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Phytotoxicity of Cd and Zn on three popular Indian mustard varieties during germination and early seedling growth

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ABSTRACT

Heavy metal toxicity is a major bottleneck to crop production throughout the world. India being a fast developing country is highly affected by heavy metal stress. Cd and Zn both pose great threat to crop production when present in excess. This work documents the differential tolerance of three cultivated varieties of *Brassica juncea* through physiological and molecular approaches at germination and early seedling growth. We observed that Zn doesn't inhibit germination whereas Cd does. But Zn effects the growth and development of the seedling after germination. Treatments of Cd (0, 0.5, 1.0, 2.0 and 4.0 mM) and Zn (0, 2.5, 5.0, 7.5 and 10 mM) doses were imparted in germinating mustard seeds. Seedling length, biomass (fresh and dry weight), metal tolerance index and chlorophyll content reduced in mustard varieties. The wrath of stress was more severe in *Pusa agrani* and less in *Pusa bold* under both Cd (2.0mM) and Zn (10.0mM) stress. The histochemical results showed H_2O_2, O_2^-, MDA, and cell death enhanced as dose was increased. *Pusa agrani* was the worst effected whereas *Pusa bold* the least. Various genes govern metal detoxification in mustards. *Pusa bold* showed better regulation in stress mediated abundance of genes depicting enhanced metal tolerance. It was found that among the three varieties *Pusa bold* was the most tolerant followed by *Pusa bahar* and *Pusa agrani* respectively. This is the first of its kind study where the effect of heavy metals has been studied and compared from molecular and varietal prospective at the germination stage.

1. Introduction

Plants suffer from various environmental stresses *viz.,* heavy metals, drought, salinity stresses, etc., which ultimately effect crop yield. Heavy metals form the major soil contaminant due to uncontrolled human activities. Increasing industrial and vehicular effluents, improper disposal of waste-water, fertilizer and pesticide, etc are mainly responsible for heavy metals contamination in soil especially Cd and Zn (Gallego et al., 2012; Adrees et al., 2015; Yousaf et al., 2016). Heavy metal phyto-toxicity brings about drastic changes in morphological, physiological, biochemical and ultra-structural architecture of plants (Ali et al., 2015; Gill et al., 2015).

Cadmium (Cd) is a toxic trace element and is ranked 7th among the top 10 toxic heavy metals. The negative impact of Cd in plants leads to complex genetic, biochemical and physiological changes leading to retarded growth and development (Tkalec et al., 2008), water and nutrient uptake, lipid peroxidation (Balen et al., 2011) and protein degradation (Tkalec et al., 2014).

Zinc (Zn) is basically an essential heavy metal, but when excess, it becomes toxic. Smelter and incinerator emissions, rubber dye, wood preservative industries, sewage sludge, pesticide and fertilizers are the main sources of Zn contamination in agricultural land (Pedler et al., 2004; Sridhar et al., 2005; Giuffré et al., 2012). Reduction in biomass production (Munzuroglu and Geckil, 2002), chlorosis, necrosis (Ebbs and Uchil, 2008), loss of photosynthetic activity (Shi and Cai, 2009), genotoxicity and disturbances in macro and micronutrients homeostasis (Jain et al., 2010) are the main evidences caused by Zn in plants. Zn phyto-toxicity is mediated by oxidative stress consorted lipid peroxidation, leading to membrane damage at cellular level (Singh et al., 2016).

Cd and Zn toxicity drastically effects *Brassica juncea* L. (Indian brown mustard) yield, an economically significant oil seed crop with global availability (Bauddh and Singh, 2012). Cd induces dose and cultivar dependent reduction in biomass (Qadir et al., 2004; Anjum et al., 2008),

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chlorosis in leaves depending on leaf age and effects chlorophyll *a* more as compared to chlorophyll *b* (Ebbs and Uchil, 2008). Xiong et al. (2006) and Sharmila et al. (2017) documented that root length, shoot dry weight, Fe and Mn content gradually decreased with increment in Cd dose in mustard. Contrastingly, it was observed that at low dose, Cd inhibit growth (Meng et al., 2009). Excess amount of Zn present in environment also cause serious problem for mustard plants. Very little works has been done on Zn toxicity in plants. Higher concentrations of Zn cause root and shoot growth inhibition in *Brassica juncea* (Prasad et al., 1999; Feigl et al., 2014). Sagardoy et al. (2009) reported that high concentrations of Zn caused decrease in biomass of *Beta vulgaris* L. Excess amount of Zn present in media causes leaf rolling and damaged roots become brownish with short lateral root. N, Mg, K and Mn concentrations decreased in all parts of plants under the influence of excess Zn. Decreased dry matter yield, chlorosis in young leaves and alternation of the periodic movement of leaves in *Glycine max* under Zn toxicity (Fontes and Cox, 1998). Similar kind of results were found in *Festuca rubra* (Powell et al., 1986), *Nigella sativa* (El-Ghamery et al., 2003), *Phaseolus vulgaris* (Cuypers et al., 2001), *Triticum aestivum* (El-Ghamery et al., 2003), *Vetiveria zizanioides* (Xu et al., 2009), and *Cajanus cajan* (Madhava Rao and Sresty, 2000). Most of these studies were done at late seedling stage thus creating a major research gap on the impact of Cd and Zn toxicity at early germination stage.

A number of plasma membrane transporter family, transcription factors and chelators also regulates the metal homeostasis in plant cells. Such as bZIP transcription factor (CdR15) and phytochelatin synthase 1 (PCS1), cation efflux transporter 2(CET2), metal transporter protein 1 (MTP1), heavy metal transporter 4 (HMA4) and glyoxylase II (GlxII). bZIP family is normally a large group of transcription factor which regulates important biological processes and also has roles to play in drought, salt and heavy metals tress tolerance (Romero-Puertas et al., 2004). MTP1 belongs to cation diffusion facilitator (CDF) family and functions as vacuolar membrane transporter to maintain homeostasis in hyperaccumulator plants. MTP1 is predominantly found in tonoplast membrane in root and shoot tissue of plants. Another transporter family is P_{1B} adenosine triphosphatase (heavy metal ATPase, HMA) which transports metal across membranes. HMA4 belongs to this family and generally present in plasma membrane. It pumps metal ions out of the cell against the gradient force. Glyoxylases acts towards detoxification of methylgloxyal which is excessively synthesized during stress. Glyoxylate system consists of two enzymes i.e., glyoxylase I and glyoxylase II. Phytochelatins binds to metals to form a low molecular weight complex which is transported into vacuoles thus preventing them to come in contact with enzymes or other active substances and thereby causing detoxification of heavy metals (Alberich et al., 2008).

