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ORIGINAL ARTICLE

Morpho-physiological and biochemical responses of *Brassica* species toward lead (Pb) stress

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Abstract

Brassica species, capable of heavy metals (HMs) hyperaccumulation, differ in their ability to accumulate and tolerate metals present in their environment. In this comparative study, the accumulation, morphological, and physiological responses of three *Brassica* species i.e., *Brassica juncea, B. napus, and B. campestris*, against lead (Pb) were examined. Plants were grown in pots under greenhouse conditions and subjected to 0, 50, 100, 150 mM concentrations of Pb for 14 days. The study revealed that 150 mM Pb concentration reduced the plant length and biomass in all the species and this decline was more obvious in *B. napus*. At 100 mM Pb concentration, plant length increased 3.5% in *B. juncea*, while decreased by 8 and 36% in *B. campestris* and *B. napus*, respectively. *B. campestris* and *B. napus* suffered from more pronounced Pb-accumulation in the root followed by shoot as compared to *B. juncea*. Pb-accumulation in 100 mM treated root of *B. campestris* and *B. napus* increased 29 and 80%, respectively as compared to *B. juncea* Pb treated root. Antioxidant enzyme catalase (CAT) activity was increased in *B. juncea* and *B. campestris* up to 150 mM concentration, while in *B. napus* activity of enzyme decreased at 100 and 150 mM Pb concentration. Phenylalanine ammonia-lyase (PAL) and nitrate reductase activity increased at 50 mM, while the polyphenol oxidase (PPO) and nitrite reductase significantly increased at 150 mM. *Brassica* species also showed more significant accumulation of amino acid, inhibition of proteins and total sugar content at 100 and 150 mM concentrations. Although all species exhibited enhanced antioxidant activity, activation in *B. juncea* was relatively higher. These results suggest that *B. juncea* is relatively more tolerant towards Pb stress as compared to *B. campestris* and *B. napus* due to reduced metal uptake and enhanced antioxidant enzyme activities.

Keywords Antioxidant enzymes · *Brassica campestris* · *Brassica juncea* · *Brassica napus* · Lead · Peroxidase · Phenylalanine ammonia-lyase · Polyphenol oxidase

Introduction

Concentration of HMs in the soil has considerably increased after the industrial revolution (Zaidi et al. 2005). The parent material, from which the soil is formed, also contains HMs but its concentration is relatively low (Herawati et al. 2000). Soil and the atmosphere are the main sources of HMs through which they enter the plant system (Uzu et al. 2010; Arshad et al. 2008). HMs accumulate in crops grown in metal polluted soil and cause harmful effects on human health after being incorporated into the food chain (Fu et al. 2008). Among the HMs that are damaging to plants, Pb is the most toxic and frequently occurring metal (Shahid et al. 2011). Anthropogenic sources of Pb include vehicles, mining, industrial activities, and agricultural activities such as use of fertilizers and pesticides. Pb adversely affects seed germination, root elongation, cell division, transpiration, and chlorophyll development (Sharma and Dubey 2005; Krzeslowska et al. 2009; Gupta et al. 2009; Gupta et al. 2010; Maestri et al. 2010). Binding of HMs ions to the sulfhydryl groups of proteins and replacement of essential cations from specific binding sites, causes inactivation of enzymes

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and production of reactive oxygen species (ROS), which in turn cause oxidative damages to lipids, proteins, and nucleic acids (Sharma and Dietz 2009). Enzymatic defence mechanism contains several enzymes which work together and protect the plant from harmful effects of ROS. Superoxide dismutase (SOD), ascorbate peroxidase (APX), CAT, guaiacol peroxidase (GPOD), and glutathione activities generally increase under metal stress (Mittler 2002; Feigl et al. 2013). Overall Pb induces harmful effects on plants at higher concentrations and decreases the crop yield and productivity.

The genus *Brassica* contains over 150 species of annual, biennial, or rarely perennial herbs mostly in North temperate parts of the world. Several species are used for human consumption, animal fodder, condiments, biofuel, and for oil production (Bancroft 2011). *B. juncea,* which is also known as mustard greens, Indian mustard, Chinese mustard, or leaf mustard, is an oilseed crop. After soybean and oil palm, mustard oil is the third most important vegetable oil. It is also widely used as a vegetable (Anuradha et al. 2012). Biodiesel production potential of *B. juncea* has recently been explored by Jham et al. (2009). Mustard seeds are used for the treatment of abdominal pain, anorexia, tumours, and diabetes (Grover et al. 2002). Extract obtained from leaf has antioxidant potential and reduces lipid peroxidation under diabetic oxidative stress (Yokozawa et al. 2003). The plant also has phytoremediation ability and removes the HMs, such as Pb from contaminated sites (Naser et al. 2012). *B. napus* or rapeseed is mainly cultivated for its oil rich seeds, but nowadays it is also grown to produce animal feeds and edible vegetable oils. Its oil is used as an effective lubricating agent and to produce soaps and plastics (Johnson 1999). *B. napus* colonizes the disturbed areas (Warwick 2010) and may increase the density of plants in ruderal habitats but it reduces crop yields when growing as a weed in agricultural fields (Gulden and Warwick 2008). *B. campestris*, or field mustard is winter annual or rotational cover crop. *B. campestris* prevents soil erosion, decreases weeds growth and soil borne pests, increases soil compaction and scavenge nutrients. It can grow under drought conditions, moderate heat, and soil with low fertility (Clark 2007). Due to agricultural and medicinal importance *Brassica* is the most economically important genus of Brassicaceae family.

