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ORIGINAL ARTICLE

Differential oxidative stress responses in *Brassica juncea* (L.) Czern and Coss cultivars induced by cadmium at germination and early seedling stage

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Abstract
Heavy metal contamination is a major trouble across the world. In India, there have been many reports of heavy metal pollution due to speedy industrialization and urbanization. The Indian brown mustard is an important oil yielding crop. However, the response of Indian mustard at germination and early seedlings stages to heavy metals like cadmium (Cd) stress is not clear. Current work renders a perceptivity into the part played by enzymatic and non-enzymatic antioxidants towards differential response of Cd (0, 0.5, and 1.0 mM doses) stress in mustard cultivars (Pusa bold, Pusa bahar, and Pusa agrani). The results show that irrespective of dose, Cd severely hamper germination and retard the early seedling growth in mustard cultivars. Pusa bold showed comparatively less reduction in seedling growth as compared to Pusa bahar and Pusa argani. Oxidative stress as measured by lipid peroxidation (MDA), hydrogen peroxide (H_2O_2), lipoxygenase (LOX), and cell death was significantly less in Pusa bold than Pusa agrani. Chlorophyll and carotenoids' content was significantly reduced in Pusa agrani compared to Pusa bold. On the other hand, antioxidant metabolites (proline, ascorbate, and glutathione) showed increased accumulation under Cd stress in Indian mustard; also was the case with antioxidant enzymes (superoxide dismutase, catalase, glutathione-s-transferase, glutathione reductase, ascorbate peroxidase, and peroxidase), which significantly ($p < 0.001$) increased in Pusa bold when compared to other two. This work brings into limelight the significant role of enzymatic and non-enzymatic antioxidants in three varieties of Indian mustard under Cd stress during germination and early seedling growth. The three cultivars in order of decreasing sensitivity to Cd: Pusa agrani > Pusa bahar > Pusa bold

Keywords Cd stress · Germination · Early seeding growth · Antioxidant enzymes · Non-enzymatic

Introduction

Heavy metal pollution in soil is a very serious concern for the living world. Speedy industrialization and urbanization have contributed to exponential increase of heavy metal concentrations in the soil which is ultimately affecting living organisms. It directly poses a negative impact on plant growth, mineral balance, metabolic processes, and yield. Cd is a non-essential heavy metal and is also considered as one of the most potent among top ten toxic heavy metals. Cd is normally noticed in the earth's crust along with zinc, lead, and copper ores. The chief source of Cd contamination in soil is excess use of fertilizer and pesticides (Cheng et al. 2014; Fagerberg et al. 2015), mining, metallurgy, electroplating, etc. When crops are cultivated in Cd contaminated soil, it easily gets absorbed due to its high mobility features and gets accumulated in different parts of plants (Aery and Rana 2003; Lux et al. 2010). Once Cd enter into plant, it reduces growth, and causes mineral nutrition imbalance and photosynthesis inhibition.

Seed germination is a crucial stage in the life cycle of plants. Cd surplus in the soil, which induces diminution in germination rate and seedling growth (Heidari and Sarani

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2011; Shanmugaraj et al. 2013; Bohra and Sanadhya 2015; He et al. 2014; Chen et al. 2011). Cd toxicity stimulates different repercussions at physiological, biochemical, morphological, and molecular levels (Shanmugraj et al. 2013; Daud et al. 2013; Fojtová and Kovãrik 2000; Kapoor et al. 2014). Cd toxicity hastens ROS synthesis in plants. In response to this oxidative burst, many non-enzymatic (proline, ascorbate, and glutathione) and enzymatic (superoxidase dismutase, catalase, peroxidase, glutathione reductase, glutathione-*s*-transferase, and ascorbate peroxidase) systems are induced in plants, for scavenging of these ROS moieties (Mobin and Khan 2007; Li et al. 2013). Previous works have mentioned that the activities of antioxidant enzymes are directly involved with plant's resistance against Cd stress (Ekmekci et al. 2008; Shah et al. 2001; Lannelli et al. 2002). Superoxide anion (O_2^-) is converted into hydrogen peroxide (H_2O_2) with the help of SOD, whereas POD acts to convert H_2O_2 into water (H_2O), and CAT breaks H_2O_2 into oxygen (O_2) and water (H_2O) molecules. On the other hand, metabolites, viz., proline, ascorbate, and glutathione, accumulate in plants in response to stressors not only to regulate osmolarity, but also assist in ROS scavenging activities (Apel and Hirt 2004; Guo et al. 2019; Murtaza et al. 2019).

Most of the work, reaction of mustard to toxicity of heavy metal has been done on late seedling stages (Vatehova et al. 2012; Gill et al. 2011a, b; Nouairi et al. 2009). A few studies were done at seed germination and early seedling growth, but no detailed studies in Indian mustard are present (Bohra and Sanadhya 2015; Marchiol et al. 2006; Bauddh and Singh 2011). Our studies provide a detailed knowledge of physiological, morphological, and biochemical changes due to Cd toxicity in three cultivated genotypes during germination and early seedling stage.

Materials and methods

Three popular varieties of Indian brown mustard (*Brassica juncea*), viz., Pusa bold, Pusa bahar, and Pusa agrani, were acquired from IARI (Indian Agricultural Research Institute) Regional station, Karanal, India. Surface sterilized seeds (in 1% (w/v) NaOCl solution for 15 min followed by washing with distilled water) were put to germination in petri-plate (Borosil, 9.0 cm in diameter) with cotton embedding and treated with solution of Cd ($CdCl_2$ salt, Sigma-Aldrich, molecular weight—183.32 and purity of 99.99%). In this experiment, four concentrations of Cd; 0.5 mM, 1.0 mM, 2.0 mM, and 4.0 mM excluding control and three varieties were selected. During the course of the experiment, 25 seeds were placed in each plate and 10.0 ml of solution was introduced into the petri-plates. Plants were grown in a growth chamber below white light with photon flux density of 52 µmol m^{-2} s^{-1} (PAR) along with a mean day and night temperature of 22/14 ± 3 °C and relative humidity of 62° ± 5%. In this experiment, total fifteen treatment combinations were repeated thrice in 45 petri-plates in a stochastic fashion.

