Original Research Article

Effect of Mercury on Seed Germination, Growth Parameters and Biochemical Characteristics of Indian Mustard (*Brassica juncea L.*) Cultivars

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A B S T R A C T

The effect of elevated concentrations of mercury on seed germination and seedlings growth responses of three Brassica juncea L. cultivars, namely Varuna, Kranti, and Pusa Jai Kisan. was evaluated. The mercury concentrations (0, 25, 50, 100, 200 µM) suppressed seed germination significantly in concentration dependent manner and differentially among the cultivars. The inhibition due to Hg was much greater after one day of treatment with lowest suppression in cv. Varuna than others. In seedling growth performance, Root growth was inhibited quite strongly, with highest and lowest inhibition in cv. Pusa Jai Kisan and Varuna respectively. Shoot growth was not affected much in all cultivars. The seedling fresh weight in general reduced. Activity of α-amylase and MDA content were differentially altered among all cultivars studied. Also, significant increase in α-amylase activity was observed in cv. Varuna when compared with other cultivars. The CAT activity was marginally promoted by Hg in cultivar Pusa Jai Kisan and Kranti. The SOD activity was not strongly influenced due to Hg treatments. The results seem to be a consequence of differential sub cellular Hg distribution and antioxidative defence resulting in constitutive activity of SOD and CAT. Findings have implication in establishment of seedlings in mercury enriched soils and in turn of phytoremediation.

Introduction

Mercury (Hg) is among the highly toxic non-essential Heavy metals (HMs) and its dispersion in the environment is considered as a serious environmental problem due to its persistent character (Liu *et al.,* 2011). Global mercury release increased remarkably with industrialization. (Kolker *et al.,* 2006) The atmospheric Hg, which undergoes oxidation reaction and deposits to the ground, increases the abundance of Hg in soil and water (Lindberg *et al.,* 2007).

In addition to this, a considerable amount of Hg is introduced into agricultural soils as fertilizers, fungicides and pesticides. In soil mercury exist in many elemental forms among which Hg^{2+} is a predominant and bioavailable forms for plants (Han *et al.,*

2006). Mercury is readily taken up by roots that accumulate most of it with a part being translocated to leaves, flowers and other developmental tissues (Sierra *et al.,* 2009). Hg import in to root cells is possibly through Fe, Cu, or Zn transporters/channels (Esteban *et al.,* 2008). High concentrations of Hg cause reduction in seedling growth, photosynthesis, nutrient uptake, transpiration rate, water uptake, chlorophyll synthesis (Boening, 2000), germination (Wang *et al.,* 2003) and respiration (Patra *et al.,* 2004), all contributing to seed toxicity and productivity loss.

Plants employ multiple strategies for cellular management of toxic metal concentrations (Sharma and Dietz 2006). They range from cytosolic chelation of heavy metal ions through different ligands to sequestration in to different cellular compartments. Heavy metals (HMs) stressed plants often exhibit enhanced level of lipid peroxidation. Strongly redox ions such as Cu^{2+} and Hg^{2+} are capable of initiating the peroxidation of lipids components of membrane system.

Data from diverse biochemical and metabolic approaches have established a firm link between cellular redox imbalance and HMs toxicity (Sharma and Dietz 2009). HMs could alter the catalytic functions of enzymes through their interaction with –SH groups, phosphate groups, and the replacement of essential ions that would alter the protein conformation (Patra *et al.,* 2004).

HMs toxicity has been reported to reduce the radical emergence via enhanced protein and carbohydrate contents, affecting the activity of peroxidases (POX) and polyphenol oxidases (PPO).

HMs supresses the oxidizing ability of roots resulting in overall lowering of carbohydrate-metabolizing enzymes–α-amylases, β-amylases, acid invertases and acid phosphatases (Singh *et al.,* 2011). HMs induce oxidative stress by stimulating the generation of ROS and concomitantly suppressing the cellular antioxidative defence (Sharma and Dietz 2006). Hg has been reported to both promote and suppress the activity of antioxidative enzymes (Pätsikkä *et al.,* 2002)by interfering and altering non enzymatic antioxidants like glutathione (GSH) and nonprotein thiols (NPSH) and also the enzymatic antioxidants like superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR) (Ortega-Villasante *et al.,* 2005). Hg has been demonstrated to induce expression of genes encoding SOD (superoxide dismutase), POD (peroxidase), and CAT (catalase) (Neumann *et al.,* 1997).

