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Assessment of phytotoxicity of zinc on Indian mustard (*Brassica juncea*) varieties during germination and early seedling growth

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ABSTRACT

The intention behind conducting this study was to evaluate the toxicological response of exposing of three popular varieties [Brassica juncea (L.)Coss and Czern]to zinc at germination and early seedling stage. The experiment was conducted at Department of Life Science of Assam University; Silcharin December 2018. The surface sterilized seeds of uniform size (of three varieties) were set for germination over cotton bedin petri-plates,treated with solution of zinc of various strengths (0.0, 2.5, 5.0 and 10.0 mM), prepared from a stock solution of zinc chloride (1.0 M). Results indicated that zinc could impose significant phytotoxicity during germination and seedling growth in all the three varieties. Among the three varieties, Pusa bold was comparatively a zinc tolerant variety,followed by Pusa bahar and Pusa agrani. An inhibition of germination from 9.34 to 17% followed by reduction in seedling length was recorded with zinc application. An enhancement in lipid peroxidation by 42.49%, hydrogen peroxide content by 88.74% was also been observed in the germinated seedlings .An increase in various antioxidant activity, SOD (1.86 fold), CAT (1.96 fold), POX (55.76%), GR (4.6 fold), GST (62%) and APx (87.96 %); as well as zinc content in the intact 7 days old seedling were recorded in all the three varieties with increasing doses of zinc.

Keywords: Indian mustard, zinc, reactive oxygen species, antioxidant, varietal difference.

INTRODUCTION

Plants have to confront diverse abiotic and biotic stresses (either alone or in combination) because of being sessile, which profoundly affect sustainable crop production from time to time. The menace of phytotoxicity from heavy metals has increased exponentially due to uncontrolled human activities, like industrial and vehicular effluents, improper disposal of waste water, fertilizers, etc (Yousaf *et al.* 2016). Zinc is one of the most important essential nutrient for plants, which helps to promote the plant productivity, growth and development of fruits. Zinc plays an important role for promoting certain metabolic reaction and it is essential for production of carbohydrate and chlorophyll. It is directly or indirectly involved in the activation of several enzymes, auxin and protein biosynthesis. Excess amount of zinc is a major problem in the soils, contaminated through mining industrial activities, smelting and long-term uses of fertilizer with sewage sludge for agricultural propose. When in higher concentrations zinc hamper the plants growth causing several abnormalities in plant morphology, and root growth inhibition (Chatterjee and Khurana, 2007). The excess amount of zinc accumulates in different edible parts of plant when grown in zinc-contaminated soil. Human body has no specific zinc storage system; thus a daily dietary intake of zinc is required to maintain the normal cellular metabolism. More than 40 mg Kg^{-1} per day consumption by human is serious threat to human health (Trumbo *et al.* 2001). The higher concentration of zinc in plants causes abnormal production of reactive oxygen species (ROS) which is responsible for including oxidative stress (Sofo *et al.* 2018). The increment of ROS in plants ultimately enhances membrane lipid peroxidation, H_2O_2, protein oxidation, RNA/DNA damage and metabolism imbalance (Alonso-Blazquez *et al.* 2015). When higher dose of zinc induces ROS production in plants, plants rapidly activates antioxidant machinery to scavenge ROS and hence prevent oxidative. Several ROS scavenging antioxidant enzymes such as ascorbate peroxidase, superoxide dismutase, peroxidase, catalase, glutathione reductase, glutathione-s-transferase and soluble non-enzymatic antioxidants such as glutathione and

ascorbate; are induced in response to excess zinc.Indian mustard (*Brassica juncea*) is most important oil seed crops grown in different parts of India and information related to its metabolism under zinc stress is very little. The present study seeks to unravel the effect of zinc concentration during germination and early stage of seedlings on some biochemical parameters.