Our intention behind conducting this experiment is to evaluate the responses of three popular mustard varieties towards Cd and Zn stress from ecotoxicology point of view. This particular study involves short duration stress treatment hence dose are high so that the responses are elicited quickly.

2. Material and methods

2.1. Variety description and experimental setup

Seeds of three varieties viz., *Pusa agrani, Pusa bahar and Pusa bold* belonging to *Brassica juncea (L.)* Czern and Coss family were collected from Indian Agricultural Research Institute (IARI) Regional station, Karanal, India. *Pusa agrani* variety is generally cultivated after rice cultivation. Seeds are medium in size (4.5g/1000 seeds) and oil content with 39–40%. The maturing time of *Pusa agrani* is 110 days. *Pusa bahar* attains normally a height of 140–150 cm. The seed weight is 4.5–5g/1000 seeds and oil content 43%. The maturing time of this variety is 108 days. *Pusa bold* variety is cultivated in most part of the country. It is a bold seeded variety with more than 6g/1000seeds weight and having 40% oil content. The maturing period time of this variety is 140 days.

Seeds were first surface sterilized in NaOCl solution for 2 min and washed with distilled water several times. The surface sterilized seeds (25 seeds/plate) were set for germination over cotton bed in petri-plates and treated with 10.0 ml of either distilled water and/solutions of Cd and Zn prepared from a stock solution of cadmium chloride ($CdCl_2$) and zinc chloride ($ZnCl_2$) respectively. In this experiment, we consider five doses for both Cd (0, 0.5mM, 1.0mM, 2.0mM and 4.0mM) and Zn (0, 2.5mM, 5.0mM, 7.5mM and 10.0mM) based on our previous trials with the same varieties and doses. 7 days after sowing (DAS), the petriplates were evaluated for various traits like seedling length, fresh weight, dry weight, chlorophyll content and metal tolerance index (MTI). Chlorophyll content was analysed according to Arnon (1949); whereas, MTI was measured comparing the rate of elongation of treated and untreated seedlings (Wilkins, 1957).

2.2. Histochemical detection of O^{2-}, loss of plasma membrane integrity, H_2O_2 and lipid peroxidation

Detection of superoxide radical, hydrogen peroxide, lipid peroxidation and membrane damage was done by nitroblue tetrazolium salt (NBT) (Rao and Davis, 1999), 3,3'-diaminobenzidine (DAB) (Ramel et al., 2009), Schiffs reagent (Awasthi et al., 2017) and Evans blue (Yamamoto et al., 2001) respectively; in 7d day old seedlings under both control and stressful environment. The whole early stage seedlings were immersed and vacuum infiltrated with 3.0 mg/ml of NBT staining solution in 50.0 mM phosphate buffer (pH 7.5), 4.0 mg/ml DAB staining solution in distilled water (pH 3.8), 10% Schiffs reagent and 0.5% Evans blue staining solution in 100ml of distilled water for 6 h. Stained seedlings was washed in distilled water for 2–3 times in case of DAB and NBT. On the other hand, for Evans blue and Schiffs reagent the seedlings were washed in 100μM $CaCl_2$ and 0.5% sodium metabisulphate solution respectively for 2–3 times. Stained seedlings was bleached in a mixture of acetic acid: glycerol: ethanol (1:1:3) for 10 min at 100 °C and stored in glycerol: ethanol (1:4) solution until photographed.

2.3. Expression analysis of heavy metal stress regulated genes

Total RNA was isolated from 100 mg of plant tissues using RNeasy Plant Mini Kit as per manufacturer's instructions (Qiagen, Germany). cDNA was synthesized using PrimeStar cDNA synthesis kit (Takara, Japan) following manufacturer's instructions. Quantitation of transcript level of different genes was done by semi -quantitative PCR. The primer sequences used for respective genes are enlisted in Table 1.

2.4. Statistical analysis

Here in this experiment, we consider three factors such as varietal differences (3 different varieties) and two stressors (Cd and Zn) with 4 doses of both the stressors and their phytotoxic impacts were evaluated in all the three different varieties. Furthermore, all the treatment combinations (48 combinations) were replicated thrice in one hundred and forty-four (144) petri plates in a randomized fashion. At 7 DAS all the data were obtained and expressed as mean value ($n=3$) followed by SE (standard error). Furthermore, to separate the effects of two stressors on different physiological and on gene expression level from varietal prospective, two-way ANOVA was performed using SPSS (Windows version 21) software at *0.05* level of significance.

3. Result

3.1. Effect of cadmium and zinc on growth

The effect of Cd and Zn doses on growth of three varieties of mustards is elaborated in Table 2 and 3. At the end of 7th days, gradual diminution in seedling lengths was observed with increase in Cd dose for all varieties. Maximum toxicity was observed in *Pusa agrani* at 4.0 mM of

Table 1
The list of different expression primer of genes in *Brassica juncea* (L.) Coss and Czern.