Phytoremediation is a green technology by which plant remove heavy metals from the contaminated soils (Kumar and Thakur 2019). Plants have species specific differences with regards to the tolerance to diverse HMs. Certain metal hyper accumulator plant species possess the ability to accumulate extraordinarily high concentration of specific metals with a low toxic influence. These plants are central to realization of phytoremediation technology (Pilon-Smits, 2005).

Several members of Brassicaceae have shown the potential for hyper accumulation, Since Indian mustard (*Brassica juncea* L.) has higher biomass and faster growth rates compared to other hyper accumulator plants, present study focuses to determine seed germination and seedling growth responses to surplus mercury concentration. With a view to reveal the likely basis of cultivar specific differences and to get insight in to the possible mechanism how plants make less severe to the mercury stress certain metabolic aspects have also been studied.

Materials and Methods

Seed source

Seeds of Indian mustard (*Brassica juncea* L.) cultivarKranti were procured from G.B. Pant University of Agriculture and Technology, Pantnagar (Uttarakhand) while Seeds of cultivar Pusa Jai Kisan and Varuna were procured from Indian Agricultural research institute (IARI), New Delhi.

Seed germination and seedling growth performance

Uniform seeds of all the cultivars were surface sterilized with 0.1% $HgCl_2$ for 5 minutes followed by thorough washing with distilled water. They were soaked in solutions (10 ml) of different concentration of Hg (25, 50, 100, 200 µM) in the form of $HgCl_2$ for 24 hours. Seeds simultaneously soaked in distilled water constituted the control. Thereafter, the seeds were transferred to Petri plates lined with double layer of Whitman's filter paper made wet with 2 ml of respected solutions of Hg concentrations. The petridishes were placed in plant growth chamber (Sanyu, Japan) at 25 ± 2^0C under continuous illumination (PAR: 40 µmol m^{-2} s^{-1}) for seed germination and seedling growth. The emergence of 2-5 mm radicle was taken as seed germination (ISTA, 1966). Seed germination was recorded for three days of incubation. After 6-day treatment, the seedling growth in terms of root length, shoot length and seedling fresh weight was measured.

α-amylase activity

α-amylase activity was determined according to the method of Filner and Varner (1967). Seeds treated with Hg were used to extract the crude enzyme by homogenizing seed tissue (100 mg) with 2 ml chilled 50 mM Tris-HCl buffer (pH 7.2) in chilled pestle and mortar.

The homogenate was centrifuged at 10,000 rpm for 10 min at 4^oC and supernatant (crude enzyme) collected. 1 ml of enzyme was incubated with 1 ml of substrate (0.15% Starch; 0.2 mM $CaCl_2$) for 10 minute at 25 ± 2^0C. 3 ml of IKI reagent (0.6% Iodine in 6% KI; 1 ml of this stock solution made to 50 ml with 0.05 N HCl) was added in the reaction mixture and incubated for 10 min. Absorbance was read at 620 nm. In control, IKI was added prior to the addition of enzyme. Total amount of starch degraded was determined with the help of a calibration curve prepared with starch.

Measurement of lipid peroxidation

Lipid peroxidation accumulated as melondialdehyde content (MDA) was estimated following the method given by Dhindsa *et al.,* (1981). In brief, the seed tissue ($\pm$ 100 mg) was homogenized with 2 ml 0.1% TCA (trichloroacetic acid). The homogenate was centrifuged at 10,000 rpm for 10 min and supernatant collected. 1 ml supernatant was reacted with 2 ml 0.05% thiobarbituric acid (TBA) in 20% trichloroacetic acid (TCA) and mixture heated at $95^{°}C$ in a water bath for 45 minutes. The reaction was terminated by rapidly cooling the reaction mixture in ice for 5 minutes. Absorbance was read at 532 nm. Correction of measurements for unspecific turbidity were performed by subtracting absorbance at 600 nm. MDA contents were determined by using the extinction co-efficient of 155 mM^{-1}cm^{-1} (Heath and Packer 1968).