Brassica species have a key role in phytoremediation as they can accumulate relatively higher amounts of toxic matter without showing any observable symptoms. In recent times, extensive studies have been conducted on the effects of HMs stress on *Brassica* species. The first visible symptoms related to Pb toxicity include stunted growth, and changes in root growth and morphology (Feigl et al. 2013). Higher concentrations of Pb significantly decreased the plant length and biomass in *B. juncea* by affecting the metabolic processes (Cu 2015; Sheetal et al. 2016; Kaur 2018). Contrary to these findings, at 250 mg/kg Pb concentrations, *B. juncea* shoot and root length increased significantly (Naaz and Chauhan 2019). Pb toxicity drastically reduces the water content of *B. juncea* plant. HMs affect the uptake of other essential elements, but *B. juncea* is able to selectively absorb essential nutrients and maintain adequate nutrition of their organs (Zaier et al. 2010). Chlorophyll content and PSII activity were increased in *B. juncea* under Cr stress due to Cr-induced stabilization of the oxygen evolving complex. In contrast to *B. juncea*, Pb stress significantly reduced the plant growth and biomass in the *B. napus* and *B. campestris* plant (Anjum et al. 2008; Ali et al. 2014a). HMs (Cd, Cr, Cu, Ni, Pb and Zn) accumulation were more pronounced in *B. napus* and *B. campestris* shoot than root (Brunetti et al. 2011). The Pb application significantly increased the ROS as well as malondialdehyde (MDA) in the leaves and roots of *B. napus* plant (Ali et al. 2014b). The essential elements were also significantly reduced after HMs toxicity (Ebbs and Kochian 1997). Reduction in the supply of these important elements can also lead to the inhibition of important enzymes used in chlorophyll biosynthesis (Ali et al. 2014c; Ahmad et al. 2015). Like any other plant, *Brassica* species also have an array of different layers of defence mechanisms comprising both enzymatic and non-enzymatic substances that reduce the HMs availability and toxicity (Mourato et al. 2012). The enzymatic system consists of several enzymes that work together to avoid the deleterious effects of ROS and other toxic species. The chelating of HMs seems to be another most important mechanism for the tolerance of *Brassica* species (Mourato et al. 2015). HMs are damaging to plants at higher concentration, but *Brassica* species have ability to combat the metal-induced toxicity by induction of different detoxification systems.

Different species of *Brassica* exhibit varying levels of HMs tolerance. However, no single experiment has been reported till date to observe the comparative tolerance level of *Brassica* species under Pb stress. This study was designed to investigate the effects of Pb on the growth of three different *Brassica* species and to assess the potential of relative tolerance of these species. Biochemical analysis and the activity of antioxidant enzymes was also checked in control and treated plants.

Material and methods

Growth conditions

Seeds of three *Brassica* species (*B. juncea*, *B. campestris* and *B. napus*) were obtained from National Agricultural Research Centre (NARC), Islamabad. Morphologically healthy seeds of three different *Brassica* species were sterilized using 25% sodium hypochlorite for 2 min. Distilled water was used for washing the seeds 3 times after the

sterilization process. Seeds were sown in earthen pots filled with soil and sand in a 3:1 ratio. The pots were placed in the greenhouse under relative humidity 55–60%, temperature 23 ± 3 °C and 16/8 day/night conditions. Three independent biological replicates were used for each experiment. For each replicate, five plants were selected for every experimental analysis.

B. juncea can tolerate as high as 250 mg/kg Pb therefore in this study sufficiently higher levels of Pb were selected to compare the extent of tolerance of these selected species. After 45 days seedlings were treated with Pb acetate $Pb(CH_3COO)_2$ as T_0: Control, T_1: Pb (50 mM), T_2: Pb (100 mM) and T_3: Pb (150 mM).

Morphological measurements

Morphological parameters including fresh and dry weights (g), shoot length, root length and number of leaves were measured after fourteen days of the treatment. Shoot lengths and root length (cm) were measured manually using a scale. For each treatment point five plants were measured.

Metal analysis

Metal uptake by plant was measured by wet acid digestion method (Wan et al. 2012). Three independent replicates were grown for metal analysis. Plant was harvested and root and shoot were separately oven dried at 60 °C for 72 h. 100 mg dried plant material was grinded into fine powder with the help of pestle and mortar and added in to 50 mL conical flask. 10 mL mixture of per chloric acid ($HCLO_4$) and nitric acid (HNO_3) was added into flask in 1:3 ratio and then left for overnight. Partially digested material was transferred to the fume hood and heated at 150 °C until brown fumes turned into white fumes. Distilled water was added to the mixture to cool and dilute it. Fully digested mixture was filtered with Whatman filter paper No. 42. Afterwards, the final volume was adjusted to 50 mL by distilled water, and the solution was used to determine the desired metal concentration.

Biochemical assay

Quantitative estimation of biochemical contents (Total soluble proteins, total free amino acids, total soluble sugar contents, activity of CAT, nitrate reductase, nitrite reductase, PAL and PPO) of *Brassica* species were determined by using UV-1100 absorption spectrophotometer. After 2 weeks of Pb application, leaves of *Brassica* species were collected. 1.0 gm leaves of each species were crushed in pestle and mortar using liquid nitrogen followed by the addition of 10 mL of 0.02 M phosphate buffer having 7 pH. Slurry was transferred into Eppendorf tubes and was centrifuged at 8000X rpm for 10 min in Hettich Zentrifugen to separate the supernatant. The supernatant was transferred to another Eppendorf tube and was used for further analysis.

Estimation of free amino acids

Total free amino acids contents were estimated by mixing 1 mL extract, 2% ninhydrin reagent (2% ninhydrin dissolved in 98 mL distilled water) and 10% pyridine (10 mL of pyridine mix in 90 mL of distilled water). The tubes were then heated in a water bath for 30 min. The optical densities were measured at 570 nm using UV-spectrophotometer (Roensen, and Johnson 1961).

Estimation of protein

Biuret method was used for the estimation of total soluble protein contents. 1 mL of enzyme extract was mixed with 1 mL of biuret reagent (mixture of reagent $CuSO_4$, NA-EDTA and KI in 5 N NaOH). After adding the required chemical and shaking vigorously, the tubes were incubated at room temperature for 25 min. The absorbance was measured at 545 nm in a spectrophotometer against an appropriate blank (Hamilton and Slyke 1943).

Estimation of total sugar

Anthrone reagent was used for estimating sugar contents in *Brassica* species. 1 mL of plant extract was mixed with 3 mL of anthrone reagent (0.2% anthrone, 80 mL H_2SO_4 and 20 mL distilled water). Test tube was heated in a water bath for 10 min, and then cooled in ice water. The optical density was observed at 620 nm using a spectrophotometer (Yemm and Willis 1954).

Estimation of nitrate reductase activity

1 mL of plant extract was added in 5 mL of 0.2 M Phosphate buffer (pH 7.0) containing 0.02 M KNO_3 and incubated at 30 °C for 30 min. Then 0.5 mL of 1% sulphonilamide and 0.5 mL of 0.02% N Ethylene diamine dihydrochloride was added and left for 20 min after which the colour was noted. Optical density was measured by spectrophotometer at 542 nm (Sym 1984).

Estimation of nitrite reductase activity

1 mL of plant extract was added in 5 mL of 0.2 M Phosphate buffer (pH 7.0) containing 0.02 M KNO_2 and incubated at 30 °C for 30 min. Then 0.5 mL of 1% sulphonilamide and 0.5 mL of 0.02% N Ethylene diamine dihydrochloride was added and left for 20 min after which the colour was noted.

Optical density was measured by spectrophotometer at 542 nm (Sym 1984).