Morphometric attributes

7 DAS (days after sowing) petri-plates were evaluated to assess the final germination percentage (FGP), germination index (GI), seedling vigor index (SVI), and moisture content percentage (MCP) along with seedling length were also measured by adopting the protocols given by Li (2008), Baki and Anderson (1973), and Moulick et al. (2016) respectively, all in triplicates.

Biochemical attributes

During the course of biochemical and thereafter for metal content analysis, doses of 2.0 and 4.0 mM Cd were not considered, for being extremely lethal to all the chosen three varieties (Chowardhara et al. 2019). At 7DAS, intact seedlings from each treatment were evaluated for chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids following Arnon (1949). Lipid peroxidation/malondialdehyde (MDA), lipoxygenase (LOX), and hydrogen peroxide content (H_2O_2) complying with Heath and Packer (1968), Williams et al. (2000), and Alexiva et al. (2001) respectively, whereas proline, glutathione, and ascorbate estimation were done according to Bates et al. (1973), Oser and Hawks (1985) and Anderson (1985), respectively.

Besides these, antioxidant enzyme profiles of seedlings germinated under Cd stress were also elucidated. To prepare enzyme extract for the same, tissue samples were grounded in 0.1 M phosphate buffer supplemented with 1 mM EDTA and 1% PVP. The grounded product was centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatant was employed to determine enzyme activity. For ascorbate peroxidase (APX) extraction, buffer additionally contained 2 mM ascorbic acid. Enzyme activity was estimated for superoxide dismutase (SOD; EC.1.15.1.1), guaiacol peroxidase (POX; EC.1.11.1.7), catalase (CAT; EC.1.11.1.6), glutathione S transferase (GST; EC.2.5.1.18), glutathione reductase (GR; EC.1.8.1.7), polyphenol oxidase (PPO; EC.1.14.18.1), and ascorbate peroxidase (APX; EC.1.11.1.11) following the protocols of Gupta et al. 1993; Chance and Maehly 1955; Habig and Jakoby, 1981; Smith et al. 1988; Mayer et al. 1966; Nakano and Asada 1981, respectively.

Estimation of membrane injury index/cell death

Cell viability was estimated spectrophotometrically by measuring Evan's blue uptake following Yamamoto et al. (2001) protocol. The intact seedling was infiltrated with

0.25% Evan's blue solution for 30 min. After that, seedlings were rinsed with 100 µM $CaCl_2$ three times to remove excess stain. The stained seedling was homogenized in 1% SDS solution and centrifuged at 12,000 rpm for 20 min. The supernatant was quantitated for OD at 600 nm.

Elemental profile of intact *Brassica* seedlings

By complying with the protocol depicted by Gill et al. (2011a, b), Cd contents of intact seedlings of three tested varieties were analyzed. At 7 DAS, intact seedlings were collected from each treatment and washed with tap water succeeded by distilled water to assure the absence of any kind of metal deposition. Intact seedlings were then oven-dried at 72 °C for 48 h and then grounded to fine powder. Before acid digestion, all apparatus (glass-wares and stainless spatulas) were dipped in freshly prepared chromic acid solution for 24 h and again oven-dried. Accurately 0.1 g of plant material from each treatment were acid digested by adding 5.0 ml of a di-acid mixture (containing perchloric acid and nitric acid) in 1:3 ratios along with reagent blank (5.0 ml of acid mixture only) replicated thrice by adopting block digestion method and then quantified the elements using atomic absorption spectrophotometer (AAS-ICE 3500).

Statistical analysis

All the obtained information was evinced as mean ($n = 3$) followed by standard error (mean ± SE) format using SPSS 21 (Windows version) software. Furthermore, difference among the various treatments was determined by employing two-way ANOVA (analysis of variance) and post hoc Tukey's HSD (honest significant difference) test at 0.05 level of significance. Origin Pro 8.5 software was employed for plotting graphs.

Results

Consequence of cadmium on germination and seedling growth

Cd stress exposure led to reduction in the final germination % (significant at $p < 0.001$ and 0.01 levels respectively) was observed, in the examined varieties in accordance with the strength of Cd. Within the three tested cultivars, Pusa bold had the highest rate of germination percentage (76%) even in the highest dose (4.0 mM) of Cd stress, as compared to the Pusa agrani and Pusa bahar varieties. Similarly, the germination index was significantly reduced (at $p < 0.001$ level) in all varieties under Cd. The seedling vigor index also showed gradual reduction dose-dependently. Pusa bold showed the highest seedling vigor index. The seedling length undergoes prominent (at $p < 0.001$ level) diminution with increase in Cd dose. Among the varieties, the growth of Pusa bold was found to be least affected (Table 1).

Measurement of Cd-induced oxidative stress and its impact

Among the varieties considered here, when analysed with respect to controls (grown in absence of Cd), Pusa bold showed least MDA accumulation (by 48.36%) than Pusa bahar (53.31%) and Pusa agrani (52.26%), respectively, grown at 1.0 mM of Cd stress (Fig. 1a). The lipoxygenase content increased profoundly with time and dose of heavy metals. The lowest activity was found in Pusa bold and highest in Pusa agrani. It showed 5.37-, 4.79-, and 3.13-fold increase in Pusa agrani, Pusa bahar, and Pusa bold at 1.0 mM of Cd, respectively (Fig. 1b). Findings from the current experiment suggest that besides MDA, LOX content, H_2O_2 was also found to increase in a linear fashion under Cd stress. At 7 DAS, the maximum level of H_2O_2 was observed in Pusa agrani (67.11%) over control under 1.0 mM Cd stress (Fig. 1c). The ion leakage phenomenon was used as cell death marker. Pusa agrani showed highest ion leakage (210%) after Pusa bahar and Pusa bold, respectively, at 1.0 mM Cd concentration (Fig. 1d).