Superoxide dismutase (SOD) activity

The activity of SOD (superoxide dismutase) was measured according to Beauchamp and Fridovich, 1971 method. Extraction buffer used was 100mM K-Phosphate (pH-7.0). Reaction mixture contained 670µl assay buffer K-Phosphate buffer (100 mM, pH 7.8), 100 µl riboflavin (130 µM), 100 µl

Methionine (13 mM), 100 µl NBT (1.26 mM), and 30 µl enzyme extract. The reaction was illuminated for 8 minute and absorbance read at 560 nm against distilled water. A 50% reduction in absorbance (as compared to blank) was taken as one-unit activity.

Catalase (CAT) activity

Treated seed tissue was homogenized with 100 mM HEPES-NaOH buffer (pH-7.4) containing 5mM ascorbate, and the homogenate was centrifuged. The supernatant was used for assay of catalase. Catalase activity was assayed polarographically using a Clarke-type O_2 electrode (Hansatech, UK). The electrode was calibrated using sodium dithionite. The reaction mixture contained 890 µl HEPES-KOH buffer100 mM (pH 7.4),10 µl of enzyme extract, and 100 µl H_2O_2 (100 mM). The reaction was initiated by adding the enzyme extract at last. The enzyme activity was calculated from the slopes recorded on graph paper by using the value of dissolved oxygen at $25^\circ C$.

All the data were recorded in triplicates. Values were then used to perform statistical analysis and create graphics in Microsoft Excel 2016. Means ± standard deviation, Standard error of the mean were calculated for all measurements.

Results and Discussion

Seed germination and seedling growth

Elevated concentrations of Hg suppressed the seed germination in all three cultivars. After one day of incubation highest Hg concentration (200 µM) showed maximum inhibition (Fig. 1) in Pusa Jaikisan (75%) followed by Kranti (65%) and Varuna (55%). In cultivar Kranti, the lower Hg concentrations (25 µM) seems to be marginally promotory for seed germination

but observed promotion was statistically insignificant. The magnitude of Hg induced inhibition was declined after one day with a considerable recovery after 3 days of incubation. After three days, the seed germination inhibition was reduced to 0, 20 and 5%, respectively in three cultivars.

On the basis of seed germination cultivar Varuna appeared to be the most tolerant to mercury toxicity. Seedling growth was inhibited by mercury in all cultivars with distinct organ and cultivar specific differences. Hg induced Suppression of root length was far greater than that of shoot length which was not affected much by Hg treatment (Fig. 2).

Lowest inhibition of root length due to Hg treatment occurred in cultivar Pusa Jai Kisan across all concentrations followed by that in cultivar Kranti and Varuna. Root growth inhibition due to 100 µM Hg was 16, 23 and 34% in cultivars Pusa Jai Kisan, Kranti and Varuna, respectively. These values increased to 29, 38 and 46%, respectively in case of 200 µM Hg (Fig. 2a). Despite of reduction in root length, the Shoot length is not affected much.

These results show strong organ specific differences (Fig. 2b). The seedling fresh weight was in general reduced due to Hg treatment. But there was no specific pattern of change in different cultivars. Thus seedling fresh weight was reduced by 10, 11 and 28% at 100 µM of Hg in cultivars Varuna, Kranti and Pusa Jai Kisan, respectively.

However, at 200 µM Hg these values became 24, 6 and 20%, respectively (Fig. 2c). Taken together data suggest cv. Pusa Jaikisan and Varuna to be the most tolerant to root length and seedling Fresh weight respectively. In order to find an explanation for Hg induced suppression of seed germination and seedling growth of *Brassica juncea* cultivars certain

metabolic parameters were measured in the seeds treated with 50 and 200 µM Hg for 24 h.

α-amylase activity

The activity of α-amylase, responsible for hydrolysis of starch and necessary for availability of mobilizable carbohydrates was evaluated. There were strong cultivars specific differences in the response of α-amylase activity to the applied Hg concentrations. Thus the activity of α-amylase in the seeds of cv. Varuna was not inhibited by Hg, instead promotion of activity was observed at both the Hg concentrations.