Estimation of CAT activity

Plant extract 0.01–0.04 mL was mixed with 3 mL of H_2O_2-phosphate buffer (35% H_2O_2, 0.067 phosphate buffer pH 7). After mixing the reagent in a tube the optical density was measured at 240 nm. Time was also observed for a decrease in absorbance from 0.45 to 0.40. Blank tube contained 3 mL of phosphate buffer. More concentrated sample solution should be used if the time of decrease is greater than 60 s (Luck 1974).

Estimation of PAL activity

The method was described by Zucker and then modified by Pendharker and Nair. Plant extract (0.3 mL) was mixed with 1 mL of 0.5% 30 mM phenylalanine. After adding 1 mL of 0.07% 200 µM Borate buffer, tubes were incubated at 40 ℃ for 1 h, afterwards 0.2 mL of 5 N HCl was added for termination of the reaction. Optical density was observed at 290 nm (Zucker 1968; Pendharkar and Nair 1975).

Estimation of PPO oxidase activity

The Decker method was used for the analysis of PPO activity. 0.1 mL of enzyme extract was mixed with 1 mL of 0.5 M phosphate buffer (K_2HPO_4, KH_2PO_4 and H_2O). Then 1 mL of 0.018% of 0.00 M tyrosine and 0.9 mL water was added. Optical density was measured at 280 nm with the help of a spectrophotometer (Decker 1977).

Statistical analysis

One-way ANOVA was used to check the statistical significance of comparisons between multiple groups. A p value of < 0.05 was considered as statistically significant. All statistical analyses were performed using SPSS (version 12.0 J; IBM Corp. Armonk, NY, USA).

Results

Plant growth characteristics

To investigate the effects of Pb on three different *Brassica* species, morphological changes (plant length and plant biomass) were analysed by treating the 45 days old plant with Pb (Figs. 1, 2). Exposure of *Brassica* species to 50, 100, and 150 mM concentrations of Pb showed significant and visible symptoms of toxicity at higher concentrations. Various growth parameters exhibit different behaviour under different Pb concentrations in all three species.

Plant morphology

Pb caused damaging effects on plants and decreased the shoot length (Fig. 1A). At 50 mM Pb concentration, shoot length exhibited non-significant changes in *Brassica* species compared to control plant. The maximum shoot length was 38.16 cm in *B. juncea* 50 mM treated plant exhibiting a 7% increase. As the Pb concentration increased, the shoot length significantly decreased in *B. napus* plants, while in *B. juncea* and *B. campestris* non-significant changes occur at 100 mM. A decrease of 31% in shoot length was recorded in *B. juncea* and 21% in *B. campestris* only at 150 mM Pb concentration. A similar pattern was recorded in the root length (Fig. 1B). Root length was increased non-significantly at 50 mM Pb concentration as compared to control plant. Maximum root length was 10.05 cm in *B. napus*. Root length was significantly decreased by 40 and 58% in *B. napus* and 28% and 37% in *B. campestris* at 100 and 150 mM Pb concentrations, respectively. While in *B. juncea* root length decreased only at 150 mM Pb concentration by 28%. Pb also affected the development of leaves in *Brassica* species (Fig. 1C). Leaf number significantly increased in *B. juncea* i.e., 20% at 50 mM Pb. As the concentration of Pb increased from 50 to 150 mM a significant decrease of 31% in leaf number was recorded. While, in *B. campestris* and *B. napus* leaf number was significantly decreased at 100 and 150 mM Pb treated plants i.e., 23% and 46% in *B. campestris* and 31% and 39% in *B. napus,* respectively.

Plant biomass

At higher concentration of Pb, plant biomass was significantly decreased (Fig. 2). The maximum shoot weight was 1.59 g in 50 mM Pb treated *B. juncea* plant (Fig. 2A). Shoot weight exhibited non-significant changes at 50 and 100 mM Pb concentration in *B. juncea* and *B. campestris* but it significantly decreased at higher concentration in both species. In *B. napus* shoot weight significantly reduced by 39% at 100 and by 55% at 150 mM Pb treated plant. Root weight exhibited tolerant behaviour in *B. juncea* plants (Fig. 2B). Therefore, non-significant changes were observed in root weight of *B. juncea* at three different stress levels. While, in *B. napus* and *B. campestris* root weight was significantly decreased as compared to the control. A similar behaviour was also exhibited by leaf weight (Fig. 2C). Maximum leaf weight was 1.5 g in 50 mM Pb treated *B. juncea* plant. Leaf weight was significantly affected by 51 and 52% in 150 mM Pb treated *B. napus* and *B. campestris* plants, respectively. Pb caused non-significant changes in *B. juncea* plant fresh and dry weight

