different at $P \leq 0.05$ applying Fisher's LSD test.

in *B. napus* under 0.25 and 0.5 mM Cd stress, respectively, whereas in *B. juncea*, it decreased by only 5 and 17%, respectively (Fig 3F).

Antioxidant enzymes

Ascorbate peroxidase and GR activities of all the tested *Brassica* seedlings increased significantly under both levels of Cd stress compared with control (Figure 4A, D). Under 0.5 mM Cd, the increase in APX activity was lower in *B. juncea*. Treatment with Cd had no effect on the MDHAR activities of the *B. campestris* seedlings, but increased MDHAR was recorded for *B. napus* and *B. juncea* under both levels of Cd treatment (Figure 4B). Both treatments of Cd stress resulted in a significant decrease in DHAR activities in *B. campestris* and *B. napus* in contrast with the control seedlings, but in *B. juncea*, a significant decrease was observed only under 0.5 mM Cd stress (Figure 4C). The activities of GPX and SOD

increased significantly in all three *Brassica* species after Cd treatment. The increase in SOD was higher in *B. juncea* than the other two species (Figure 5A, B). Cadmium stress resulted in a decrease in CAT activities in *B. campestris*, whereas in *B. napus* and *B. juncea*, no significant difference was observed compared with the control seedlings (Figure 5C).

Glyoxalase system and methylglyoxal level

The activity of Gly I increased in all three *Brassica* species compared with the control seedlings, whereas Gly II activity decreased in *B. campestris* and *B. napus* under both Cd doses, but no significant difference was observed in *B. juncea* (Figure 6A, B). A substantial increase in MG was observed under both levels of Cd stress compared with the control seedlings, but MG accumulation was lower in *B. juncea* compared with the other species (Figure 6C).

Discussion

Brassica species are known as hyperaccumulators of heavy metal (Prasad and Freitas, 2003), but their metal accumulation capacities are not the same. According to Nouairi et al. (2006; 2009), *B. juncea* possesses a greater potential for Cd accumulation than *B. napus*. They reported that levels of non-protein thiols and phytochelatin increased greatly in leaves of *B. juncea* by increasing Cd supply, but no change was observed in *B. napus*. Plants grown in heavy metal-contaminated medium showed higher metal accumulation in the roots than the shoots (Srivastava et al., 2014; Ahmad et al., 2015; Nahar et al., 2016; Rahman et al., 2016). In our present study, Cd exposure resulted in the accumulation of Cd in the root and shoot tissues of all three *Brassica* seedlings, with the highest accumulation showing in *B. juncea*. The root tissues accumulated higher Cd content than the shoot tissues of all three *Brassica* species.

Cadmium stress reduces the growth, development, and productivity of plants by disrupting different physiological processes (Sanita di Toppi and Gabbriellini, 1999), which were reflected in the findings of our study. In the present study, FW and DW of all three *Brassica* species decreased under Cd stress in a dose-dependent manner. On the other hand, plant height decreased significantly under 0.5 mM CdCl₂ stress in *B. campestris* and *B. napus*, whereas no significant difference was observed in *B. juncea*. Therefore, in terms of growth, *B. campestris* was more sensitive to Cd stress. Growth reduction in response to Cd stress has also been reported for mustard (Iqbal et al., 2010), mung bean (Nahar et al., 2016), rice (Rahman et al., 2016), and for various *Brassica* species after exposure to excess Zn or Cu (Ebbs and Kochian, 1997).

Different abiotic stresses reduce leaf RWC and increase Pro content in wheat (Hasanuzzaman and Fujita, 2013), mung bean (Nahar et al., 2016) and rice (Rahman et al., 2016). Toxic metals increase accumulation of Pro and hamper plant water balance (Ahmad et al., 2015). Free Pro in plants acts as an osmoprotectant, metal chelator, and antioxidant. Therefore, Pro protects plants from free radical damage and improves phytochelatin synthesis by maintaining a favorable cellular environment and Cd sequestration (Gill and Tuteja, 2010). Accordingly, for identifying the tolerance level of plants under any stress, leaf RWC and Pro content are very important factors. In our study, leaf RWC decreased and Pro content increased in all three *Brassica* seedlings with the increase in stress level, but the decrease in leaf RWC was lower and the increase in Pro content was higher in *B. juncea*, suggesting that *B. juncea* is more tolerant to Cd stress than the other two species.