Sl. No	Genes	Primer Sequences	Length (bp)	Amplicon Size (bp)	Tm(°)	Reference
1	*BjUBQ9*	Fw-GAAGACATGTTCCATTGGCA	20	150	55	Ruby chandna et al., 2012
		Rev-ACACCTTAGTCCTAAAACCACCT	23		55	
2	*BjCdR15*	Fw-CCAAGCGCATATCAGCGACA	20	176	54	Bhuiyan et al. (2011)
		Rev-GGCGGAAACCTCCAATCCAC	20		56	
3	*BjCET2*	Fw-ATGCTTCCATGCGCAAGCTC	20	172	54	Bhuiyan et al. (2011)
		Rev-CCAGCAGCCCACAAGGAGAA	20		56	
4	*BjMTP1*	Fw-ATGCTTCCATGCGCAAGCTC	20	172	54	Bhuiyan et al. (2011)
		Rev-CCAGCACCCACAAGGAGAA	20		56	
5	*BjHMA4*	Fw-CACAGGATAAAGCTAGCGAG	20	113	57	–
		Rev-CAAAGGATGACCACAAATTGCC	20		53	
6	*BjGLx II*	Fw-TCAGAGAAGCAGCCGTCGTT	20	155	56	–
		Rev-CATGTTGCAGAGGAACCGCC	20		58	
7	*BjPCS1*	Fw-TTCCGGCAGGAAACGATGTG	20	175	54	Bhuiyan et al. (2011)
		Rev-AGCAGCTGAAGTTGGCGTCG	20		56	

Table 2
Impact of Cd stress upon germination and seedling growth and metal content in (intact seedlings) at 7 DAS (days after sowing).

Variety	HM*	HM level (mM)	Relative Seedling length (cm) (RSL)	Relative Fresh Weight (gm) (RFW)	Relative Dry Weight (gm) (RDW)	Relative Metal Tolerance Index (RMTI)	Relative Total Chlorophyll
Pusa agrani	**Cd**	0.5	67.47 ± 1.42^{cd}	46.35 ± 0.38^{c}	32.46 ± 0.41^{d}	50.93 ± 0.64^{d}	46.97 ± 0.88^{e}
		1.0	84.15 ± 1.78^{e}	67.97 ± 0.65^{f}	45.64 ± 0.74^{f}	73.32 ± 0.79^{f}	49.82 ± 0.63^{f}
		2.0	92.78 ± 0.31^{f}	73.70 ± 0.94^{g}	49.67 ± 0.50^{f}	84.11 ± 0.33^{g}	–
		4.0	94.28 ± 0.63^{f}	89.46 ± 0.09^{h}	66.13 ± 0.83^{h}	89.49 ± 0.23^{h}	–
Pusa bahar	**Cd**	0.5	60.84 ± 1.28^{c}	43.86 ± 0.31^{c}	27.22 ± 0.93^{c}	60.75 ± 0.23^{c}	27.14 ± 0.43^{c}
		1.0	82.31 ± 1.48^{e}	61.59 ± 0.65^{e}	39.67 ± 0.90^{e}	80.68 ± 0.33^{f}	46.68 ± 0.45^{e}
		2.0	92.29 ± 2.10^{f}	76.57 ± 0.57^{g}	40.39 ± 0.20^{e}	90.54 ± 0.24^{g}	–
		4.0	93.07 ± 0.85^{f}	84.79 ± 0.36^{h}	52.75 ± 0.22^{g}	92.76 ± 0.28^{g}	–
Pusa bold	**Cd**	0.5	52.81 ± 3.75^{b}	29.89 ± 0.42^{b}	18.37 ± 0.17^{b}	67.16 ± 0.65^{b}	15.41 ± 0.32^{b}
		1.0	74.20 ± 0.66^{d}	47.24 ± 0.43^{c}	21.78 ± 0.53^{bc}	84.34 ± 0.22^{e}	29.30 ± 0.51^{d}
		2.0	84.58 ± 1.42^{e}	57.83 ± 0.87^{e}	30.69 ± 0.35^{d}	91.76 ± 0.35^{f}	–
		4.0	89.80 ± 1.82^{ef}	74.82 ± 0.47^{g}	38.62 ± 0.20^{e}	94.56 ± 0.01^{g}	–
Source of variation			***F-value***				
Cd *Stress*			219.087***	3.376***	1.058***	3.662***	687.696***
Variety			32.076***	1.136***	1.316***	589.961***	1.068***
Cd *Stress* x Variety			1.787^{ns}	20.60***	31.842***	34.104***	112.957***

(1) 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 *** indicates that values were significant at $p < 0.05, 0.01$ and 0.001 levels, respectively.

Table 3
Impact of Zn stress upon germination and seedling growth and metal content in (intact seedlings) at 7 DAS (days after sowing).

Variety	HM*	HM level (mM)	Relative Seedling length (cm) (RSL)	Relative Fresh Weight (gm) (RFW)	Relative Dry Weight (gm) (RDW)	Relative Metal Tolerance Index (RMTI)	Relative Total Chlorophyll
Pusa agrani	**Zn**	2.5	76.63 ± 0.72^{c}	56.58 ± 0.74^{d}	16.67 ± 0.64^{e}	72.38 ± 0.33^{b}	51.74 ± 0.15^{e}
		5.0	82.34 ± 0.51^{d}	60.68 ± 0.55^{e}	19.26 ± 0.09^{f}	78.01 ± 0.37^{c}	54.04 ± 0.86^{e}
		7.5	86.40 ± 0.60^{f}	66.65 ± 0.30^{g}	22.69 ± 0.65^{g}	80.55 ± 0.39^{d}	–
		10.0	87.78 ± 0.17^{f}	69.46 ± 0.25^{h}	24.62 ± 0.58^{g}	82.58 ± 0.14^{d}	–
Pusa bahar	**Zn**	2.5	76.35 ± 0.34^{c}	48.71 ± 0.42^{b}	12.03 ± 0.58^{d}	76.97 ± 0.28^{c}	29.06 ± 0.69^{c}
		5.0	80.57 ± 0.09^{d}	56.82 ± 0.42^{d}	16.08 ± 0.40^{e}	82.75 ± 0.52^{d}	46.68 ± 0.45^{d}
		7.5	84.68 ± 0.34^{e}	62.97 ± 0.20^{f}	$18,20 \pm 0.20^{f}$	84.64 ± 0.30^{e}	–
		10.0	85.60 ± 0.37^{e}	63.39 ± 0.33^{f}	23.99 ± 0.35^{g}	85.57 ± 0.36^{e}	–
Pusa bold	**Zn**	2.5	72.13 ± 0.48^{b}	46.96 ± 0.23^{b}	4.84 ± 0.39^{b}	76.39 ± 0.28^{c}	27.14 ± 0.43^{b}
		5.0	77.57 ± 0.30^{c}	49.60 ± 0.27^{c}	7.22 ± 0.44^{c}	81.49 ± 0.63^{d}	32.52 ± 0.43^{c}
		7.5	81.24 ± 0.30^{d}	58.28 ± 0.31^{e}	9.91 ± 0.66^{c}	86.27 ± 0.68^{e}	–
		10.0	83 ± 0.25^{e}	60.21 ± 0.49^{e}	16.40 ± 0.54^{e}	87.67 ± 0.18^{e}	–
Source of variation			***F-value***				
Variety			140.899***	552.543***	544.080***	159.470***	898.324***
Zn *Stress*			369.774***	714.044***	242.910***	358.208***	349.452***
Zn *Stress* x Variety			2.448*	10.665***	5.539**	2.389*	107.610***

(1) 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 *** indicates that values were significant at $p < 0.05, 0.01$ and 0.001 levels, respectively.