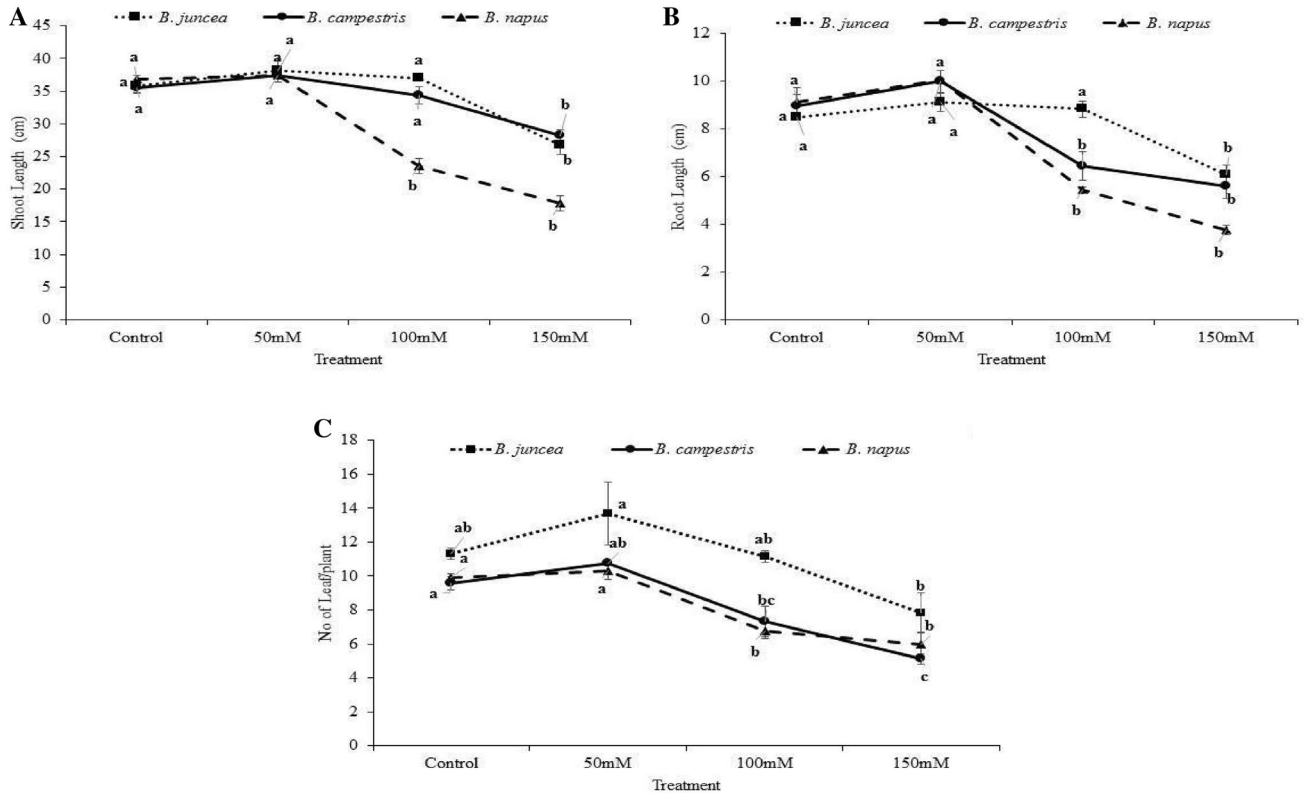

Fig. 1 Effects of different concentrations of Pb Control (0 mM), T1: Pb (50 mM), T2: Pb (100 mM) and T3: Pb (150 mM) on growth parameters. **A** Shoot length **B** root length and **C** no of leaf/plant in *B. juncea*, *B. campestris* and *B. napus*. Values are shown as the mean of three replicates ± SE. Means followed by the same small letters are not significantly different at $p \leq 0.05$

(Fig. 2D, E). Fresh and dry weight of 50 and 100 mM Pb treated *B. juncea* plants were greater than control plants. Fresh and dry weight was significantly affected at 100 and 150 mM Pb concentrations in *B. campestris* and *B. napus*.

Pb uptake

To investigate the causative agent for the different responses of *Brassica* species Pb uptake by plants were measured after fourteen days of Pb treatment (Fig. 3). Pb uptake by plant root and its transportation towards shoot significantly affected the plant growth and metabolism. Uptake of Pb was increased in plant roots as the concentration of Pb was increased from 50 to 150 mM (Fig. 3A). *B. juncea* exhibit a non-significant difference at 50 and 100 mM but at 150 mM, Pb concentration increased significantly in plant roots as compared to other two Pb treatments. While, in *B. campestris* and *B. napus* Pb uptake was increased significantly as the concentration of Pb increased. A Similar behaviour was also observed in the transportation of Pb from root to shoot (Fig. 3B). Maximum Pb contents were present in 150 mM Pb treated *B. napus* plant.

Biochemical traits

Biochemical traits were analysed to investigate the role of enzymatic and non-enzymatic antioxidant under the Pb stress (Figs. 4, 5) Total amino acid contents are one of the most important Physiological traits. Amino acid content significantly increased in *B. campestris* and *B. napus* at all three different treatments (Fig. 4A). While, in *B. juncea* treated plant non-significant changes occurred in the amino acid contents as compared to control plant. Maximum increase was at 150 mM i.e., 75.6% in *B. juncea*, 65% in *B. campestris* and 30% in *B. napus*. Pb significantly decreased the total soluble protein contents in the *B. napus* plant at all three different stress levels (Fig. 4B). While in *B. juncea* and *B. campestris* Pb induced non-significant changes at 50 and 100 mM Pb concentrations as compared to control. At 150 mM Pb concentration, protein contents decreased by 24% in *B. juncea* and 26% in *B. campestris*. Total sugar contents also exhibited similar behaviour (Fig. 4C). Maximum sugar content was recorded in the control plants of *Brassica* species. Sugar content was decreased under the stress conditions. In 50 and 100 mM *B. juncea* and *B. campestris* treated plants non-significant changes were observed in sugar content. While at 150 mM, sugar contents were significantly