Effect of cadmium on photosynthetic pigments

Pusa agrani showed the highest reduction (57.15%) of chlorophyll *a*, and then Pusa bahar (42.97%) and Pusa bold (33.74%), respectively, to 1.0 mM Cd stress at 7DAS (Fig. 2a). Similar phenomenon was observed in case of chlorophyll *b*, total chlorophyll, and carotenoids on exposure to Cd stress (Fig. 2b–d).

Effect of Cd on proline accumulation

The proline content significantly increased in all the three varieties of Indian mustard compared to respective controls on Cd treatment. Here, Pusa bold (4.62 fold) showed the highest accumulation, whereas Pusa agrani (2.54-fold) less amount of proline under 1.0 mM Cd stress at 7DAS (Fig. 3a).

Effects on ascorbate and glutathione accumulation

Ascorbate content heightened with the enhancement in Cd dose in all the studied varieties. Among the varieties considered here, Pusa bold experienced the highest increase in ascorbate content in a dose-dependent manner whereas, Pusa agrani showed the least ascorbate concentration (Fig. 3b). Reduced and oxidized glutathione showed significant increase with increase in concentration of Cd. Pusa agrani,

Table 1 Consequences of Cd toxicity on germination, seedling growth, and metal content (in intact seedlings) at 7 DAS (days after sowing)

Cultivar	Cd dose (mM)	Final germination % (FGP)	Germination index (GI)	Seedling vigor index (SVI)	Seedling length (cm)	Moisture content % (MCP)	Metal content (MC) (ppm)
Pusa agrani	0.0	98.66 ± 0.66^{bc}	21.76 ± 0.18^{b}	1353.96 ± 0.46^{d}	13.85 ± 0.14^{d}	91.85 ± 1.18^{c}	0 ± 0^{a}
	0.5	89.66 ± 0.33^{b}	20.22 ± 0.56^{ab}	403.61 ± 1.80^{c}	4.50 ± 0.16^{c}	87.74 ± 0.70^{bc}	3.31 ± 0.01^{cd}
	1.0	81.33 ± 0.66^{ab}	18.47 ± 0.07^{ab}	177.98 ± 2.86^{b}	2.19 ± 0.23^{ab}	80.06 ± 0.74^{bc}	6.36 ± 0.07^{e}
	2.0	73.33 ± 0.33^{a}	17.11 ± 0.74^{ab}	73.38 ± 4.01^{ab}	1.0 ± 0.05^{a}	78.00 ± 0.55^{b}	–
	4.0	37.33 ± 0.66^{a}	12.23 ± 0.10^{a}	29.45 ± 2.79^{a}	0.79 ± 0.08^{a}	69.78 ± 1.18^{ab}	–
Pusa bahar	0.0	91.33 ± 0.66^{bc}	24.06 ± 0.91^{b}	1251.50 ± 0.64^{cd}	13.62 ± 0.31^{d}	92.00 ± 1.18^{c}	0 ± 0^{a}
	0.5	90.0 ± 0.0^{b}	21.52 ± 0.18^{ab}	480.02 ± 0.54^{c}	5.33 ± 0.18^{c}	89.03 ± 0.70^{c}	2.41 ± 0.009^{c}
	1.0	86.33 ± 0.33^{b}	19.53 ± 0.59^{ab}	178.74 ± 1.01^{b}	2.46 ± 0.17^{ab}	86.99 ± 0.74^{bc}	4.59 ± 0.03^{d}
	2.0	74.66 ± 0.66^{ab}	17.28 ± 0.62^{ab}	76.29 ± 2.85^{ab}	1.04 ± 0.25^{a}	78.03 ± 0.55^{b}	–
	4.0	59.33 ± 0.33^{ab}	12.37 ± 0.11^{a}	44.43 ± 1.56^{a}	0.93 ± 0.09^{a}	68.64 ± 0.77^{ab}	–
Pusa bold	0.0	94.33 ± 0.33^{c}	22.64 ± 0.59^{b}	1245.35 ± 0.39^{cd}	13.38 ± 0.35^{d}	86.91 ± 0.46^{b}	0 ± 0^{a}
	0.5	92.66 ± 0.66^{b}	21.68 ± 0.16^{ab}	583.56 ± 3.05^{c}	6.30 ± 0.46^{c}	85.88 ± 2.87^{b}	0.90 ± 0.002^{b}
	1.0	88.33 ± 0.33^{b}	19.72 ± 0.34^{ab}	305.18 ± 3.40^{bc}	3.45 ± 0.17^{b}	84.70 ± 3.15^{b}	3.01 ± 0.003^{cd}
	2.0	84.66 ± 0.66^{ab}	17.9 ± 0.62^{a}	173.85 ± 1.15^{b}	2.05 ± 0.13^{ab}	82.57 ± 2.21^{b}	–
	4.0	75.33 ± 0.66^{ab}	13.69 ± 0.23^{a}	100.59 ± 5.52^{ab}	1.35 ± 0.20^{a}	75.31 ± 2.55^{ab}	–
Source of variations		*F* value					
Variety		4.35*	0.18	10.49***	7.45**	0.922	3.79***
Cd stress		24.68***	22.86***	704.86***	667.08***	39.91***	2.232***
Cd stress × variety		3.86**	0.5	3.46**	2.23*	2.33*	1.042***

Values refer to the mean value ($n = 3$) followed by letter case; values with identical letter case in a column are not significantly different at $p < 0.05$

*, **, and *** indicate that values were significant at $p < 0.05$, 0.01, and 0.001 levels, respectively

Pusa bahar, and Pusa bold showed 4.68-, 7.36-, and 7.95-fold increase in reduced glutathione, respectively, under 1.0 mM of Cd treatment with respect to control (Fig. 3c, d). The results of oxidized glutathione and total glutathione showed similar trend as of reduced glutathione. GSH/GSSG ratio under 1.0 mM of cadmium stress did not show much deviation from what observed in control condition (Fig. 3f).