In contrast, the α-amylase activity was inhibited due to Hg treatment in cv. Kranti and Jai Kisan. The magnitude of inhibition was greater in case of cultivar Jai Kisan than that in cv. Kranti. Thus the α-amylase activity in case of cv. Kranti inhibited by 24 and 30% due to 50 and 200 µM Hg, respectively. This inhibition was 40 and 76% respectively in case of cv. Jai Kisan (Fig. 3a).

Lipid peroxidation

Lipid peroxidation measured in terms of Malondialdehyde (MDA) contents in the seeds of *Brassica juncea* cultivars was differentially altered due to Hg treatment in different cultivars. Thus in cultivar Varuna MDA contents was substantially increased at both the concentrations of Hg. An increase of 54 and 34 % was noted at 50 and 200 µM Hg, respectively.

However, in other two cultivars MDA contents were either not changed due to Hg or they were reduced. Thus in cultivar Kranti, MDA contents remained generally unchanged at 50 µM Hg but were reduced by 20% at 200 µM Hg. In contrast, in case of cultivar Jai Kisan MDA contents were reduced by 33% at 50 µM but not changed at 200 µM Hg (Fig. 3b).

Superoxide dismutase and catalase activity

In addition to the lipid peroxidation, the activity of two antioxidative enzymes namely superoxide dismutase (SOD) and catalase (CAT) were monitored in Hg treated seeds. The SOD activity was not strongly influenced due to Hg treatment. At 50 µM Hg SOD activity was promoted by 4 and 8% in cultivar Kranti and Varuna, respectively but was inhibited by 11% in case of cv. Jai Kisan.

At 200 µM Hg SOD activity was promoted to varying degree in different cultivars. Thus a promotion of 9, 28 and 15% was observed in cultivars Jai Kisan, Kranti and Varuna respectively (Fig. 4a). The CAT activity was marginally promoted by Hg in cultivar Jai Kisan and Kranti. The degree of promotion ranges from 2-12% in these cultivars. In contrast the CAT activity was suppressed due to Hg in case of cultivar Varuna. An inhibition of 30% was observed at both concentrations of Hg (Fig. 4b).

Seed germination was suppressed by Hg in a concentration dependent manner. Hg has inhibitory effects have been reported by many workers (Jeliazkova *et al.,* 2003; Ling *et al.,* 2010; Jagastheeswari and Ranganathan, 2012). The Hg- induced suppression of seed germination was evident only after one day of the treatment specially at highest (200 µM) Hg concentration used where cv. Varuna proved significantly more tolerant to Hg than cultivars Kranti and Pusa Jaikisan.

The type and magnitude of HMs effects seed germination appeared to be determined by the degree of permeability of seed coat to HM ions in different species (Wierzbicka and Obidzińska, 1998). Apparently, Hg uptake by seeds occurred in this study that is evident from suppression of seed germination. However, it is not clear whether the differential seed germination suppression by

Hg was related to differential Hg uptake as we have not measured the Hg contents of seed.

The root elongation growth parameter has been employed for the assessment of HM toxicity (Sharma and Dietz 2009) and for assigning the relative HM sensitivity / tolerance to different genotypes of a species. In the analysis of seedling growth performance marked organ specific differences in response to Hg were observed. Thus the root elongation growth was suppressed much more in case of all cultivars as compared to shoot elongation growth. Such differences are consistent with the retention of the bulk of metal ions in the roots (Jagastheeswari and Ranganathan, 2012; Thakur and Sharma, 2016).

Furthermore, the Hg induced root suppression was significantly lowest in case of cv. Pusa Jai Kisan then in others. Obviously, the Hg tolerance of different *Brassica juncea* cultivars varied depending upon the parameter considered. Thus on the basis of performance of seed germination cv. Varuna was significantly most tolerant while on the basis of root length inhibition cv. Pusa Jai Kisan was most tolerant.