Cd in contrast to *Pusa bold* under Zn stress. As such Zn didn't effect germination but showed severe impact on seedling growth in the three varieties. (Figs. 1 and 2).

3.2. *Effect of cadmium and zinc on biomass*

In case of fresh and dry weight, both Cd and Zn stress, induce reduction after 7 days. Most prominent and significant effect of Cd was found in *Pusa agrani*, where fresh and dry weight were found to be decreased by 89.46% and 66.13% at 4.0 mM, respectively. Whereas *Pusa*

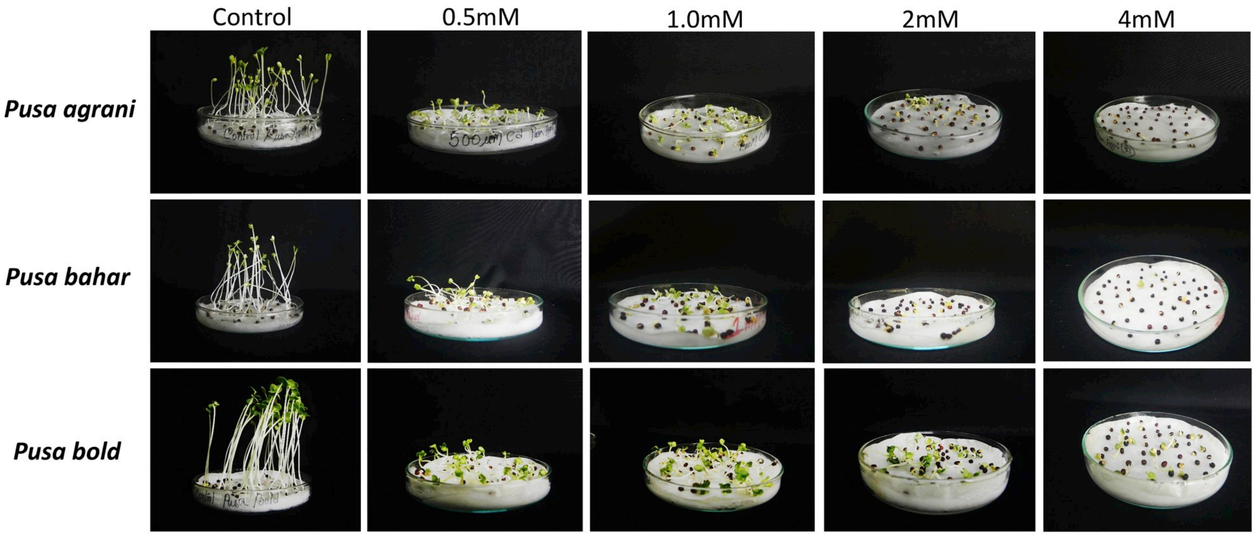

Fig. 1. Physiology of germinating seeds due to cadmium excess. 25 seeds where allow to germinate in cadmium (0, 0.5, 1.0, 2.0, and 4.0 mM) containing stratum for 7 days and its effects on physiology recorded. Experiment was repeated at 3 times where 25 germinating seeds were taken as a unit.

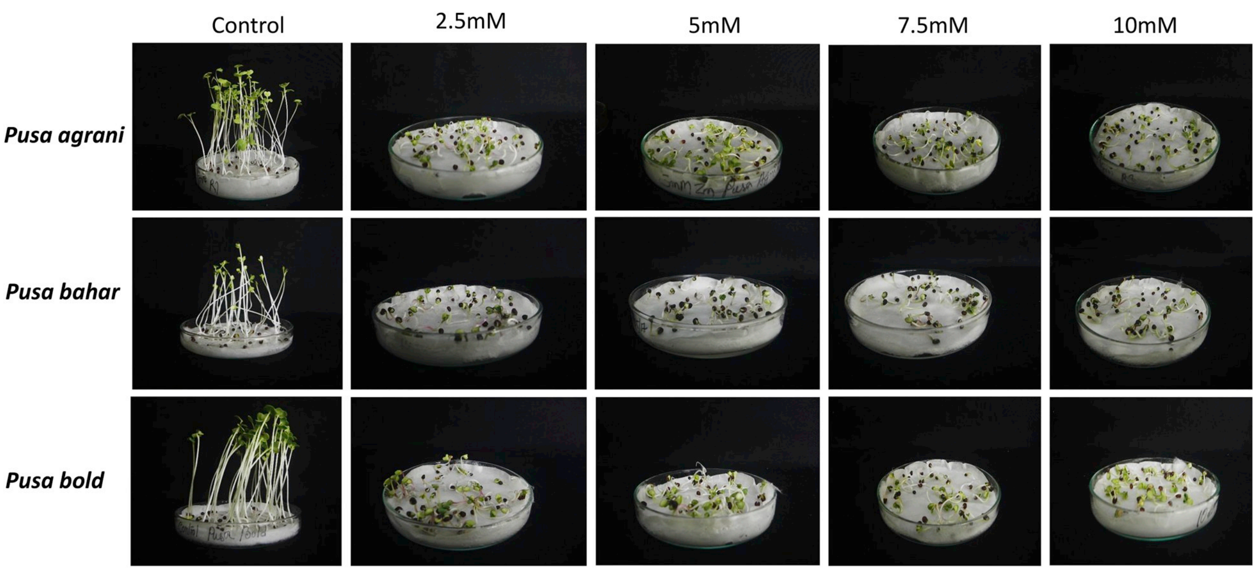

Fig. 2. Physiology of germinating seeds due to zinc excess. 25 seeds where allow to germinate in zinc (0, 2.5, 5.0, 7.5, and 10.0 mM) containing stratum for 7 days and its effects on physiology recorded. Experiment was repeated at 3 times where 25 germinating seeds were taken as a unit.