Effects of cadmium stress on antioxidant enzymes activities

The antioxidant enzymes have crucial roles to play in precluding oxidative stress by detoxification of free radicals. Higher activity denotes better ROS scavenging and, hence, better survival instincts. SOD activity increased by 1.59-, 2.02-, and 2.34-fold in Pusa agrani, Pusa bahar, and Pusa bold, respectively, at 1.0 mM Cd stress, with respect to controls (Fig. 4a). POX activity was also found to increase in a dose-dependent and variety irrespective manner in a highly significant way (at $p < 0.001$ level) for Pusa bold. Among the varieties, an enhancement by 78.34%, 69.49%, and 42.02% in Pusa bold, Pusa bahar, and Pusa agrani can be seen under 1.0 mM Cd stress, respectively, at 7 DAS (Fig. 4b). Similar to previous trend, a moderate-to-high significant (at $p < 0.01$–0.001 level) enhancement in CAT activity can be seen in all the studied varieties in a concentration-dependent mode under Cd stress. At 7 DAS, highest activity of CAT was recorded in Pusa bold (2.04 fold) and least in Pusa agrani (1.38 fold) at 1.0 mM Cd stress (Fig. 4c). The Cd stress on mustard seedlings increased GST activities at early seedling stage. The highest activities were recorded in Pusa bold and Pusa bahar varieties. At 7 DAS, the relative activity of GST was recorded as 69.91%, 74.83%, and 75.13% at 1.0 mM of Cd for Pusa agrani, Pusa bahar, and Pusa bold, respectively (Fig. 4d). GR activities in Pusa agrani, Pusa bahar, and Pusa bold increased by 1.6-, 1.69-, and 2.14-fold, respectively, at 1.0 mM dose of Cd. Pusa agrani relatively showed lesser increase in GR activity (Fig. 5a). Exposure to heavy metals significantly enhanced the functioning of PPO in all tested cultivars. Among the three varieties, though Pusa bold experienced highest PPO activity in a dose-dependent manner, but it was not significant compared to other two. The APX activity significantly heightened with enhancement in the concentrations of Cd. Among the three varieties, Pusa bold showed the highest activity of APX on Cd treatment at 7 DAS (Fig. 5c).

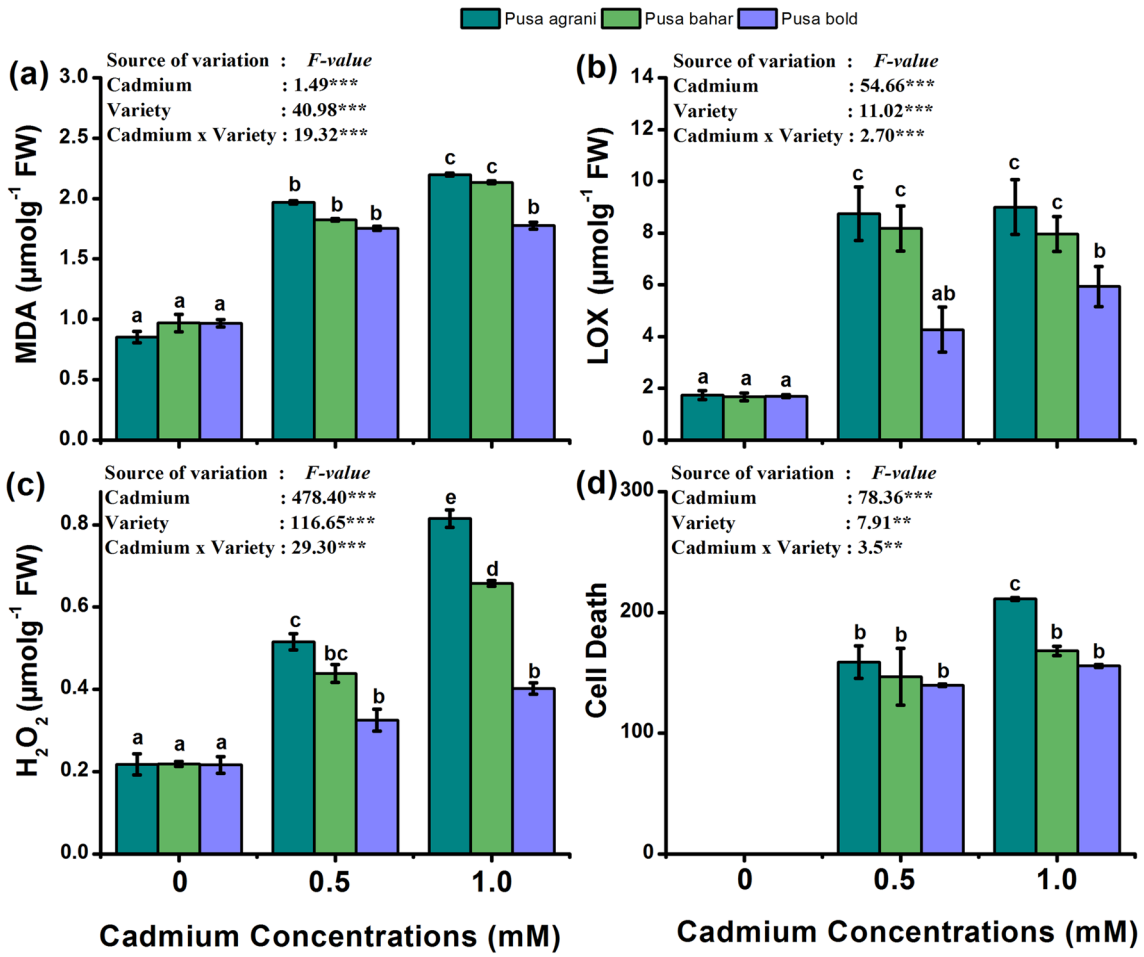

Fig. 1 Impact of Cd stress on **a** MDA, **b** LOX, **c** H_2O_2, and **d** cell death at 7 DAS. Each vertical column represents mean ± SE ($n = 3$) value. Column-bearing same letter cases are not significantly different at $p < 0.05$ level. *, **, and *** indicate that the F values are significant at 0.05, 0.01, and 0.001 levels, respectively

Determination of cadmium content in plant tissue

The atomic absorption spectrometry data for all the varieties showed an enhancement in Cd content when exposed to stress (Table 1). Accumulation of Cd was observed to be more in 1.0 mM Cd treatments. Whereas, among the varieties considered here, the order of Cd content lies in the order Pusa bold < Pusa bahar < Pusa agrani.