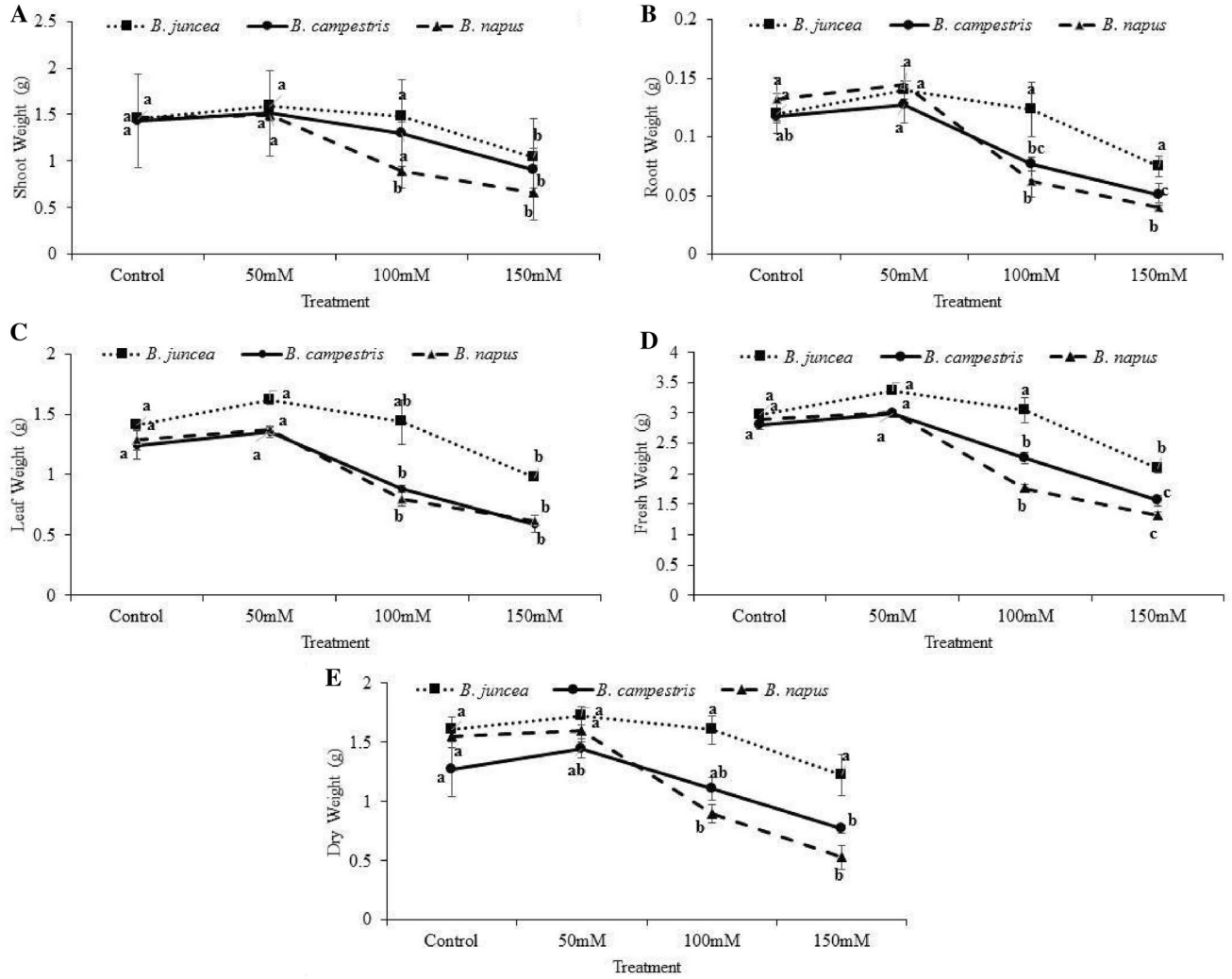

Fig. 2 Effects of different concentrations of Pb Control (0 mM), T1: Pb (50 mM), T2: Pb (100 mM) and T3: Pb (150 mM) on growth parameters. **A** Shoot length **B** Root length **C** Leaf weight and **D**, **E** Fresh and Dry weight in *B. juncea*, *B. campestris* and *B. napus*. Values are shown as the mean of three replicates ± SE. Means followed by the same small letters are not significantly different at $p \leq 0.05$

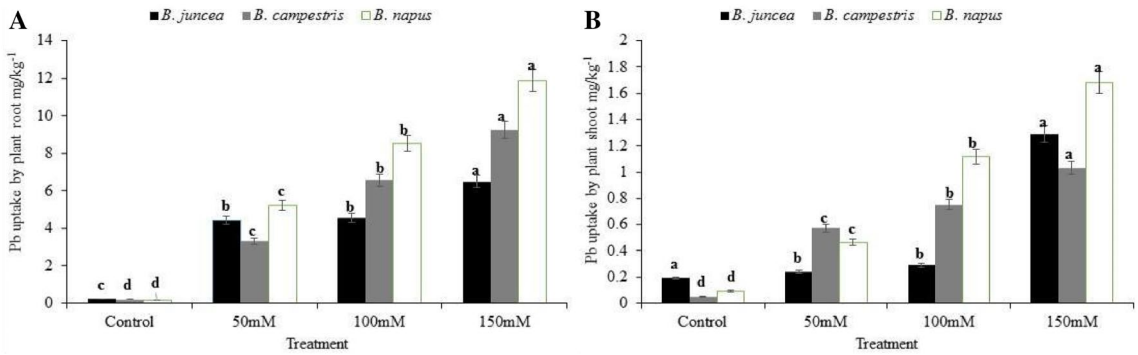

Fig. 3 Accumulation of Pb a in root and b shoot of *B. juncea, B. campestris* and *B. napus* plant after Pb treatment. Values are shown as the mean of three replicates ± SE. Means followed by the same small letters are not significantly different at $p \leq 0.05$

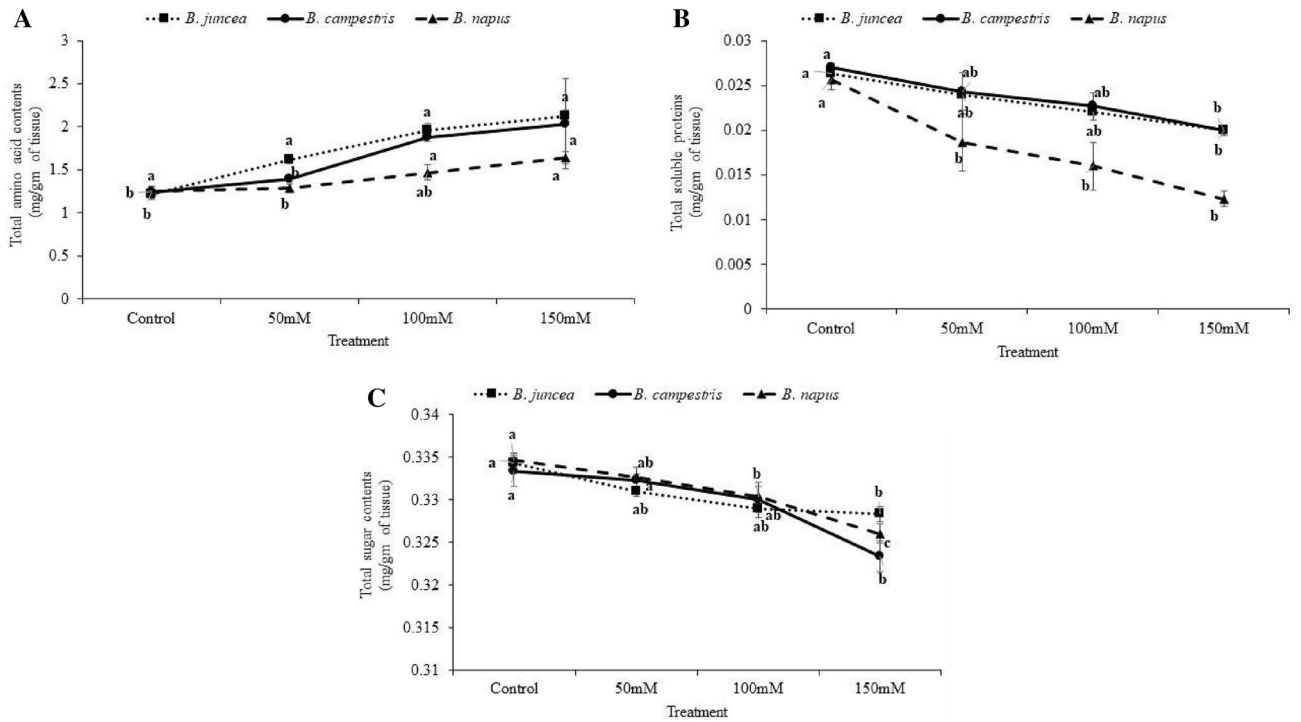

Fig. 4 Effects of different concentrations of Pb Control (0 mM), T1: Pb (50 mM), T2: Pb (100 mM) and T3: Pb (150 mM) on **A** amino acid content **B** protein content and **C** sugar contents in *B. juncea, B. campestris* and *B. napus*. Values are shown as the mean of three replicates ± SE. Means followed by the same small letters are not significantly different at $p \leq 0.05$

decreased. In *B. napus,* sugar content at 100 and 150 mM Pb concentrations was significantly lower than the control.