bold observed 74.82% and 38.62% of relative fresh and dry weight at 4.0 mM of Cd,. Similar kind of result was found in case of Zn where *Pusa agrani* show highest reduction of fresh and dry weight (69.46% and 24.62%) at 10.0 mM of Zn. On the other hand, *Pusa bold* showed less reduction of fresh (60.21%) and dry (16.40%) weight at 10.0 mM of Zn. Three varieties when compared, *Pusa bold* showed less reduction of fresh and dry weight and *Pusa agrani*, the highest respectively under both Cd and Zn treatments (Table 2, 3).

3.3. *Effect of cadmium and zinc on metal tolerance index*

The MTI drastically decreased with increase in the concentrations of both Cd and Zn in all the three varieties. Within the varieties, *Pusa agrani* depicted the least MTI (89.94%) at high dose of Cd and *Pusa bold* showed the highest (94.56%). Similar results were obtained when treated with Zn (Table 2, 3).

3.4. *Effect of cadmium and zinc on pigment system*

Cd and Zn profound adverse effect on pigment system of *Brassica juncea* (Table 2 and 3). Under Cd stress, *Pusa agrani* showed highest total chlorophyll reduction (49.82%) at 1.0 mM Cd treatment and *Pusa bold* showed minimum reduction in total chlorophyll content (29.30%) at 1.0 mM Cd treatment. Similarly, result was found under Zn stress in the three cultivated varieties. A maximum reduction of total chlorophyll was profoundly in *Pusa agrani* (54.04%) where *Pusa bahar* show mid reduction of total chlorophyll (46.68%) and *Pusa bold* showed least reduction of total chlorophyll content (32.52%) at 5.0 mM Zn (Table 2, 3).

3.5. *Histochemical detection of O_2^-, H_2O_2, lipid peroxidation and plasma membrane integrity*

Under normal physiological condition, the varieties showed minimal ROS accumulation and cellular damage as depicted from intensity of coloration in stained samples. But when exposed to Cd and Zn stress, the seedlings suffered from severe oxidative stress. Higher accumulation of O_2^- (dark blue spots) and H_2O_2 (dark brown spots) (Figs. 3 and 4) were clearly seen in young seedlings under both Cd and Zn stress respectively, as compared to control. Here, *Pusa agrani* showed higher accumulation of O_2^- and H_2O_2 was seen at 1.0 mM Cd and 5.0 mM Zn stress condition; whereas *Pusa bold* clearly showed lesser accumulation of O_2^- and H_2O_2 as compare to other two varieties (Fig. 3).

On other hand, lipid peroxidation (pink spots) and cell death (blue spots) was also clearly detected in the 7DAS old seedlings. *Pusa agrani* showed highest lipid peroxidation and cell death as seen at 1.0 mM Cd and 5.0 mM Zn stress respectively. Whereas *Pusa bold* showed less lipid peroxidation and cell death. Comparison among the three cultivated varieties under Cd and Zn stress, showed lesser amount of lipid peroxidation, H_2O_2, O_2^- and cell death as compared to *P. bahar* and *Pusa agrani* (Figs. 3 and 4).

3.6. *Expression analysis of heavy metal stress regulated genes*

Normalization of cDNA was done with housekeeping gene UBQ9. Total seven genes were selected for expression analysis upon treatment with Cd and Zn viz., *Bj*CdR15, *Bj*CET2, *Bj*CET3, *Bj*MTP1, *Bj*GlxII, *Bj*HMA4, and *Bj*PCS1 in all the three studied varieties. The genes selected are heavy metal transporters and stress response elicitors to deduce the impact of heavy metal treatment on their expression. Expression of all genes were found to be up-regulated under both Cd and Zn stress but the degree of up-regulation varied among the three varieties. The activities of *Bj*CdR15 was highest in *Pusa bahar* and least in *Pusa agrani* under both Cd and Zn stress (Fig. 5). . The activities of CET2 was found to be significantly enhanced in three cultivate mustards under Cd treatment, whereas for Zn, significant enhancement was observed in *Pusa agrani* and *Pusa bahar*. For *Bj*MTP1 under both Cd and Zn stress *Pusa bold* showed drastic increase in transcript abundance when compared to the other two (Fig. 5). In case of *Bj*HMA4, the highest expression of HMA4 was found in *Pusa agrani* and least in *Pusa bold* as compared to control respectively. *Bj*GlxII expression pattern was found to be similar to that of *Bj*HMA4. For *Bj*PCS1, drastic change in transcript abundance was observed for all the three varieties when treated with both Cd and Zn (Figs. 5 and 6).

4. Discussion

Cd and Zn toxicity poses serious threat to crop production worldwide. Through this study the physio-molecular aspect of Cd and Zn toxicity at germination stage has been brought to limelight. The findings from the analysis of various physiological parameters i.e., seedling length, fresh and dry weight and heavy metal tolerance index of mustard plants established that the Cd and Zn has inhibitory effect on these parameters in all the three studied varieties (Table 1 and 2). Similar results were found in different cultivate crops under different heavy metals stress when applied after germination *Oryza sativa* L. (Panda et al., 2011), *Fragaria x ananassa* cv. Camarosa (Muradoglu et al., 2015), *Elsholtzia argyi* (Li et al., 2015), *Zea mays* L. (Anjum et al., 2015), under Cd stress where *Beta vulgaris* L. (Sagardoy et al., 2009) under Zn stress.

Cd cause diminution in fresh and dry weight with increment in dose and time. Such results were inferred in different plants under Cd stress at post germination stage; *Brassica juncea* (Qadir et al., 2004, Ahmad et al., 2011), *Raphanus sativa* (Anuradha and Rao, 2007), *Solanum nigram* (Deng et al., 2010), *Oryza sativa* (Huang et al., 2018; Panda et al., 2011). Similarly, for Zn also diminution in fresh and dry weight was observed.