It was interesting to note that despite strong suppression of root growth shoot growth remains generally unaffected in all the cultivars. Hg has inhibitory effect on shoot growth parameter is reported by many studies (Jagastheeswari and Ranganathan, 2012; Mondel *et al.,* 2015). The lack of Hg effect on shoot growth in *Brassica juncea* in present study suggests a tolerance towards Hg through restricted root to shoot translocation at least at seedling stage. Our results of shoot growth parameters are consistent with the results of Rodríguez-Alonso *et al.,* 2019 where no significant differences were observed among the Hg treatments on above-ground parts in *Quercus ilex L.* seedlings. Consequently, the seedlings are able to maintained photosynthetic functions even in the presence of Hg ions. The seedling fresh weight was in general reduced due to Hg treatment. But there was no specific pattern of change in different cultivars. Taken together data suggest cultivar Pusa Jaikisan and Varuna, significantly most tolerant to root length and seedling Fresh weight respectively.

The Hg induced root growth inhibition could also be explained on the basis of changes in hydrolytic enzymes and redox parameters. Activity of Enzyme α-amylase responsible for breakdown of polysaccharide reserves was studied in seeds. The data concerning the α-amylase activity suggest that the differential Hg tolerance in terms of seed germination performance was related to the differential effect of Hg on starch hydrolysis. The α-amylase activity was not inhibited in most tolerant cv. i.e. Varuna but was inhibited in other cultivars. Besides, hydrolytic enzymes and redox metabolism Hg might have modified the GA_3 levels necessary for induction of α- amylase activity and in turns the availability of mobilizable sugars (Sahu *et al.,* 2012).

HMs induced phytotoxicity is often described among other reasons to the cellular redox imbalance (Schutzendubel and Polle, 2012; Sharma and Dietz, 2009). In response to membrane lipid peroxidation, specific cellular metabolic circumstances and repair capacities may promote cell survival or induce cell death through constitutive antioxidants defence systems or signalling pathways activation that regulates antioxidants proteins resulting in an adaptive stress response (Sharma and Kumar 2015). Accordingly, we have measured the lipid peroxidation, SOD and CAT activity in Hg treated seeds. The observed changes in these parameters in present study indicate that Hg imposed an oxidative stress to varying

degrees in the seeds of these cultivars. Lipid peroxidation in terms of Malondialdehyde (MDA) contents in the seeds of *Brassica juncea* cultivars was differentially altered due to Hg treatment in all cultivars. Cultivar Varuna proved to be most tolerant than its counterparts to elevated Hg concentrations. Our findings of MDA increase in Hg treated seeds conform to those in barley (Sharma *et al.,* 2004) under Cd exposure, tomato (Cho and Park 2000) under Hg exposure and Indian mustard (Thakur and Sharma 2016) under Ni exposure.

Superoxide dismutase (SOD) activity was not markedly affected in Varuna and Kranti but was significantly inhibited in cv. Jai Kisan so this finding seems to be in contrast to that of Cho and Park (2000) where the results showed a marked increase in SOD activity in the Hg treated tomato seedlings. The CAT activity was marginally promoted by Hg in cultivar Pusa Jai Kisan and Kranti. Under relatively low levels of SOD and CAT activity the ROS, such as superoxide anions and H_2O_2 will increase in concentration. This might account for the observed growth inhibition to some extent. In addition to this the differential Hg tolerance of the *Brassica juncea* cultivars might be related to the differences in subcellular allocation of Hg ions.
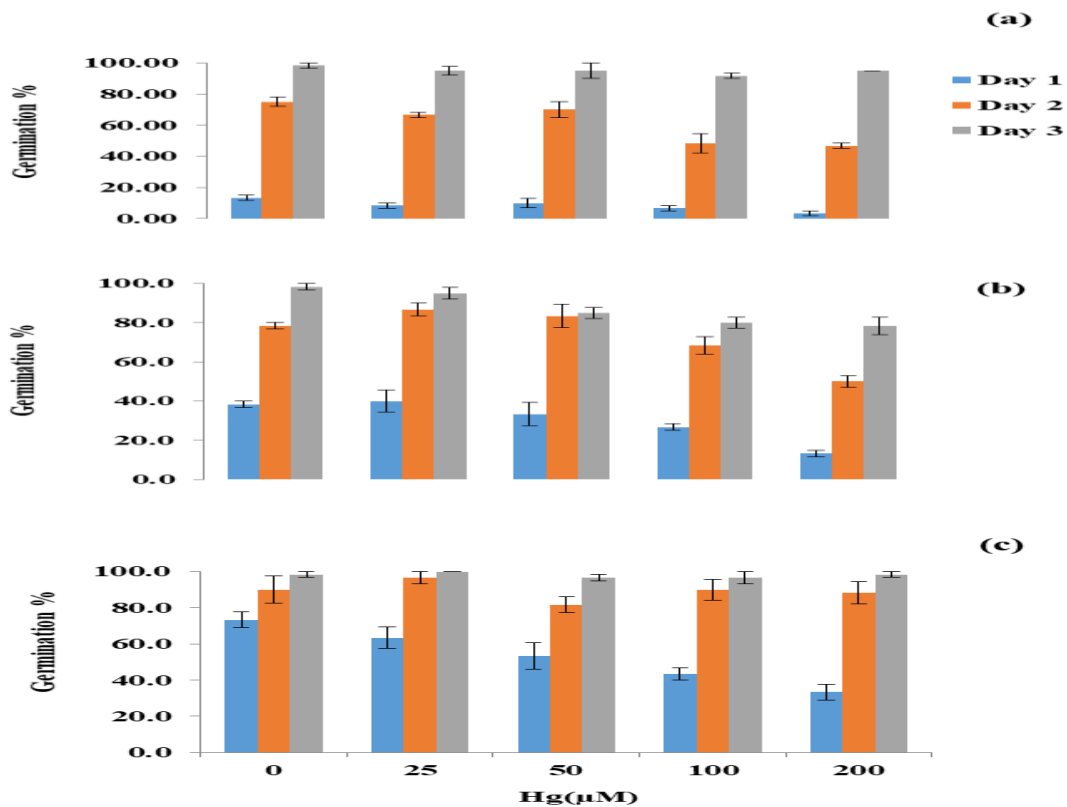