Chlorophyll content decreases as a result of inhibition of protochlorophyllide reduction and aminolevulinic acid synthesis (Stobart et al., 1985). Cadmium stress adversely affect photosynthesis by altering the photosystem II (Baszynski, 1986), decreasing amount of plastoquinone in the chloroplast (Krupa et al., 1992) and disrupting the calvin cycle (Weigel, 1985). In the present study, a significant reduction in the content of chl *a* and chl *b* was noticed in the leaves of all three *Brassica* species, but the rate of reduction was lower in *B. juncea*. A similar result was obtained by Nouairi et al., (2006), in which the total chl content reduction of *B. juncea* was significantly lower than *B. napus*.

Abiotic stresses at the molecular level accelerate the production of ROS such as ¹O₂, O₂^{•-}, H₂O₂, and OH[•] (Hasanuzzaman et al., 2012a). In our study, excessive production of H₂O₂ and O₂^{•-} was recorded under different doses of Cd in all the tested *Brassica* species. Cadmium toxicity resulted in the production of ROS, which in turn

converted fatty acids to toxic lipid peroxides, and caused membrane damage in cadmium-sensitive plants (Nouairi et al., 2009). In contrast to *B. campestris* and *B. napus*, an increased rate of MDA production was lower in the leaves of *B. juncea* (compared with its control) under both Cd treatments despite its high cadmium accumulation. Similar results were previously reported in different species of *Brassica* (Nouairi et al., 2006; 2009). The lower rate of MDA production in *B. juncea* seedlings seems to be closely related to heavy-metal tolerance in plants (Howlett and Avery, 1997). Therefore, these results clarify that all three *Brassica* plants have different defense strategies against Cd stress as well as oxidative stress. Lipoxygenase, an iron-containing enzyme responsible for membrane degradation (Macri et al., 1994), is an indicator of oxidative damage (Ali et al., 2005). Singh and Shah, (2014) reported that Cd causes oxidative damage in rice plants by increasing LOX activity. In our study, LOX activity increased with an increasing concentration of Cd in all three *Brassica* species, but compared with control, the rate of increase was lower in *B. juncea*. Higher LOX activity under Cd stress is supported by a higher level of MDA (Rahman et al., 2016). As in our study, the rate of increasing lipid peroxidation (MDA) resulting from Cd stress was lower in *B. juncea*, which was partly due to lower LOX activity in the same species, making the plant more tolerant than *B. campestris* and *B. napus*.