MATERIALS AND METHODS

Seeds of three varieties viz., *Pusa agrani, Pusa bahar* and *Pusa bold* were collected from Indian Agricultural Research Institute (IARI) Regional station Karnal (Haryana). Seeds were first surface sterilized in 1% (w/v) NaOCl solution for 15 minutes and washed with distilled water several times. Then the sterilized seeds of uniform size were set for germination over cotton bed in petri-plate and treated with solution of zinc prepared from a stock solution of zinc chloride (1.0M). Four doses of zinc (2.5, 5.0, 7.5 and 10.0 mM) excluding control were evaluated in a randomized fashion with three replicates. Twenty-five seeds were placed in every plates and 10.0 ml of solution (either zinc/ distilled water) was introduced into the glass petri-plates. Final germination percentage (FGP), germination index (GI), and finally seedling vigor index (SVI), seedling length were measured by following protocols of Li. (2008); Abdu;-Baki and Anderson (1973) and Moulick *et al.* (2016), respectively. Selected stress induced biochemical attributes such as lipid peroxidation expressed malonaldehyde (MDA) content, lipoxygenase (LPO), hydrogen peroxide (H_2O_2) and finally cell viability estimation was carried out by adopting the methodology described by Heath and Packer (1968); Williams *et al.* (2000); Alexieva *et al.* (2001) and Romero-Puertuas *et al.* (2004), respectively. Zinc-treated seedlings were washed thoroughly with water order to remove metals adsorbed to the surface. They were oven-dried at 72°C for 2 days and acid digested by following the methodology of Moulick *et al.* (2018). Then digested material was used to quantify for the metal content using atomic absorption spectrometer (model AAS-ICE 3500). Moreover, to authenticate the metal quantification process rice flour SRM (1568a) or standard reference material (obtained from NIST, USA) was also digested and quantified along with the sample (s). Our finding regarding SRM suggests a 95.8% recovery of SRM after quantification. The metal treated samples were homogenized in 0.1M phosphate buffer containing 1% PVP and 1mM EDTA. The supernatant was employed for enzyme assays. For ascorbate peroxidase (APx) extraction buffer was additionally supplemented with 2mM ascorbic acid. The activities of Superoxide dismutase, Guaicol peroxidase, Glutathione-S-transferase, Glutathione reductase, and Ascorbate peroxidase were measured according to the methodology provided by Gupta *et al.* 1993); Chance and Maehly (1955); Habig *et al.* (1981); Smith *et al.* (1988) and Nanko and Asada (1981), respectively.The data as mean (n=3) were analysed followed by standard error (mean ± SE) format using SPSS 21 (Window version) software. Further, difference among the various treatments was determined by employing *Two Way ANOVA* (Analysis of Variance) & Post Hoc *Tukey's* HSD (Honest significant difference) test at *0.05 level* of significance.

RESULTS AND DISCUSSION

Effect of zinc treatment on germination and seedling growth

Upon exposure to zinc stress, a noteworthy reduction in the final germination was observed, irrespective of varietal differences in dose dependent fashion. *Pusa bold* maintained the higher final germination (90.66±3.71 %) even in 10mM zinc when compared to *Pusa agrani* (87.33±0.66%) and *Pusa bahar* (83±4.04%). Germination index was also effect, through was not statistically significant. No profound change in germination speed was observed. The seedling vigor index also reduced dose dependently. *Pusa bold* showed highest seedling vigor index even at 10mM zinc in comparison to other two varieties. Moisture content also varied dose dependently in the three varieties. The effect of zinc on seedling growth was found to be enormous. Among the varieties the growth of *Pusa bold* had least effect (Table 1). Inhibition of seed germination under zinc stress may be considered as absence of necessary/ sufficient protective arrangement during this particular stage (germination) of plant's life cycle. Our findings also indicated that though zinc influenced growth and development, but didn't

affect the germination rate in a significant manner (Table 1). Seedling length was significantly reduced as concentrations of zinc increased in all three varieties due to high dose of zinc whichinhibit the cell division. Seedling length at 10 mM was significantly decreased in *Pusa agrani* whereas *Pusa bold was* found to have less effect on seedling length as compared to other varieties (Table 1). Similar result was found in *Raphanus sativa*. by Ramakrishan and Rao, (2013).

Table 1: Impact of zinc stress upon germination, germination index, seedling vigor index, seedling length, moisture content and metal content (Intact seedlings) at 7 (day after sowing)