The metal tolerance index (MTI) is one of the most prominent tool for determination of stress tolerance potential of genotypes (Wilkins, 1957, 1978). Our finding results showed that *Pusa bold* has high capacity of metal tolerance rather than *Pusa bahar* and *Pusa agrani* under Cd and Zn stress (Table 1 and 2).

Previous findings of Lu and Zhang (2000); Tanyolac et al. (2007) indicated that, pigments (chlorophyll *a*, *b* and carotenoid) system is highly sensitive to heavy metals and other adverse conditions in higher plants. Chlorophyll content has been regarded as a trustworthy stress induced biomarker to evaluate heavy metal induced phyto-toxicity in different field crops, (Moulick et al., 2017, 2018a, 2018b; 2018c). Results shows that both Cd and Zn can inhibit chlorophyll content in accordance with the doses in all the tested varieties (Table 1 and 2) , supports the findings of Dhir et al. (2008). The significant reduction in chlorophyll content might be a consequence of excess accumulation of Zn and Cd in seedlings leaves, which in turn may resulted into either (a) reduction in chlorophyll biosynthesis, (b) increase in chlorophyll degradation, (c) replacement of magnesium bivalent-ion from chlorophyll with either Zn or Cd due to having similar oxidation state or even by (d) stimulating membrane (thylakoid) damage (Meitei and Prasad, 2014; Küpper and Andresen, 2016) yet to be confirmed. Reduction in chlorophyll content might have resulted into decrease in photochemical

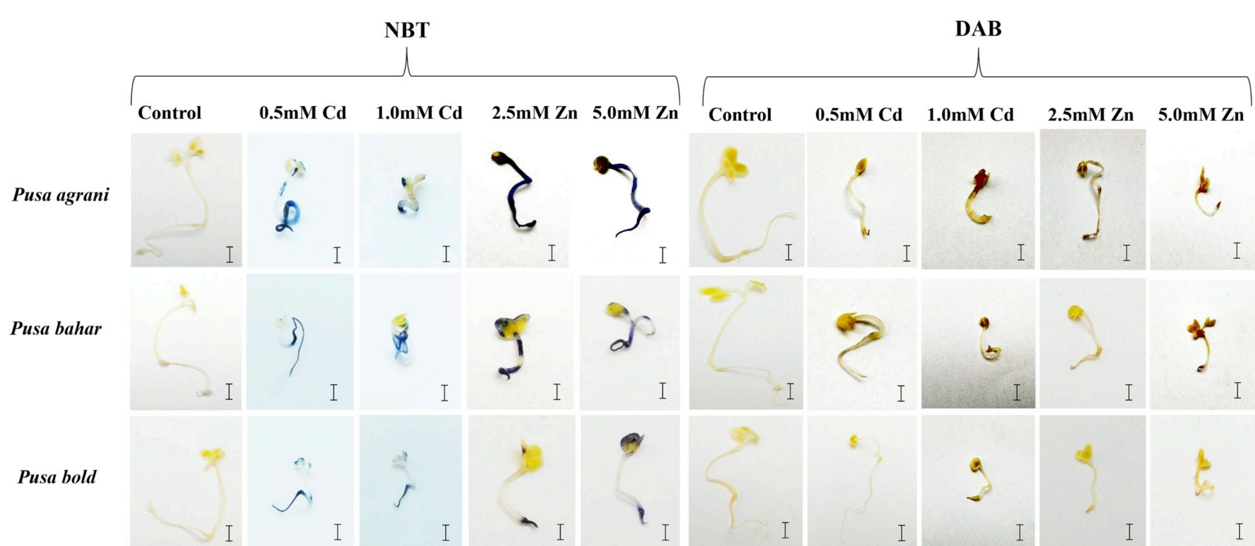

Fig. 3. Histochemical detection of O_2^- and cell death: Histochemical detection of O_2^- by NBT and H_2O_2 by DAB staining in three varieties of *Brassica juncea* (*Pusa agrani, Pusa bahar* and *Pusa bold*) under control condition as well as cadmium and zinc stress.

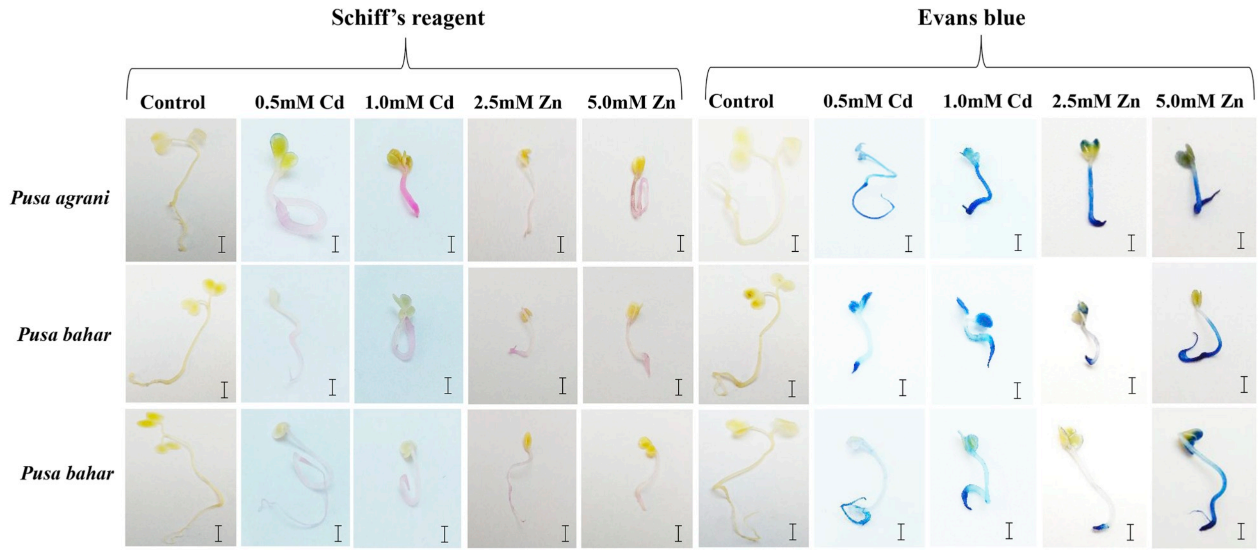

Fig. 4. Histochemical detection of H_2O_2 and lipid peroxidation: Histochemical detection of lipid peroxidation by Schiff's and cell death by Evans blue staining in three varieties of *Brassica juncea* (*Pusa agrani, Pusa bahar* and *Pusa bold*) under control condition as well as cadmium and zinc stress. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