To scavenge ROS, plants possess a well-organized, efficient non-enzymatic (AsA, GSH, α -tocopherol, phenolic compounds, alkaloids, and non-protein amino acids) and enzymatic (SOD, CAT, APX, GR, MDHAR, DHAR, GPX, POX, etc.) antioxidative defense system (Gill and Tuteja, 2010; Mittler et al., 2004). To maintain cellular redox potential for abiotic stress tolerance, the components of the AsA-GSH cycle perform a significant role (Foyer and Halliwell, 1976; Pang and Wang, 2008; Mahmood et al., 2010; Pang and Wang, 2010; Anjum et al., 2011). Ascorbate is a vital water-soluble antioxidant that reacts with different ROS including H₂O₂, O₂^{•-}, and ¹O₂. It can also scavenge OH[•] at diffusion-controlled rates (Smirnoff, 2005). Changes in AsA, DHA, and the AsA/DHA ratio are part of the antioxidant defense mechanism of plants (Alam et al., 2013). In our present study, the change in AsA and DHA contents was not same in all three *Brassica* species under both levels of Cd stress. Under the 0.5 mM dose of Cd, the rate of reduction of AsA, the AsA/DHA ratio, and the increasing rate of DHA was lower in *B. juncea* than *B. campestris* and *B. napus*, because the increase in APX activity was lower in *B. juncea*, which might render the species more able to scavenge ROS than the other species under Cd stress. Glutathione is often considered an important intracellular antioxidant of the AsA-GSH cycle, and the GSH/GSSG ratio in particular has a vital function in the redox status and stress signaling processes. Therefore, higher GSH/GSSG is considered to support improved abiotic stress tolerance including toxic metal stress (Gill and Tuteja, 2010; Hasanuzzaman et al., 2012a). Cd stress significantly increased the GSH levels in all three *Brassica* species, compared with the control seedlings, and the highest increase was observed in *B. juncea*. GSSG content also increased in all three species, much like GSH, with an increasing dose of Cd, but the lowest rate of increase was recorded in *B. juncea*. That is why the lowest reduction in the GSH/GSSG ratio was recorded in *B. juncea* under Cd stress. An increased level of GSSG may be due to the oxidation of GSH to GSSG during the scavenging process of ROS (Nahar et al., 2016). Toxic metals increase GSH content, which scavenges ROS with the help of GPX and GST (Hasanuzzaman et al., 2012a; Szalai et al., 2009). In the

present study, GPX increased significantly compared with the control plants in all the *Brassica* species tested.

In plant cells, SOD constitutes the frontline defense against ROS and it catalyzes and converts $O_2^{\cdot-}$ to H_2O_2 (Hasanuzzaman et al., 2012a). Previous research findings showed that Cd stress increases SOD activity (Nahar et al., 2016; Rahman et al., 2016). In our study, SOD activity increased significantly in all three *Brassica* species after Cd treatment, but the rate of increase in SOD activity was higher in *B. juncea* than the other two species, which shows that *B. juncea* has a greater capacity to prevent ROS. Catalase converts H_2O_2 to H_2O and O_2 , which contributes to preventing oxidative damage (Sánchez-Casas and Klesseg, 1994), but in our study, Cd stress resulted in a decrease in CAT activities in *B. campestris*, whereas in *B. napus* and *B. juncea*, no significant difference was observed compared with the control seedlings.

The four enzymes (APX, MDHAR, DHAR, and GR) of the AsA-GSH cycle work with AsA, GSH, and NADPH and together detoxify H_2O_2 in a series of cyclic reactions and again regenerate AsA and GSH (Hasanuzzaman et al., 2012b). Ascorbate peroxidase catalyzes the reduction of H_2O_2 to H_2O by using AsA (Wang et al., 2006; Xu et al., 2008). On the other hand, GR regenerates AsA and GSH to maintain cellular balance with the help of MDHAR and DHAR (Mishra et al., 2013). Our present study revealed that the APX and GR activities of all the tested *Brassica* seedlings increased significantly under both levels of Cd stress compared with control, and the increase in APX activity was lower in *B. juncea* under higher Cd levels. Cd treatment had no effect on MDHAR activity in the *B. campestris* seedlings, but increased MDHAR was recorded for *B. napus* and *B. juncea* under both levels of Cd treatment. A significant decrease in DHAR activities was recorded in *B. campestris* and *B. napus* in contrast with the control seedlings, but in *B. juncea* a significant decrease was observed under only 0.5 mM Cd stress, which corroborate with GSH content of *B. juncea* because under mild stress GSH content increased rate was significantly higher than the other two species. With the GSH-GSSG pool, *B. juncea* showed higher upregulation of antioxidative enzymes. Similar findings were observed in Cd-treated rice plants (Rahman et al., 2016).