Discussion

Germination of seed is a crucial phase in the life cycle of any plant which is highly dependent on variety of environmental factors (Seneviratne et al. 2017; Anjum et al. 2015). Suppression of seed germination under Cd stress may be considered as the absence of necessary/sufficient protective arrangement during this particular stage (germination) of plants' life cycle. At this junction (during germination), for the first time, plants come into contact with external environment. If any kind of stressor exists (biotic/abiotic such as Cd here), it makes germination and seedling growth (early developmental stage) more prone to inhibition (Liu et al. 2012a, b). Generally, heavy metals bear toxic effects on ecosystem, especially in agro-ecosystem. Cd had direct impression on germination, growth, and development of mustard plants. The consequences of Cd on germination were scored as FGP, GI, and SVI which had been earlier recorded in different plants, e.g., mulberry (Chen et al.2019), bread wheat (Bouziani et al. 2019), *Ocimum basilicum* (Singh and Lal, 2018), *Oryza sativa* (He et al. 2014), *Picea omorika* (Prodanovic et al. 2016), and *Suaeda salsa* (Liu et al. 2012a, b) with similar responses. Current findings regarding a decline in FGP, GI, and SVI in all the tested varieties in accordance with stress indicate significant phytotoxicity due to Cd.

If ROS persists for longer duration within the plant cell, it results in undesired consequences like intensification in

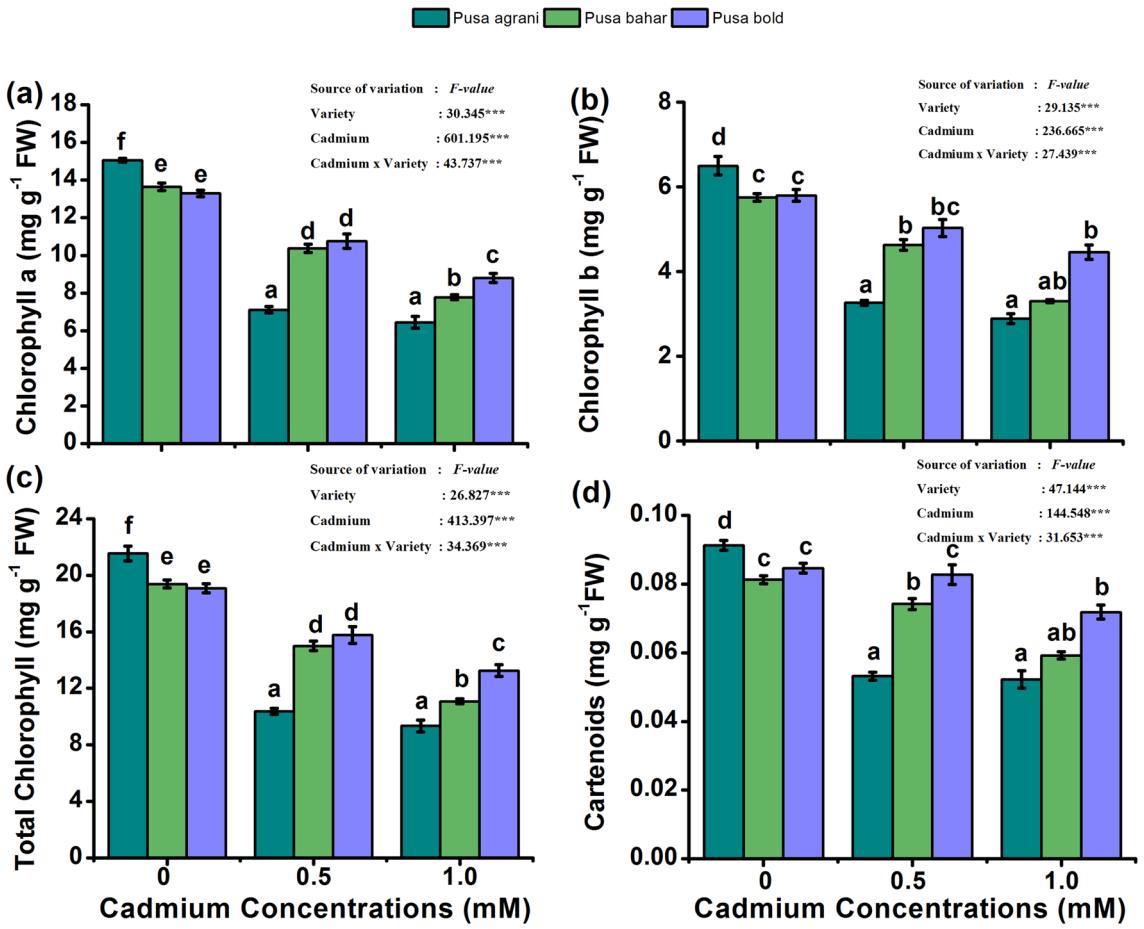

Fig. 2 Impact of Cd stress on **a** chlorophyll *a*, **b** chlorophyll *b*, **c** total chlorophyll, and **d** cartenoids at 7DAS. Each vertical column represents mean ± SE ($n = 3$) value. Column-bearing same letter cases are not significantly different at $p < 0.05$ level. *, **, and *** indicate that the *F* values are significant at 0.05, 0.01, and 0.001 levels, respectively

MDA content (lipid peroxidation), and subsequent loss of ions from the cell which ultimately results in cell death. MDA, H_2O_2, and LOX (associated with lipid peroxidation) content/activity has been employed as a reliable indicant of stress (Aravind et al. 2003; Zhou et al. 2008; Zhang et al. 2016; Samma et al. 2017; Borgohain et al. 2019). The results show marked enhancement in MDA, H_2O_2, and LOX content/activity irrespective of varietal and stressor (Cd) differences in a linear fashion, indicating that Cd have the potential to significantly disrupt the ROS homeostasis in all the tested varieties. Results also show that, from varietal prospect, MDA, H_2O_2, and LOX content/activity follows the order Pusa bold < Pusa bahar < Pusa agrani. Cell death due to loss of membrane integrity has been depicted through enhancement in uptake of EB staining here. EB staining is a commonly employed tool to measured cell death for membrane degradation in numerous crops like *Oryza sativa* (Choudhury and Panda 2004), *Pisum sativum* (Yamamoto et al., 2001), and *Nicotiana tabacum* when exposed to aluminum (Zhang et al. 2016). With respect to control, cell death was more prominent when exposed to Cd in all the cultivars. Among the varieties considered here, the effect was more striking in Pusa agrani whereas least in Pusa bold.