Fig.1 Time-course of seed germination of *Brassica juncea* cultivars, (a) Pusa Jai Kisan, (b) Kranti, (c) Varuna as affected by Hg. Data are arithmetic means ± S.E., n=3 (each replicate comprised 20 seeds)
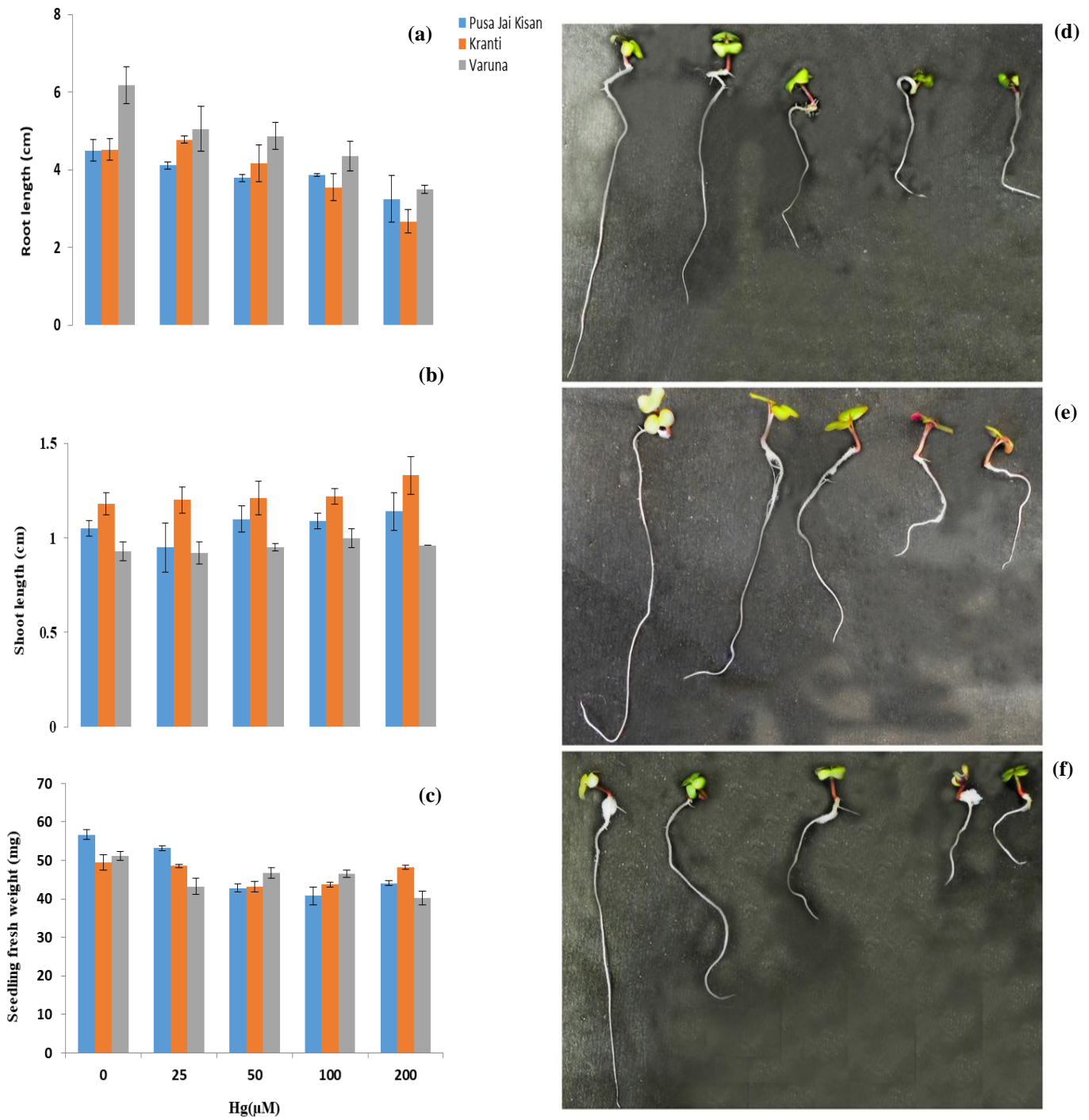
Fig.2 Effect of Hg on seedling growth after 6d, of different *Brassica juncea* cultivars, (a) Root length, (b) Shoots length, (c) seedling fresh weight. 6d-old seedlings of cvs. Pusa Jai Kisan (d), Kranti (e), and Varuna (f). Data are arithmetic means ± S.E., n=30
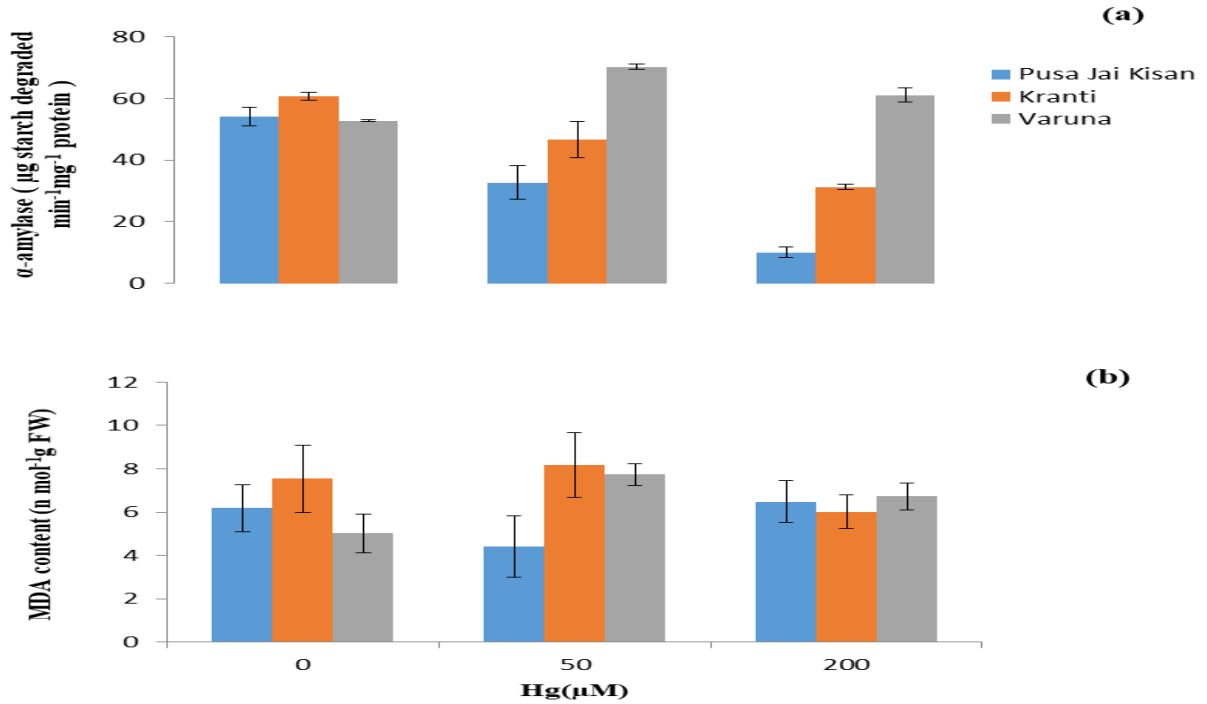