The MG level markedly increases in different plants under abiotic stress including Cd stress (Hasanuzzaman et al., 2012c; Suhartono et al., 2014; Nahar et al., 2016; Rahman et al., 2016), which is similar to the findings of our present study. Upregulation of the MG detoxification system or glyoxalase system is very important for plants to improve stress tolerance against toxic MG or MG-induced oxidative stress (Yadav et al., 2005a). The enzymes of the glyoxalase system, Gly I and Gly II, can detoxify MG effectively in a two-step reaction. In the first step, MG is converted to SLG by using the Gly I enzyme, where GSH acts as co-factor, and in the second step, SLG is converted to D-lactate by using the Gly II enzyme, where GSH is recycled back (Yadav et al 2005b; Mustafiz et al., 2010). In the present study, Gly I activity increased in all three *Brassica* species, whereas Gly II activity decreased in *B. campestris* and *B. napus* under both 0.25 and 0.5 mM Cd doses, but no significant difference was observed in *B. juncea*. The increase in Gly I activity and the decrease in Gly II activity were also observed in mung bean (Nahar et al., 2016) and rice (Rahman et al., 2016) under Cd stress. Compared with the control seedlings, a marked increase in MG was observed in all three species of *Brassica* under both levels of Cd stress, but the increase in MG production was lower in *B. juncea* than the other two

species. Lower MG levels in *B. juncea* are supported by the higher efficiency of its glyoxalase system.

Materials and methods

Plant materials and stress treatments

Uniform sized seeds of three *Brassica* species (*Brassica campestris* L. cv. BARI Sharisha 9, *Brassica napus* L. cv. BARI Sharisha 13 and *Brassica juncea* L. cv. BARI Sharisha 16) were selected and surface sterilized with 70% ethanol followed by washing several times with sterile distilled water. Then sterilized seeds were sown in petri dishes (9 cm) lined with six layers of filter paper moistened with 10 ml of distilled water for germination and kept for 2 days in germinator. Each petri dish contained 60 germinated seedlings and those were grown under controlled conditions (light, $350 \mu\text{mol photon m}^{-2} \text{s}^{-2}$; temperature, $25 \pm 2^\circ\text{C}$; relative humidity, 65–70%) in growth chamber; 5,000-fold diluted Hyponex solution (Hyponex, Japan) was applied as nutrient every day according to necessity. Cadmium was added to the nutrient medium as CdCl_2 in two concentrations: 0.25 mM and 0.5 mM on ten days old seedlings. Control seedlings were grown in Hyponex solution only. After three days of Cd treatment, leaves were harvested and used for studying various morphological and physiological parameters. The experiment was conducted following Completely Randomized Design (CRD) with nine treatments and it was repeated three times under the same condition.

Measurement of growth parameters

Plant height was taken from each treatment and expressed as cm. Ten randomly selected fresh seedlings from each treatment were weighted, recorded and considered as fresh weight (FW). Dry weight (DW) was determined after drying the seedlings at 80°C in oven for 48 h. Both DW and FW were expressed in milligram (mg).

Measurement of relative water content

Relative water content (RWC) was measured according to Barrs and Weatherly (1962) using following formula:
$$\text{RWC (\%)} = \frac{(\text{FW} - \text{DW})}{(\text{TW} - \text{DW})} \times 100$$
 [Here TW means turgid weight]

Measurement of Cd content

Cadmium content was determined by using an atomic absorption spectrophotometer (Hitachi Z-5000, Japan). The plant samples were oven dried at 80°C for 72 h. The dried samples from root and shoot (0.1 g) were ground and digested separately with acid mixture ($\text{HNO}_3:\text{HClO}_4 = 5:1$ v/v) for 48 h at 80°C . Then absorbance of sample was recorded from atomic absorption spectrophotometer and Cd content of shoot and root was calculated using standard curve of known concentration.

Measurement of chlorophyll content

Chlorophyll (chl) content was measured following the method of Arnon (1949) after extraction of plant leaves by 80% v/v acetone.

Measurement of proline content

Proline (Pro) content was measured according to Bates et al. (1973) using acid ninhydrin.