Variety	Zinc level (mM)	Final germination %	Germination index (GI)	Seedling vigor index (SVI)	Seedling length (cm)	Moisture Content % (MCP)	Metal Content (mg Kg^{-1})
Pusa agrani	0	91.3 ± 0.6^{bc}	21.7 ± 1.1^{b}	1373.9 ± 25.1^{d}	13.7 ± 0.2^{d}	91.8 ± 1.1^{c}	1.85 ± 0.02^{bc}
	2.5	93.3 ± 0.6^{bc}	22.3 ± 0.03^{b}	323.4 ± 18.0^{bc}	3.2 ± 0.18^{bc}	88.1 ± 4.8^{bc}	4.73 ± 0.07^{d}
	5.0	90.6 ± 1.7^{b}	21.6 ± 0.4^{ab}	244.2 ± 19.8^{b}	2.4 ± 0.1^{b}	73.3 ± 4.8^{ab}	4.99 ± 0.05^{d}
	7.5	90 ± 2.0^{b}	21.7 ± 0.2^{ab}	188.2 ± 6.6^{b}	1.8 ± 0.06^{ab}	53.8 ± 2.2^{a}	-
	10	87.3 ± 0.6^{b}	22.6 ± 1.2^{ab}	169.2 ± 2.5^{ab}	1.6 ± 0.02^{ab}	48.6 ± 0.7^{a}	-
Pusa bahar	0	92.6 ± 3.3^{bc}	24.0 ± 3.0^{b}	1196.3 ± 26.6^{cd}	13.5 ± 0.3^{d}	92 ± 1.1^{c}	2.61 ± 0.01^{c}
	2.5	88 ± 2.3^{b}	19.7 ± 0.5^{ab}	275.4 ± 6.8^{bc}	3.1 ± 0.07^{bc}	78.0 ± 3.0^{b}	4.31 ± 0.04^{d}
	5.0	84.6 ± 1.3^{b}	19 ± 0.3^{ab}	206 ± 3.6^{b}	2.3 ± 0.04^{b}	68.9 ± 2.9^{a}	5.35 ± 0.04^{de}
	7.5	85.3 ± 1.3^{b}	19.2 ± 0.1^{ab}	183.5 ± 4.3^{b}	2.0 ± 0.04^{b}	65.5 ± 3.9^{a}	-
	10	83 ± 4.0^{ab}	18.1 ± 0.4^{ab}	172.3 ± 0.4^{ab}	1.9 ± 0.004^{bd}	65.1 ± 0.1^{a}	-
Pusa bold	0	92.6 ± 1.3^{c}	22.6 ± 0.5^{b}	1226.4 ± 94.3^{cd}	13.0 ± 1.0^{d}	86.91 ± 0.4^{b}	2.31 ± 0.04^{c}
	2.5	92.6 ± 1.7^{c}	21.1 ± 0.6^{ab}	350.3 ± 9.7^{bc}	3.7 ± 0.1^{bc}	107.6 ± 6.4^{d}	4.09 ± 0.03^{d}
	5.0	87.3 ± 4.0^{b}	20.8 ± 0.1^{ab}	235.6 ± 3.3^{b}	2.9 ± 0.1^{b}	102.0 ± 2.2^{cd}	5.32 ± 0.03^{de}
	7.5	88.6 ± 2.4^{b}	20.3 ± 0.1^{ab}	235.6 ± 3.3^{b}	2.5 ± 0.03^{b}	80.8 ± 0.7^{b}	-
	10	90.6 ± 3.7^{b}	20.7 ± 0.8^{ab}	211.6 ± 4.3^{b}	2.2 ± 0.04^{ab}	76.6 ± 8.9^{b}	-
Source of Variations					*F*-Values		
Variety		7.1**	4.97**	6.35**	1.70	39.4***	0.52
Zinc stress		4.97**	3.08**	850.37***	85.3.48**	35.08***	0.004
Variety x Zinc stress		0.55	0.14	2.80**	1.14	6.50***	0.002

values with identical letter case in a column are not significant by at p <0.05
*F- values. *, ** and *** indicates significant at p < 0.05, 0.01 and 0.001 levels, respectively*

Lipid peroxidation, Lipoxygenase, Hydrogen peroxide and Cell death

Under zinc stress at 7 DAS (day after sowing), the order of MDA accumulation in intact seedlings lies in the order of *Pusa bold* (28.8%) <*Pusa bahar* (42.89%) <*Pusa agrani* (55.79%), then their respective controls. H_2O_2 was also found to increase in a linear fashion under zinc stress. At 7 DAS, the *Pusa agrani* showed highest H_2O_2(90.34%) content, followed by *Pusa bold* (87.32%) and *Pusa bahar* (88.58%) (Table 2). The lipoxygenase (LOX) activity increased profoundly with time and dose of zinc. Among the varieties, the lowest activity was found in *Pusa bold* and highest in *Pusa agrani*. LOX activity was highest in *Pusa agrani* at 5 mM zinc. A noteworthy increase by 6.1, 6.0 and 2.2 times was noted in *Pusa agrani, Pusa bahar* and *Pusa bo*ld, respectively (Table 2). Zinc induced cell death in seedling, but with moderate intensity (Table 2). Finding of Kupper and Andrsen, (2016) regarding zinc induced phytotoxicity, suggests that zinc stress can result into consequences like disruption of cellular metabolism, membrane damages (increase in lipid peroxidation) and lingering photosynthesis etc. in Brassica species. The current investigation supports the above findings, irrespective of varietal differences and stress in a linear fashion. Excess zinc in the environment may itself trigger ROS mediated disruption of cellular homeostasis, which may even result in drastic reduction in yield as well as oil content (Table 2). If excess ROS persists for longer duration within the plant cell, it results in undesired consequences like intensification in MDA content (lipid peroxidation) and subsequent loss of ions from the cell and finally leads to cell death. MDA, H_2O_2 and LOX (associated with lipid peroxidation) content has been used as a reliable indicator of stress. Results shows that, from varietal prospect, the MDA, H_2O_2 and LOX content follows the order *Pusa agrani*>*Pusa bah*ar >*Pusa bold*.