Plant pigments (Chl *a*, *b* and carotenoids) are stress sensitive. Various reports have mentioned that pigments are highly sensitive to heavy metals (Lu and Zhang 2000; Ekmekci et al. 2008). Chlorophyll content has been considered as important stress stimulated biomarker to measure heavy metal phytotoxicity in various crops (Moulick et al. 2017, 2018). Cd stress can lead to a decrease in chlorophyll content in a linear fashion in all the tested varieties, as earlier observed by Shi and Cai (2008).

The prominent diminution in chlorophyll capacity might be the consequence of aggregation of Cd in seedling leaves, which later inhibit the chlorophyll biosynthesis process, stimulate chlorophyll reduction, and cause alternation of magnesium bi-valent ion from chlorophyll with Cd, as it bears alike oxidation state or even by facilitating membrane (thylakoid) damage (Parmar et al. 2013; Kupper and Andresen 2016). The reduction of photochemical

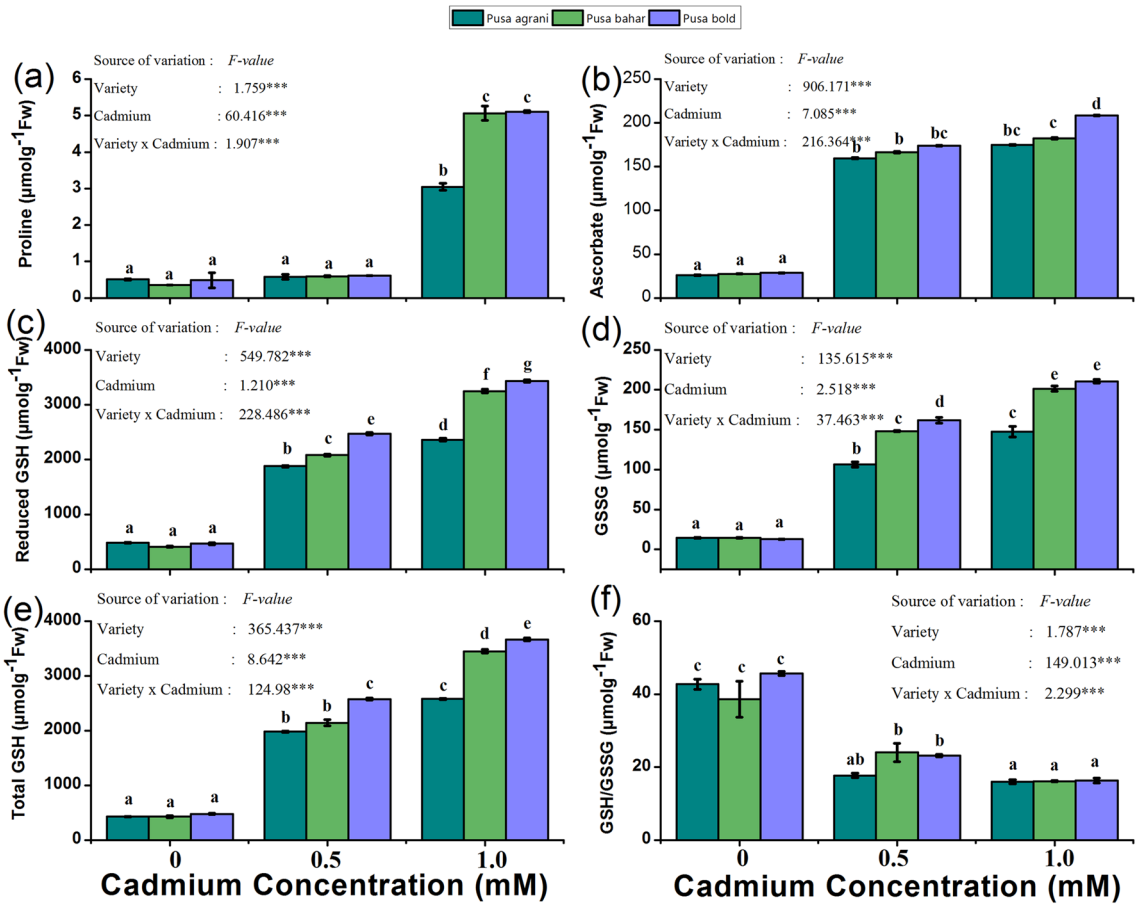

Fig. 3 Impact of Cd stress on **a** proline, **b** ascorbate, **c** reduced glutathione, **d** oxidized glutathione, **e** total glutathione, and **f** ration of GSH and GSSG at 7 DAS. Each vertical column represents mean ± SE ($n = 3$) value. Column-bearing same letter cases are not significantly different at $p < 0.05$ level. *, **, and *** indicate that the *F* values are significant at 0.05, 0.01, and 0.001 levels, respectively

function ultimately leads to diminution in seedling growth in all cultivars due to the degradation of chlorophyll content. Besides these, a significant increment in the functioning of non-enzymatic antioxidants and carotenoids was found. Previous statement was supported by Dias et al. 2013; Jali et al. 2016; Nath et al. 2017 detecting that greater efforts of plants towards ROS quenching activity to withstand excess Cd-induced imbalance of cellular machinery in a significant manner, applicable to all the tested varieties.