Histochemical detection of hydrogen peroxide and superoxide

The hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot-}$) were localized histochemically by staining of *Brassica* leaves according to the method described in Chen et al. (2010) with slight modification. Leaves were stained in 0.1% 3-diaminobenzidine (DAB) and 0.1% nitrobluetetrazolium chloride (NBT) solution for 24 h under dark condition for H_2O_2 and $O_2^{\cdot-}$, respectively. Incubated leaves were then immersed in boiling ethanol solution for bleaching. After that brown spots was appeared due to the reaction of DAB with H_2O_2 and dark blue spots was appeared due to the reaction of NBT with $O_2^{\cdot-}$. Finally photographs were taken by placing the leaves on glass.

Measurement of lipid peroxidation

The level of lipid peroxidation was measured by estimating MDA content according Heath and packer (1968) with slight modifications (Hasanuzzaman et al., 2012b). The leaf samples (0.5 g) were homogenized in 3 ml 5% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at $11,500\times g$ for 15 min. One ml supernatant was mixed with 4 ml of thiobarbituric acid (TBA) reagent (0.5% of TBA in 20% TCA). The reaction mixture was heated at $95^\circ C$ for 30 min in a water bath and then quickly cooled in an ice bath and centrifuged again at $11,500\times g$ for 10 min. The absorbance of the colored supernatant was measured at 532 nm and was corrected for non-specific absorbance at 600 nm. The content of MDA was calculated by using the extinction coefficient of $155\text{ mM}^{-1}\text{ cm}^{-1}$ and expressed as $\text{nmol of MDA g}^{-1}\text{ FW}$.

Determination of hydrogen peroxide content

Hydrogen peroxide was determined according to the method of Yu et al. (2003) using 0.1% $TiCl_4$ in 20% H_2SO_4 (v/v).

Measurement of methylglyoxal level

Methylglyoxal was measured following the method of Wild et al. (2012) using sodium dihydrogen phosphate and N-acetyl-L-cysteine.

Extraction and measurement of ascorbate and glutathione

Fresh leaves (0.5 g) were homogenized in 3 ml ice-cold 5% meta-phosphoric acid containing 1 mM Ethylenediaminetetraacetic acid (EDTA) using mortar and pestle. Homogenates were centrifuged at $11,500\times g$ for 12 min at $4^\circ C$, and the supernatant was collected for analysis of AsA and GSH. Ascorbate content was determined following the method of Hunag et al. (2005) with some modifications. The supernatant was neutralized with 0.5 M K-Buffer (pH 7.0) and the oxidized fraction were reduced by 0.1 M dithiothreitol. Total and reduced AsA was assayed spectrophotometrically at 265 nm in 100 mM K-P buffer (pH 7.0) with 0.5 unit of ascorbate oxidase (AO). A specific standard curve with AsA was used for quantification. Oxidized AsA (DHA) was calculated by subtracting AsA from total AsA. The GSH pool was assayed according to

previously described methods (Yu et al., 2003) with modifications as described by Paradiso et al. (2008). Aliquots (0.2 mL) of supernatant were neutralized with 0.3 ml of 0.5 M K-phosphate buffer (pH 7.0). Based on enzymatic recycling, GSH is oxidized by 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) and reduced by nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of GR and GSH content was evaluated by the rate of absorption changes at 412 nm of 2-nitro-5-thiobenzoic acid (NTB) generated from the reduction of DTNB. Oxidized glutathione was determined after removal of GSH by 2-vinylpyridine derivatization. Standard curves with known concentrations of GSH and GSSG were used. The content of GSH was calculated by subtracting GSSG from total GSH.

Determination of protein

The protein concentration of each sample was determined according to Bradford, (1976) using BSA as a protein standard.