Table 2: Content of MDA, H2O2, LOX and Cell death in Indian mustard under zinc stress at 7 DAS (day after sowing)

Variety	Zinc level (mM)	MDA(µmol g^{-1} Fw)	H_2O_2 (µmol g^{-1} Fw)	LOX (µmol g^{-1} Fw)	Cell death
	0	0.95±0.04^{a}	0.14±0.007^{a}	2.54±0.17^{a}	-
Pusa agrani	2.5	2.06±0.15^{c}	1.08±0.09^{c}	9.0±0.89^{c}	64.33±7.24^{b}
	5.0	2.25±0.16^{c}	1.19±0.06^{c}	10.36±0.56^{c}	90.99±2.96^{c}
	0	0.92±0.04^{a}	0.13±0.001^{a}	1.89±0.05^{a}	-
Pusa bahar	2.5	1.6±0.01^{b}	0.74±0.01^{ab}	8.85±1.04^{c}	59.67±3.02^{b}
	5.0	1.63±0.01^{b}	0.83±0.01^{b}	10.08±0.91^{c}	71.37±1.16^{b}
	0	0.91±0.02^{a}	0.12±0.006^{a}	1.66±0.14^{a}	-
Pusa bold	2.5	1.1±0.01^{b}	0.52±0.05^{b}	4.21±0.95^{b}	54.84±2.20^{b}
	5.0	1.52±0.02^{b}	0.55±0.08^{b}	4.31±1.14^{b}	58.17±2.35^{b}
Source of Variation		*F*-value			
Variety		40.73***	35.58***	42.58***	16.45***
Zinc Stress		99.63***	89.05***	88.05***	138.3***
Variety x Zinc stress		10.33***	5.65**	4.65*	7.39***

Zinc content in plant

The data showed an enhancement in zinc content when exposed to stress (Gaur *et al.* 2018). The trend was found to be as *Pusa bold* > *Pusa bahar* > *Pusa agrani* under 5 mM dose. The findings indicate that the accumulation of zinc in intact seedlings was in accordance to the doses, and presence of zinc in excess correlates with the phytotoxicological consequences in a significant way in all the varieties. Zinc accumulation might have caused significant imbalance in ROS production and can be taken as a possible justification behind toxicity symptoms during the germination and subsequent seedling growth.

Effect of zinc stress on antioxidant enzyme activities

The antioxidant enzymes have important roles to play in preventing oxidative stress by detoxification of free radicals. A noteworthy increase in antioxidant enzyme activity was observed under zinc toxicity, irrespective of varietal differences suggesting that varieties employ a considerable effort to detoxify ROS induced upon exposure to zinc stress. SOD activity increasedby 1.7, 1.8 and 2.1 fold respectively in *Pusa agrani, Pusa bahar* and *Pusa bold* (Table 3). These findings are in agreement with those of Sofo *et al.* (2018) and Kaya *et al.* (2018). POX activity was also increased in a dose dependent and variety manner in a highly significant way. Among the varieties, an enhancement by 67.3% (*Pusa bold*), 58.3% (*Pusa bahar*) and 41.7% in P*usa agrani* was noted under zinc stress respectively at 7 DAS (Table 3). Similar to previous trend, a moderate to highly significant enhancement in CAT activity was noted in all the varieties in a dose dependent manner under zinc stress at 7 DAS. Similar result was also observed for CAT, *Pusa agrani* (1.9 fold) showed less activity as compared to *Pusa bold* (2.1 fold) and *Pusa bahar* (1.9 fold). CAT is generally located in peroxisomes and mitochondria while POX is located in cytoplasm, membrane, vacuole, apoplast, extracellular space and cell wall. Sofo *et al.* (2018) depicted that POX is activated by heavy metal induced oxidative stress and is more efficient than CAT which was also observed in this study, support our findings.