Metabolites plays a crucial part in plant abiotic stress responses. Proline, ascorbate, and glutathione are three main metabolites which plays a crucial role during heavy metals stress in plants. Sun et al. (2007) mentioned that free proline combines with Cd to form a non-toxic Cd proline complex. Our results showed that enhancement in Cd dose causes a significant increase in proline content under Cd stress. Similar result was found in different plants under Cd stress, i.e., *Solanum melongena* (Sun et al. 2007), *Malva parviflora* (Zoufan et al. 2018), *Arachis hypogsea* (Dinakar et al. 2009), and *Groenlandia densa* (Yilmaz and Parlak 2011).

GSH-AsA cycle is a major antioxidant system in plants which is responsible for neutralization of ROS moieties (Khan et al. 2019). Our finding shows a significant enhancement in AsA-GSH under Cd stress especially in Pusa bold, as compared to other two cultivated Indian mustard varieties.

Antioxidant enzymes also have a crucial part in ROS scavenging and quenching activities to mitigate oxidative damage caused by too heavy metals. A marked increase in antioxidant enzymes under Cd toxicity irrespective of varietal differences suggests that the studied varieties employ a considerable effort to detoxify ROS induced upon exposure to Cd stress. The present study detected enhancement in functioning of SOD under Cd stress, matches with the findings of Srivastava et al. (2014); Zayneb et al. (2015) who reported about similar observations in *Oryza sativa* (L.) and *Trigonella foenumgraecum* respectively. Irfan et al. (2014) also reported identical enhancement in SOD activity in *Brassica juncea,* but the stress was important at late seedling stage. CAT is generally situated in peroxisomes and mitochondria, while POX is located cytoplasm, membrane,

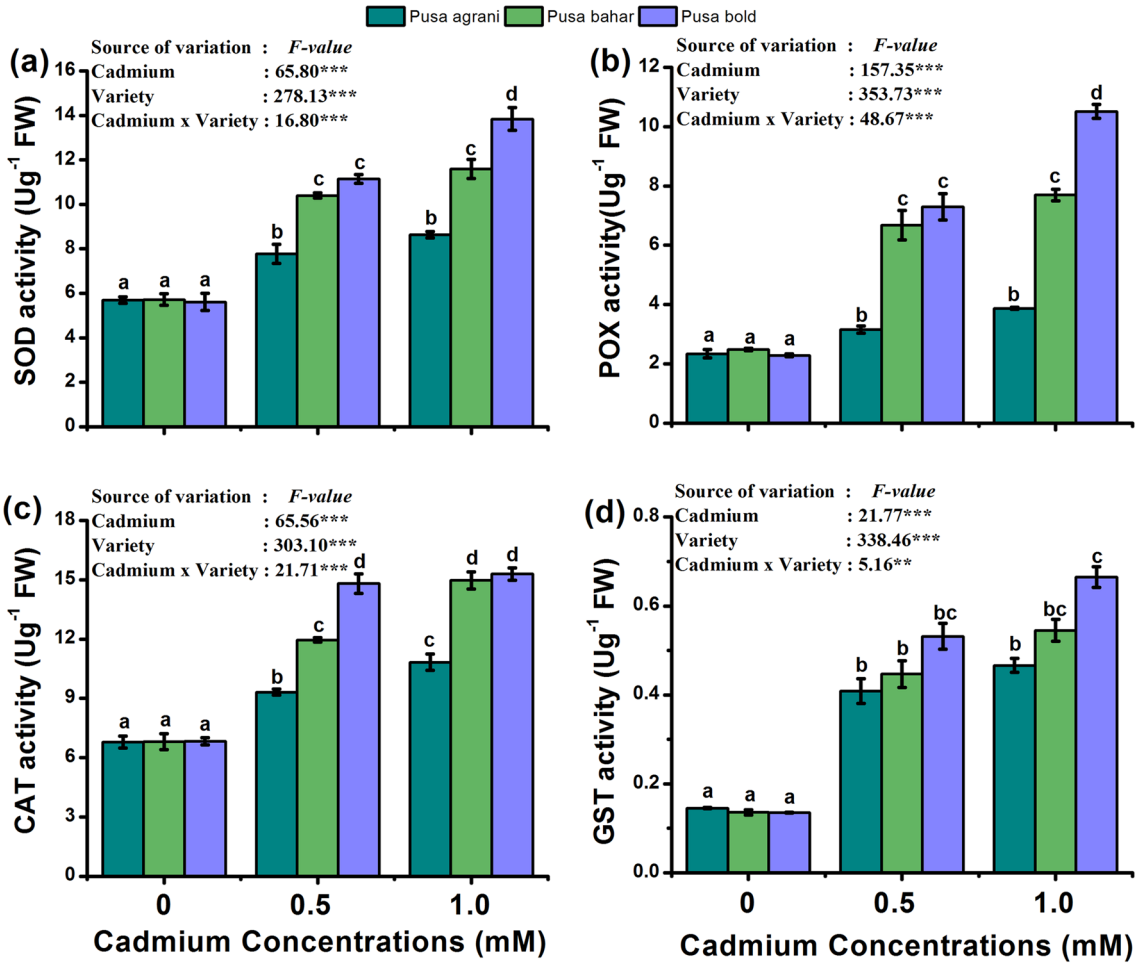

Fig. 4 Impact of Cd stress on **a** SOD, **b** POX, **c** CAT, and **d** GST at 7 DAS. Each vertical column represents mean ± SE ($n = 3$) value. Column-bearing same letter cases are not significantly different at $p < 0.05$ level. *, **, and *** indicate that the F values are significant at 0.05, 0.01, and 0.001 levels, respectively