Enzyme extraction and assays

Leaf tissue (0.5 g) was homogenized in 1 ml of 50 mM ice-cold K-P buffer (pH 7.0) containing 100 mM KCl, 1 mM AsA, 5 mM β -mercaptoethanol and 10% (w/v) glycerol using a pre-cooled mortar pestle. The homogenates were centrifuged at $11,500\times g$ for 10 min and the supernatants were used for determination of enzyme activity. $0-4^\circ C$ temperature was maintained for performing all the activities.

Lipoxygenase (EC 1.13.11.12) activity was determined following the method of Doderer et al. (1992) by monitoring the increase in absorbance at 234 nm using linoleic acid as a substrate. Superoxide dismutase (EC 1.15.1.1) activity was measured based on xanthine-xanthine oxidase system following the method of El-Shabrawi et al. (2010). Catalase (EC: 1.11.1.6) and GR (EC: 1.6.4.2) activities were assayed following the method of Hasanuzzaman et al. (2012b) by monitoring the decrease of absorbance at 240 and 340 nm, respectively. Ascorbate peroxidase (EC: 1.11.1.11) and DHAR (EC: 1.8.5.1) activities were determined according to the method of Nakano and Asada (1981). Assay mixture for APX contained 50 mM K-P buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2 and 0.1 mM EDTA. Reaction buffer of DHAR contained 50 mM K-P buffer (pH 7.0), 2.5 mM GSH, 0.1 mM EDTA and 0.1 mM DHA. Monodehydroascorbate reductase (EC: 1.6.5.4) activity was measured following the method as described in Hossain et al. (1984). The reaction mixture of MDHAR contained 50 mM Tris-HCl buffer (pH 7.5), 0.2mM NADPH, 2.5mM AsA and 0.5 unit of AO. Glutathione peroxidase (EC: 1.11.1.9) activity was assayed by the method of Elia et al. (2003). The reaction mixture consisted of 100 mM K-P buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide (NaN_3), 0.12 mM NADPH, 2 mM GSH, 1 unit GR and 0.6 mM H_2O_2 (as a substrate). Glyoxalase I (EC: 4.4.1.5) activity was determined following the method of Hasanuzzaman et al. (2012b). Assay mixture of Gly I contained 100 mM K-P buffer (pH 7.0), 15 mM magnesiumsulphate, 1.7 mM GSH and 3.5 mM MG. Glyoxalase II (EC: 3.1.2.6) activity was determined according to Principato et al. (1987) with reaction mixture contained 100 mM Tris-HCl buffer (pH 7.2), 0.2 mM DTNB and 1 mM S-D-lactoylglutathione (SLG).

Statistical analysis

The data were subjected to analysis of variance (ANOVA) and the mean differences were compared by a Duncan's multiple range test (DMRT) using XLSTAT v. 2015 software (Addinsoft, 2015) from three replicates. Differences at $P \leq 0.05$ were considered significant.

Conclusion

The results of our study show that Cd exposure to different *Brassica* species resulted in differences in Cd accumulation, growth, oxidative damage, and antioxidant defense. Growth suppression due to Cd accumulation was observed in all three *Brassica* species with increasing Cd concentration in the growth medium, but no significant decrease in plant height was observed in *B. juncea* even though it had the highest level of Cd of the tested species. Proline content, lipid peroxidation, ROS production, MG accumulation, and chl degradation increased with the increasing dose of Cd, whereas leaf RWC decreased in all three tested species. The level of lipid peroxidation, ROS production, MG accumulation, chl degradation, and leaf RWC reduction were lower and the increase in Pro content was higher in *B. juncea*. Better performance of the antioxidant and methyl glyoxalase systems under both levels of Cd stress was observed in *B. juncea* compared with the other tested species even though it accumulated the highest level of Cd in its roots and shoots. Considering Cd accumulation capacity and physiological attributes, we conclude that of the studied *Brassica* species, *B. juncea* is a relatively tolerant species to Cd toxicity.

Acknowledgments

This research was funded by the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. We thank Mr. Dennis Murphy, United Graduate School of Agricultural Sciences, Ehime University, Japan, for a critical review and editing the English of the manuscript.

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