Activity of enzymes

Pb induced significant changes in antioxidant enzyme activities under different concentrations in all three species. Pb significantly decreased the activity of nitrate and nitrite reductase in *B. napus* at 100 and 150 mM concentrations (Fig. 5A, B). While, in *B juncea* and *B. campestris* activity of nitrate and nitrite reductase decreased only at higher concentration. The nitrite activity reduced by 78% in *B. juncea* at 150 mM whereas it reduced up to 44 and 46% in *B. campestris* and *B. napus,* respectively. At lower concentration, the decrease in enzyme activity was non-significant. CAT is the major enzyme under stress conditions. Its concentration was significantly increased in *B. juncea* and *B. campestris* (Fig. 5C). While, in *B. napus* concentration of enzyme decreased non-significantly at higher Pb concentrations. At 150 mM the CAT activity increased by 81% in *B. juncea*, 68% in *B. campestris*, and 10% in *B. napus*. PAL activity also increased significantly in all three *Brassica* species whereas a non-significant increase in PPO activity was observed at 50 mM Pb concentration (Fig. 5d, e). As the concentration of Pb increased, a non-significant decrease in PPO activity was observed in *B. juncea* and *B. campestris*. While, in *B. napus* PPO activity significantly decreased at higher Pb concentration (Fig. 6).

Discussion

Heavy metals are continuously being incorporated into the environment due to rapid industrialization and urbanization. This raises serious concerns owing to their toxicity (Sethy and Shyamasree 2013). Pb is one of the most toxic HMs and its concentration in soil continues to increase as it is extensively use in various industries (Hamid et al. 2010). It is toxic even at low concentrations as its exposure causes serious physiological, biochemical, and morphological changes in plants (Ali et al. 2013, 2014b). The present study was aimed to compare the response of three species of *Brassica* i.e., *Brassica juncea*, *Brassica napus*, and *Brassica campestris* against various treatments of Pb using morphological and biochemical markers.

Morphology

Shoot length decreased at 150 mM in all three species i.e., 51% in *B. napus*, 30% in *B. juncea*, and 20% in *B. campestris*. In *B. napus* and *B. campestris* shoot length also decreased at 100 mM concentration. Pb-induced

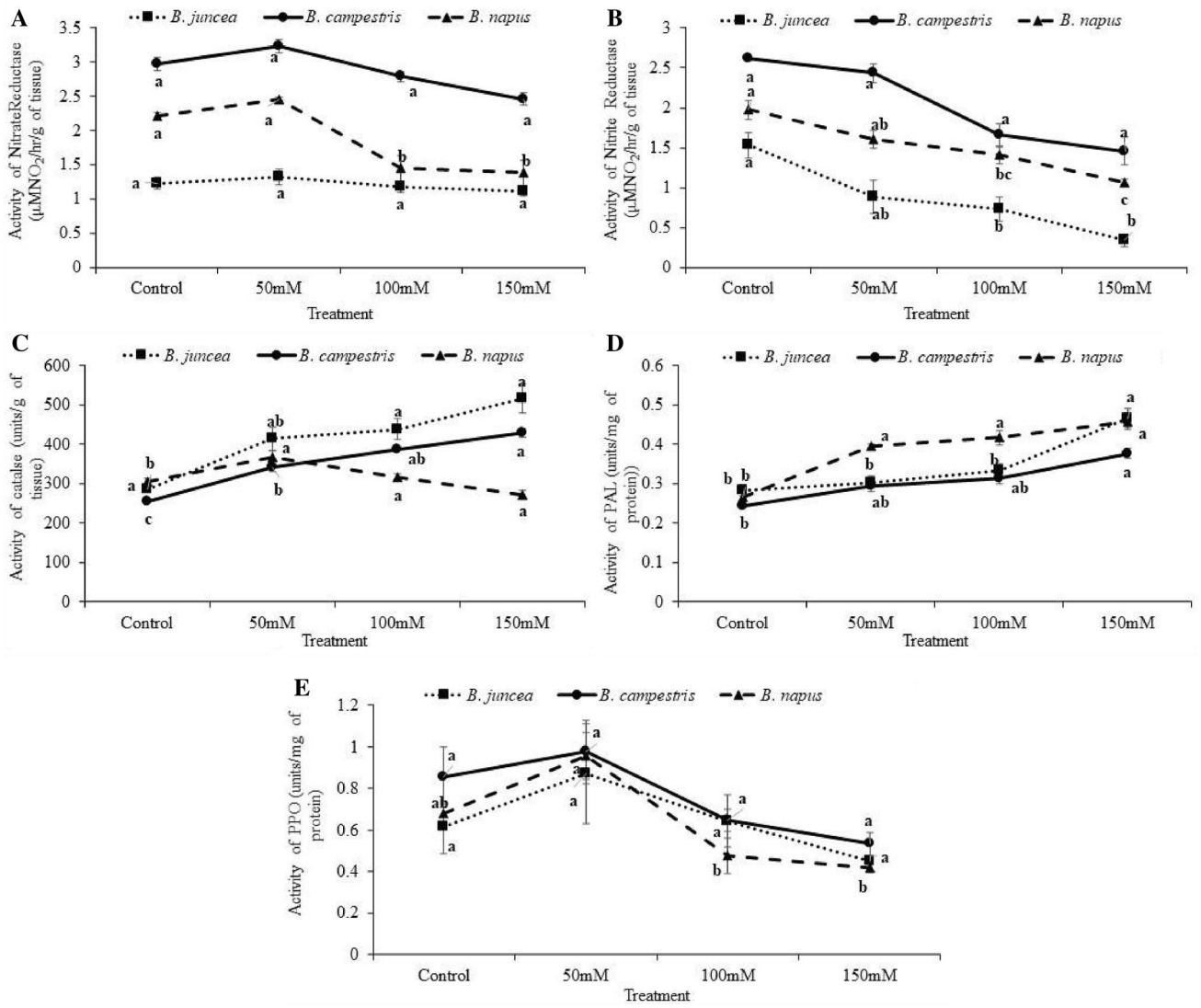

Fig. 5 Effects of different concentrations of Pb Control (0 mM), T1: Pb (50 mM), T2: Pb (100 mM) and T3: Pb (150 mM) on enzyme **A**, **B** nitrate and nitrite reductase activity **C** catalase activity and **D**, **E** PAL and PPO activity in *B. juncea, B. campestris* and *B. napus*. Values are shown as the mean of three replicates ± SE. Means followed by the same small letters are not significantly different at $p \leq 0.05$