vacuole, apoplast, extracellular space, and cell wall. Wang et al. (2008) depicted that POX is activated by heavy metal hastened oxidative stress and is more efficient then CAT which was also observed in this study. Prodanovic et al. (2016) and Mohamed et al. (2012) also detected enhancement in CAT activity when exposed to Cd stress in *Picea omorika* and *Brassica juncea* spp., respectively. Cd stress led to enhancement in APX functioning in the three cultivated varieties. Along with the above-mentioned antioxidant enzymes involved directly to combat ROS induced fluctuation in various cellular domains, a considerable increase (compare to the control) in the activity of PPO was also observed. The enhancement in the functioning of PPO in all the studied cultivars exposed to Cd suggests that these varieties employ phenolic compound and metal chelators to withstand heavy metal-induced toxicity. PPO activity in all three varieties increased dose-dependently. Some plant species showed that the activity of PPO under heavy metals stress significantly increased when compared to control (Wang et al. 2008; D'souza and Devaraj 2012). PPO is not directly involved in stress response, but helps in the synthesis of key phenolic compounds, which acts as ROS removers and metal chelators. GST catalyzes GSH binding to xenobiotic and thus plays a vital role in detoxification processes (Davis and Swanson 2001). Several endogenously produced reactive metabolites react with GSH in the presence of GST to produce a conjugate (Nagalakshmi et al. 2001). These conjugates are transported into vacuoles for further degradation and thus protect the plants from oxidative injury (Mohanpuria et al. 2007). GST activity increased with increment in dose of Cd in a dose-dependent and variety independent fashion. GST activity in Cd-induced stress has also been found to increase in *Eichhornia crassipes* and *Salvinia auriculata* (Vestena et al. 2011). Similar to GST, GR activity was also found to increase under Cd stressed condition in all the three varieties during germination and early stage seedlings as compared to control. Similar result was found by Panda et al. (2011) in *Oryza sativa* and Mishra et al. (2008)

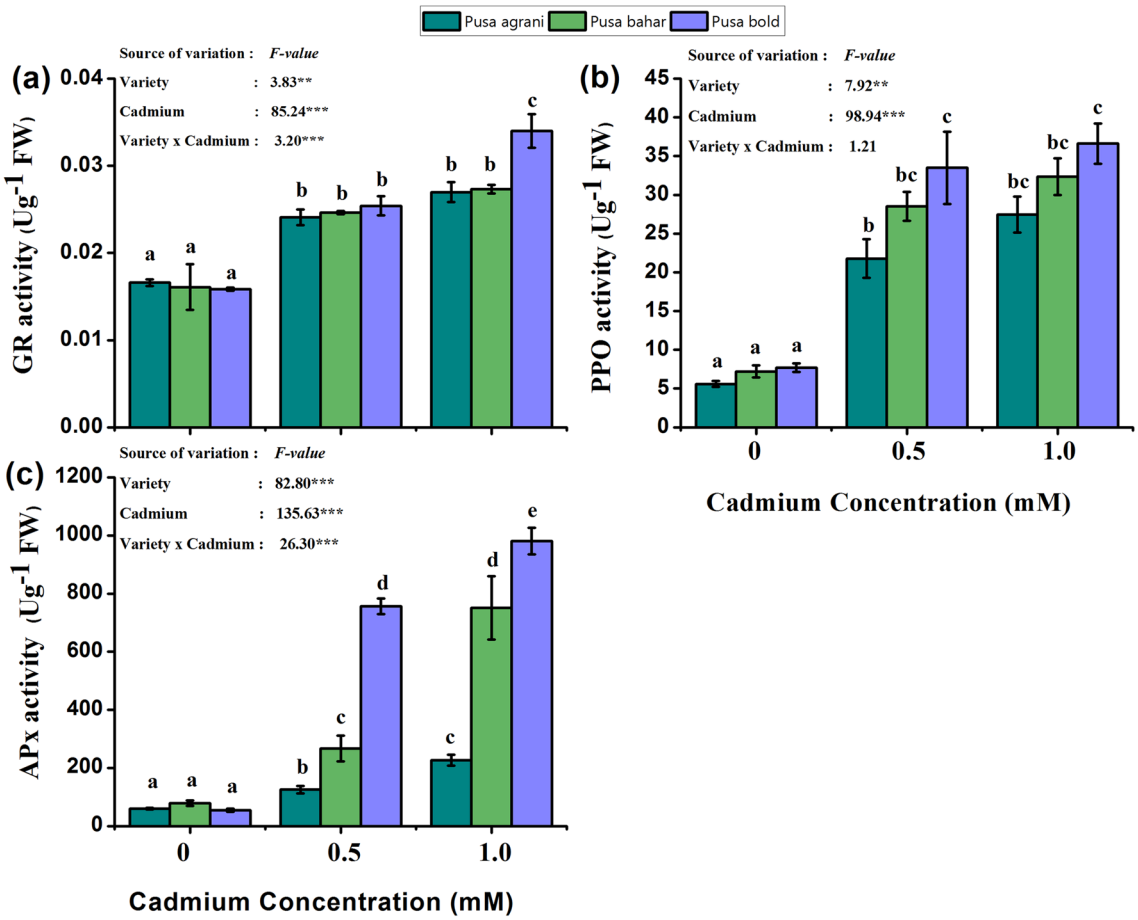

Fig. 5 Impact of Cd stress on **a** GR, **b** PPO, and **c** APx at 7 DAS. Each vertical column represents mean ± SE ($n = 3$) value. Column-bearing same letter cases are not significantly different at $p < 0.05$ level. *, **, and *** indicate that the F values are significant at 0.05, 0.01, and 0.001 levels, respectively

in *Ceratophyllum demersum* L., but oppose the results of Mobin et al. (2007) who detected a decrease in the activity of GR in *Brassica juncea* (L.).

Conclusion

Our experiment demonstrated differential stress response in the three genotypes of Indian mustard under Cd exposure on the basis of morphological, physiological, and biochemical mechanisms at germination stage. The findings depicted that Cd toxicity led to heavy injury in Indian mustard seedlings and also that some defense mechanisms were activated to protect from damages. Pusa bold exhibited more tolerance among the three cultivated varieties. The main reason behind the Cd stress tolerance for Pusa bold was less oxidative stress due to increased enzymatic and non-enzymatic antioxidants. Our results finding give a broad range of implications of Cd stress on Indian mustard at germination as well as early seedling growth stage.

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Author contribution statement BC, BS, and SKP designed experiment. BC and PB performed the experiments. BC wrote manuscript. PB, BS, and JPA analyzed the data and edited manuscript.

Compliance with ethical standards

Conflict of interest No conflict among the authors.

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