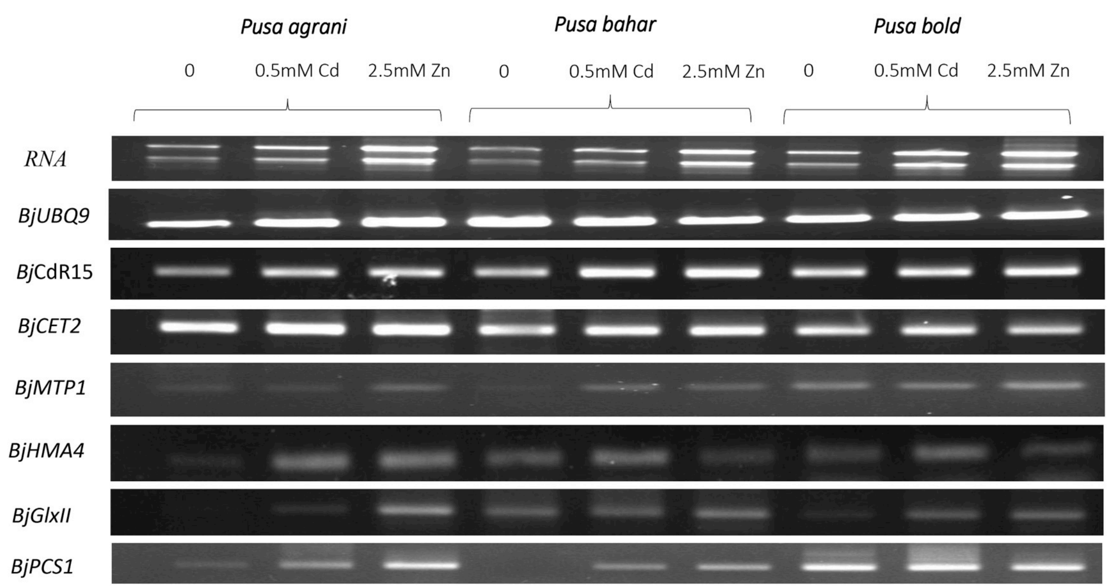

Fig. 5. Semi-quantitative PCR of *BjCdR15, BjCET2, BjMTP1. BjHMA4, BjGlxII* and *BjPCS1* under cadmium and zinc stress: Semi-quantitative PCR of *BjCdR15, BjCET2, BjMTP1. BjHMA4, BjGlxII* and *BjPCS1* under cadmium and zinc stress at early stages of seedling of three cultivated varieties of *Brassica juncea* (*Pusa agrani, Pusa bahar* and *Pusa bold*). The cDNA quantity was normalized with *BjUBQ9*.

function including gas exchange process which can be taken as a justification for reduction in seedling growth in all the tested varieties. Prior to this investigation authors like Arenas-Lago et al. (2016) and Kumar et al. (2012) also reported the similar reduction in chlorophyll content and can be attributed to the investment of greater efforts towards ROS quenching activities by seedlings subjected to Cd and Zn stress, in a variety irrespective manner.

Stressors including heavy metal stress induces excess production of ROS. Now if plant cell cannot eliminate the ROS may leads to some fatal consequences. If adequate measures are not adapted by the plants, with the ROS mediated enhancement in MDA and other toxic metabolite content leads to cell death. ROS induces not only lipid peroxidation which in turn directly effects cell membrane. ROS accumulation also causes oxidative damage by producing lipid-derived radicals (Montillet et al., 2005). Histochemical study showed that increasing the concentrations of Cd and Zn ultimately increases lipid peroxidation (Fig. 4). Many plants were found to speed up lipid peroxidation like *Nicotiana tabacum* L. (Ye et al., 2016), *Oyrza sativa* L. (Srivastava et al., 2014) under Cd stress. Cell death occurs as a result of significant alternation in membrane integrity further confirmed by increased uptake of Evans blue staining here (Fig. 4). Evans blue staining is a widely used tool to visualize stress induced membrane damage in a wide range of crops like in *Pisum sativum* (Yamamoto et al., 2001) and *Nicotiana tabacum* under aluminum (Zhang et al., 2016); *Medicago sativa* under copper (Samma et al., 2017); *Cucumis sativus* L. under pharmaceutical and personal care products (Sun et al., 2018). Compare to the control, cell death was higher when treated with Cd and Zn in all the varieties. Though Zn treatments also showed similar trend, but was less severe than that of Cd induced cell death. Among the varieties considered in the current investigation, the adverse effects were more marked in *Pusa*