Fig.3 Effect of Hg (24 h treatment) on (a) α-amylase activity and (b) lipid peroxidation in terms of MDA content in the seeds of *Brassica juncea* cultivars. Data are arithmetic means ± S.E., n=3
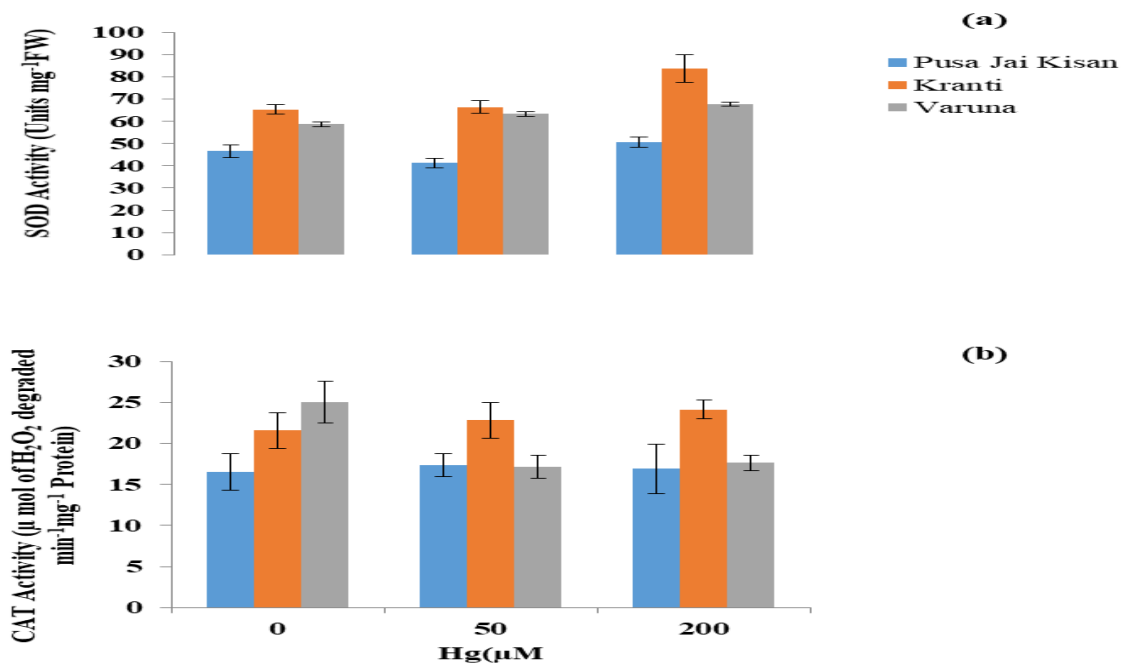
Fig.4 Effect of Hg (24 h treatment) on (a) SOD activity and (b) Catalase activity content in the seeds of *Brassica juncea* cultivars. Data are arithmetic means ± S.E., n=3

In conclusion Hg induced suppression of seed germination and seeding growth seems to be linked to the altered hydrolytic enzymes and redox metabolism. Findings suggest strong organ and cultivar specific differences in response of different *Brassica juncea* cultivars to the applied Hg concentrations. The differential responses of *Brassica juncea* cultivars to lipid peroxidation and alpha amylase activity seems to be a consequence of differential sub cellular Hg distribution and antioxidative defence resulting in constitutive activity of SOD and CAT. The findings have implications for establishment of seedlings in mercury rich soils which is a prerequisite for effective phytoremediation

Acknowledgements

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