reduction in shoot length of *Brassica* species at higher concentration was also reported by Pratima and Mathad (2016), Kaur (2018), Sheetal et al. (2016) and Helal et al. (2016). Higher concentrations of Pb effect the mitotic process which may result in the decrease in shoot length (Srivastava et al., 2011). Root to shoot transportation of Pb was higher in *B. napus* and *B. campestris* as compared to *B. juncea* which resulted in the reduction in shoot length being more pronounced in the former two species. On the other hand, in *B. juncea,* shoot length increased at 50 and 100 mM Pb concentration because of low Pb concentration in shoot. Presence of relatively lower concentration of Pb in shoot also indicates towards a tolerant behaviour in *B. juncea* as Pb was not actively translocated from root to shoot. Cu (2015) also reported an increase in shoot length of *B. juncea* at lower Pb concentration.

Root is adversely affected under Pb stress as it is the first organ which comes in direct contact with the components present in the soil (Kumar et al. 1995). The root growth was reduced in *B. napus* and *B. campestris* at 100 and 150 mM Pb concentrations i.e., 58.5% in *B. napus* and 37% in *B. campestris*. Reduction in root length of *Brassica* species was also reported by Ali et al. (2015), Helal et al. (2016) and Li et al. (2018) under Pb, Cd and Cr stress. This decrease in root length was due to higher Pb uptake by plant roots, which disturbs the barrier function and selective permeability of plasmalemma, and tonoplast (Seregin et al. 2004). After entering the root Pb also affects the mitotic apparatus

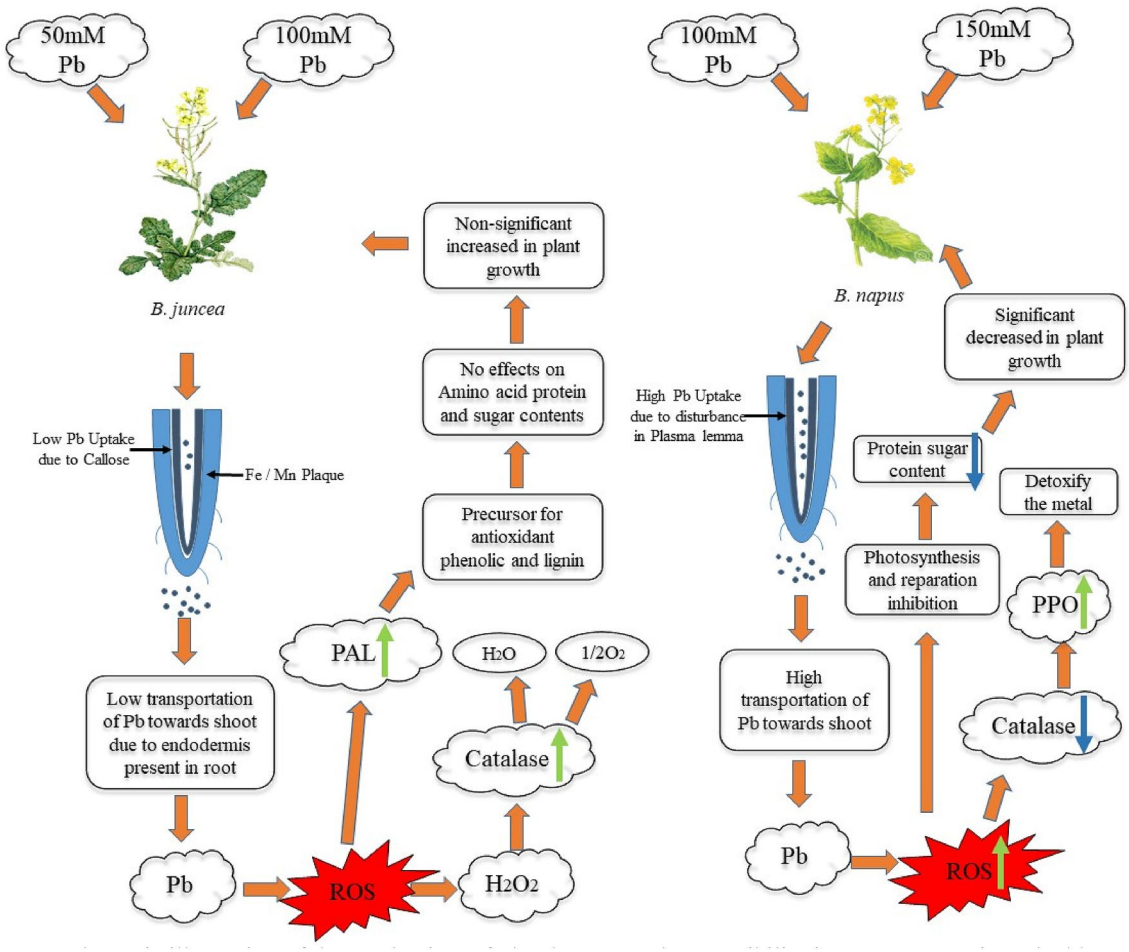

Fig. 6 Schematic illustration of the mechanism of Pb tolerance and susceptibility in *Brassica* species. The blue and green arrows indicate changes in enzymatic and non-enzymatic antioxidant activities (downward arrows indicate decrease and upward arrows indicate increase) under the Pb stress, respectively

and decrease root length. On the contrary in *B. juncea,* lower uptake of Pb by plant roots caused the root length to increase at 50 and 100 mM Pb concentration. The root endodermis acts as a barrier in the transport of Pb from root to shoot (Seregin and Lvaniov 1997). The callose present between cell wall and plasma membrane act as an additional barrier against Pb uptake.

The decrease in the number of leaves was also more pronounced in *B. napus* and *B. campestris* as compared to *B. juncea* particularly at 100 mM Pb concentration. This decrease in leaf number can be attributed to the toxic effects of HMs on chlorophyll contents, gas exchange parameters, stomatal conductance, and photosynthetic rate (Balakhnina et al. 2005; Wahid et al. 2007; Ali et al 2014c). Boroumand et al. (2012) and Kanwal et al. (2014) also reported that *B. napus* shows visible symptoms of toxicity when exposed to Pb.