The zinc stress on mustard seedlings increased GST activities at early seedling stage. The highest GST activities were recorded in *Pusa bold* and *Pusa bahar* varieties. At 7 DAS, the relative activity of GST was recorded as *Pusa agrani* < *Pusa bahar* < *Pusa bo*ld at 5 mM dose (Table 3). GST catalyzes GSH binding to xenobiotic and thus play a vital role in detoxification process. Several endogenously produced reactive metabolites react with GSH to produce a conjugate. These conjugates are transported into vacuoles for further degradation and thus protect the plants from oxidative injury. GST activity increased with increment in dose of zinc in a dose dependent and variety independent fashion. Similar to GST, GR activity was found to increase under the zinc stressed condition in all three varieties during germination and early stage seedlings as compared to

control. Even at higher concentration of 5 mM, GR activity increased significantly for *Pusa agrani, Pusa bahar* and *Pusa* bold by 1.9, 2.0 and 2.1 fold respectively. The APx activity significantly increased with increase in the concentrations of zinc. When compared among the three varieties, *Pusa bold* showed highest activity of APx in seedlings at 7 DAS. *Pusa agrani, Pusa bahar* and *Pusa bold* was observed to show 86.5%, 83.8% and 93.6% increase at 5 mM dose respectively. Increasing pattern of APx activity was also observed in *Triticum aestivum* (Li *et al.* 2013). APx is known to catalyse the first step ascorbate-glutathione cycle by reducing AsA to monodehydroascorbate with the oxidation of H_2O_2 to H_2O (Apel and Hirt, 2004).

Table 3: Activities of SOD, POX, CAT, GST, GR and APx in Indian mustard under zinc stress at 7 DAS (day after sowing)

Variety	Zinc level (mM)	SOD (μg^{-1} Fw)	POX (μg^{-1} Fw)	CAT(μg^{-1} FW)	GST(μg^{-1} FW)	GR(μg^{-1} FW)	APx (μg^{-1} FW)
	0	4.71±0.26^{a}	2.01±0.13^{a}	7.78±0.30^{a}	0.24±0.002^{a}	0.016±3.85^{a}	60.22±2.64^{a}
Pusa agrani	2.5	8.24±0.46^{b}	3.22±0.13^{b}	10.48±0.16^{b}	0.52±0.05^{b}	0.02±2.50^{ab}	430±48.54^{a}
	5.0	9.32±0.25^{b}	3.72±0.27^{b}	14.91±0.24^{d}	0.55±0.08^{b}	0.027±1.80^{c}	464±53.61^{ab}
	0	5.44±0.14^{a}	2.18±0.03^{a}	7.81±0.40^{a}	0.23±0.002^{a}	0.016±0.002^{a}	79.09±9.6^{a}
Pusa bahar	2.5	9.67±0.22^{bc}	4.77±0.20^{c}	12.58±0.39^{c}	0.74±0.01^{bc}	0.022±4.80^{b}	380±26.85^{a}
	5.0	10.82±0.29^{c}	5.80±0.41^{c}	15.09±0.29^{d}	0.83±0.01^{b}	0.027±3.47^{c}	689.27±23.54^{b}
	0	5.91±0.38^{a}	1.86±0.05^{a}	7.48±0.18^{a}	0.24±0.001^{a}	0.015±1.70^{a}	54.36±5.45^{a}
Pusa bold	2.5	10.03±0.11^{bc}	5.08±0.22^{cd}	12.81±0.33^{c}	1.08±0.09^{c}	0.024±1.40^{bc}	756.84±26.68^{b}
	5.0	11.08±0.28^{c}	6.50±0.25^{d}	15.97±0.35^{d}	1.19±0.06^{c}	0.040±1.39^{d}	981.52±45.78^{c}
Source of Variation				*F*-value			
Variety		14.59***	39.77***	8.91**	48.27***	36.92***	61.10***
Zinc stress		255.64***	178.15**	457.98***	128.27***	219.94±***	316.51***
Variety x Zinc stress		4.48*	11.83***	5.99**	11.73***	21.74***	21.49***

The current study brings into limelight the differential response mechanism of Indian mustard to zinc stress on short-term exposure during germination stage. This study for the first time clearly depicts that strong anti-oxidative response mechanism to differential heavy metal accumulation in early seedling stage. Out of the three varieties for differential tolerance, *Pusa bold* showed better zinc tolerance capability owing to its better anti-oxidative stress response mechanism 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.

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