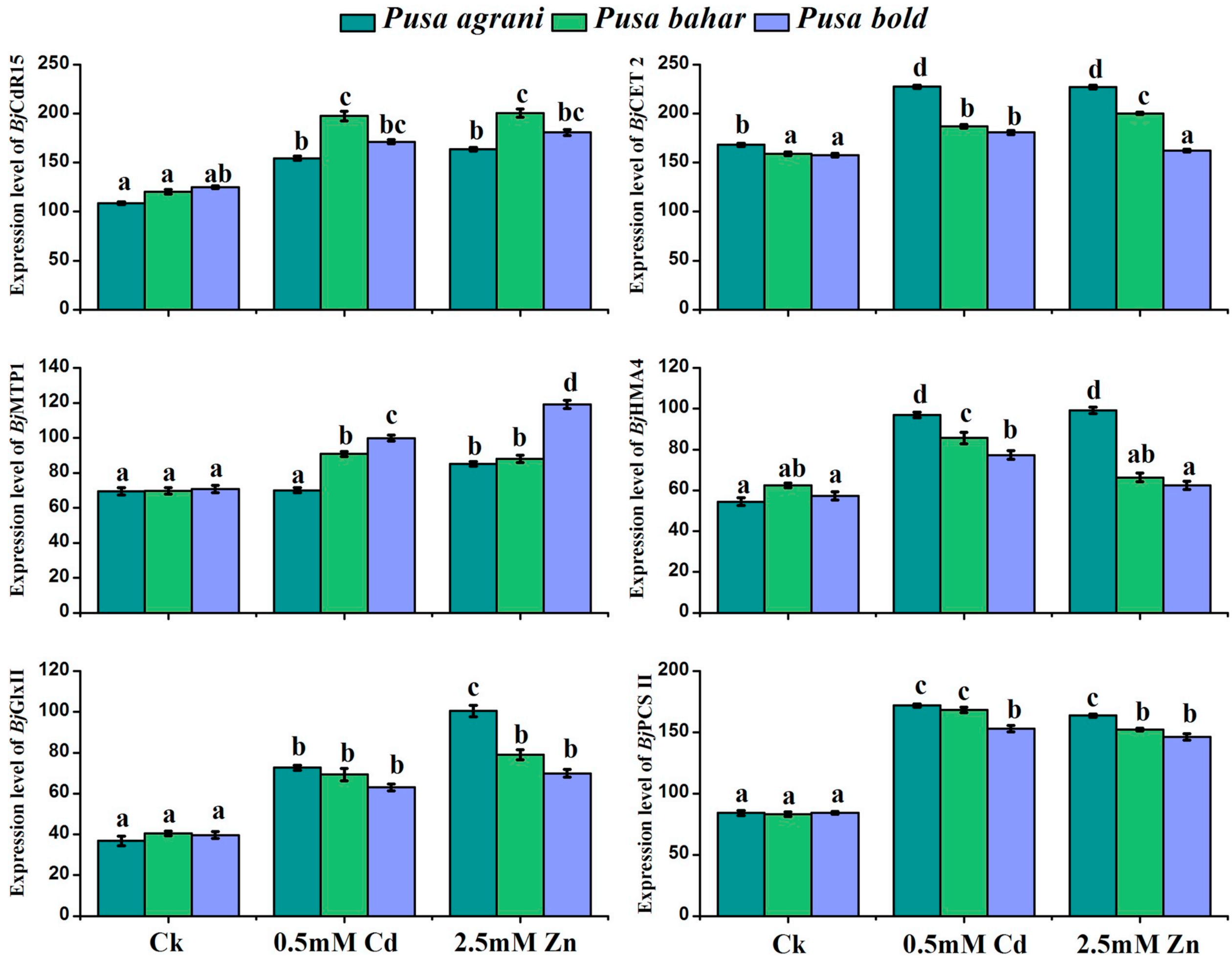

Fig. 6. Relative expression of *BjCdR15, BjCET2, BjMTP1. BjHMA4, BjGlxII* and *BjPCS1* under cadmium and zinc stress: Relative expression was scored by scanning the band intensity with Image J software. Each vertical column represents mean ± SE (n = 3) value. Column bearing same letter cases are not significantly different at $p < 0.05$ level.

agrani whereas least in *Pusa bold.*

The plants are governed by numerous genes which play significant role to detoxify heavy metal through sequestration. Few of these genes (*Bj*CdR15, *Bj*CET2, *Bj*MTP1, *Bj*HMA4, *Bj*Glx II, *Bj*PCS1 and *Bj*UBQ9 studied here) were chosen to monitor their expression pattern in Indian mustard under the influence of Cd and Zn stress (Bhuiyan et al., 2011; Chandna et al., 2012). Furthermore, among the studied genes, *Bj*CdR15 helps in regulation of Cd uptake from roots and transport from root to shoot (Farinati et al., 2010). The transcript abundance of this gene increased with dose in all three varieties. CET2 is responsible for deposition of heavy metals in leaves (Xu et al., 2009). CET2 was most expressed in the sensitive variety *Pusa agrani*. MTP falls in the cation diffusion facilitator family. Normally *Bj*MTP1 is localized in the plasma membrane and responsible for transport of heavy metal to vacuole for detoxification (Muthukumar et al., 2007). Although this gene expression is less in all the varieties, the tolerant *Pusa bold* showed the highest expression. *Bj*HMA4 is expressed in roots and play a critical part in heavy metal homeostasis and tolerance in plants (ZHANG et al., 2011). HMA4 showed variable expression with dose among varieties. The role of GlxII in *Brassica juncea* is very important for detoxification of Cd and Zn induced toxicity. Generally, glyoxylase plays many key functions in plants growth and development under normal conditions (Hasanuzzaman and Fujita, 2013). The transcript abundance increased with stress dosage. *Bj*PCS1 is prominently expressed in root and play a role for metal detoxification in plant cell. PCS interact with metals to form complexes which is then transported into vacuoles and hence reduce the toxic effect (Chang et al., 2012). This gene also increased with stress dosage but highest expression in *Pusa bold.* It was interesting to note that the genes which are directly responsible for vacuolar deposition of heavy metals (MTP and PCS) are highly expressed in the tolerant variety though it showed lesser accumulation when metal content was analysed (Fig. 5). This might be explained by saying that the vacuolar deposition reached its limit which led to leaching out.

5. Conclusion

The current study brings into limelight the differential response mechanism of Indian mustard to Cd and Zn stress on short-term exposure during germination stage. This particular study for the first time clearly depicts expression profile of various transporters as well as heavy metal chelators leading to differential heavy metal accumulation in early seedling stage. Out of the three studied varieties evaluated for differential tolerance, *Pusa bold* seems to have better Cd and Zn tolerance capability at germination stage whereas *Pusa agrani* was the least tolerant. As the mustard (oil and other products) is widely used and consumed by large population therefore, it is obvious that transmission of toxic chemicals should be minimized as much as possible. In future if further work can be carried out then not only ecotoxicological aspects of Cd and Zn will emerge out but from agronomical prospect will also get an edge to combat such situation.

Conflicts of interest

The authors declare no conflicting interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101349.

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