Plant biomass has been considered as a prerequisite measurement to assess the extent of abiotic stress. Present study depicted that plant biomass decreased in *B. napus* and *B. campestris* at 100 and 150 mM Pb concentration. These findings are supported by Kanwal et al. (2014) who also reported a decrease in *B. napus* biomass under higher Pb exposure. The decrease in plant biomass under higher Pb exposure may be due to the reason that Pb affects root and mineral uptake which in turn affects plant metabolism and ultimately decreases biomass (Breckle 1991; Islam et al. 2008; Gopal and Rizvi 2008; Singh et al. 2010; Sharma and Dubey 2005). HMs stress might also inactivate the photosystem II, enzymes of carbon reduction cycles and cause photosynthesis inhibition which ultimately results into biomass reduction (Gill et al. 2015). While in *B. juncea* plant biomass was increased at 50 and 100 mM Pb concentration which indicates that *B. juncea* has more tolerance towards increasing Pb concentrations than other two species. This might be due to more efficient uptake of other metals in *B. juncea* under Pb stress.

Biochemical traits

Measurement of amino acid content is a useful tool to trace the toxicity of HMs, as they tend to accumulate under stress conditions and help in osmotic adjustments and stabilize the structure of macromolecules and organelles (Kasai et al. 1998). In present investigation, amino acid contents were increased in three *Brassica* species in response to increasing Pb concentration. Maximum amino acid content was recorded in 150 mM Pb treated *B. juncea* plants. Amino acid level increases under Pb exposure in accordance with the stress level (Ahmad and Jhon 2005; Ahmad et al. 2006; 2008). The increase is due to metal chelation in the cytosol by high affinity ligands which is a metal detoxification and tolerance mechanism. These ligands may be amino acids and organic acids (Hall 2002). It has been also suggested that amino acids have a role in osmotic adjustment at the cellular level and enzyme protection by stabilizing the structure of macromolecules and organelles. Storage proteins also have an important role in growth and development of seedling. During the seed germination, variety of proteases degrade the storage protein and convert them into soluble peptides and free amino acids to provide the energy and support the growth (Schlereth et al., 2001). Many researchers have reported decrease in protein content of *Brassica* species after exposure to HMs stress (Singh and Sinha 2005; John et al. 2009). In the present study, the protein content decreased in all three species, however, it was more pronounced in *B. napus* at higher Pb concentration. Under Pb stress protein content decreased due to increased protease enzyme activity, which induced lipid peroxidation and fragmentation of protein under oxidative stress (Stiborova et al1987; Palma et al. 2002). Protein degradation also contributes to amino acid accumulation in metal stressed plant (Chen et al. 2003). Soluble sugar is the major constituent that helps in direct detoxification of ROS and maintaining the osmotic potential (Sharma and Dietz 2006; Kavi-Kishor and Sreenivasulu 2013; Keunen et al. 2013). Ali et al. (2015) also confirmed the protective role of total sugar content in *B. napus* under the Cd stress. HMs are known to affect plant sugar content through ROS induced oxidative stress. Monireh et al. (2011) found that increasing concentration of Pb significantly decreased the total sugar content in *B. napus*. In the current study, total sugar content also decreased in all *Brassica* species but it was more pronounced in *B. napus* due to stimulation of respiration rate and photosynthesis inhibition (Ouzounidou 1995).

Oxidative burst

Plants have developed antioxidant defence mechanisms to decrease the oxidative damage caused by HMs including Pb (Ruciska-Sobkowiak and Pukacki 2006). Pb toxicity can either induce their synthesis or may decrease the activity of these enzymes. This Pb induced inhibition of enzyme activity depends upon plant species, duration of treatment, and the Pb concentration (Islam et al. 2008; Gopal and Rizvi 2008). Decrease in the enzymatic activity is due to affinity of -SH group for Pb (Gupta et al. 2009; Sharma and Dubey 2005). Similarly in the present study nitrate activity was significantly decreased in *B. napus* at higher concentration due to disorganization of chloroplast structure. Metal stress at enzyme production sites causes water stress, which in turn, either reduces NADH supply or causes reduction in NO (Kumar et al. 2008). The nitrite reductase was significantly decreased in *Brassica* species under Pb toxicity due to reduced carbon fixation, low uptake of NO_3^- by roots and translocation in the xylem (Rai et al. 2004). CAT is the major enzyme which reduce the oxidative stress by converting the H_2O_2 into water and oxygen (Miller et al. 2008). A significant correlation was found between increase in the CAT activity and metal stress. In our findings, the CAT activity was significantly increased in *B. juncea* and *B. campestris* under Pb stress. Szollosi et al. (2009), Nouairi et al. (2006) and Goncalves et al. (2013) also reported increase in CAT activity in *B. juncea* at higher metal exposure. HMs induce increased transcription of CAT gene which results in increased synthesis of CAT enzyme. Contrarily, CAT activity was decreased in *B. napus* which might be responsible for its susceptible behaviour against Pb stress. PAL is one of the branch point enzyme and functions in the plant phenyl propanoid biosynthetic pathway to deaminate the amino acid L-phenylalanine forming trans-cinnamic acid and ammonia (McInnis et al. 2009). PAL activity was significantly increased in three *Brassica* species with the increasing Pb concentration. The increased PAL activity was due to enhanced phenolic metabolism, which produces precursors for antioxidant phenolics and lignin, to reduce the oxidative stress caused by Pb (Dai et al. 2006; Kovacik et al. 2007). PPO is another enzyme associated with defence mechanism and catalyses the oxidation of phenols to quinones (Martins and Mourato 2006). Activity of PPO non-significantly changed in *B. juncea* and *B. campestris* at all Pb concentrations which indicate a tolerant mechanism in these species. While in *B. napus,* which exhibited a susceptible behaviour, the PPO activity was significantly changed suggesting its role in the synthesis of phenolic compounds. These compounds play an important role in metal detoxification.

Conclusions

The current study indicate that prominent differences were observed in three *Brassica* species in response to Pb stress. Higher Pb concentrations negatively affected the different morphological characteristics in all three *Brassica* species.

At low concentration *B. juncea* showed the highest tolerance level as compared to the other two *Brassica* species. The major tolerance strategy of *B. juncea* relies on low uptake of Pb by root and its translocation towards the shoot. While the other two species are less tolerant to Pb due to higher accumulation of Pb in the root and its translocation toward the shoot. Different biochemical parameters also varied in *Brassica* species under Pb stress. Higher Pb concentration caused protein degradation which resulted in an increase in free amino acid level. Pb also affected photosynthetic processes, ultimately reducing sugar contents. To cope with this stressful condition, all species exhibited higher antioxidant enzyme activity. Our study highlights the significance of enzymatic and non-enzymatic antioxidant activities in *Brassica* species under Pb stress. Nevertheless, for a better understanding of this tolerance mechanism, further investigations on genomic and proteomic level will be deeply insightful.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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