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Amelioration of Lead Toxicity in Radish (*Raphanus sativus* L) Plants by Brassinolide

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Abstract

The present study was designed to evaluate the effect of lead (Pb) and combine effect of Pb with brassinolide (BL) on the growth, metabolite contents and enzyme activities in radish plants. Pb suppressed the growth of the radish plants, reduced net photosynthesis rate and soluble sugar levels. Activities of nitrate reductase in radish plants were also reduced under Pb stress. However seed treatment with BL mitigated the adverse effects of Pb and stimulated the growth of radish plants. The growth promotion was associated with enhanced rate of photosynthesis and elevated levels of reducing and total sugars and nitrate reductase activities in radish plants under Pb stress. Under Pb stress, reactive oxygen species (ROS) levels and lipid peroxidation were increased in radish plants, which were significantly inhibited by BL application. The activities of antioxidant enzymes such as peroxidase, ascorbate peroxidase and superoxide dismutase were increased during metal stress. Treatment with BL further increased the activity of ascorbate peroxidase, catalase and superoxide dismutase. The BL treatment also enhanced glutathione reductase activity under stress. From the results it can be concluded that BL could play a positive role in the alleviation of oxidative stress caused by ROS by enhancing antioxidant defense system, thus improving the tolerance of radish plants to Pb stress.

Keywords: Brassinosteroids, catalase, nitrate reductase, peroxidase, photosynthetic rate, plant growth.

INTRODUCTION

Soil contamination with heavy metals is now a worldwide problem, leading to agricultural losses and hazardous health effects. Cultivation of different crops on metal contaminated soil of peri-urban lands have become a major concern today as these crops are said to be the major source of heavy metal contamination of human-beings through various food chains [1]. When these toxic metals (Pb, Cd, and Hg) are present in the soil at very high levels and absorbed by the food and fodder crops, they hamper their growth, productivity and quality [2].

Several industries, vehicular and agricultural activities contribute to heavy metal contamination of agricultural lands in peri-urban areas [3].

Lead (Pb) is a major heavy metal pollutant in both terrestrial and aquatic ecosystems. Significant increase in the Pb cause sharp decrease in crop productivity thereby posing a serious problem for agriculture [4].

Plant growth regulators (PGRs) play an important role in modern agriculture and proper application of certain PGRs improves agriculture and thereby benefits environment [5]. Recently, increasing interest has been developed in utilizing PGRs with multiple functions in agriculture management. PGRs could not only regulate plant growth but also enhance resistance to various environmental stresses.

Brassinosteroids (BRs) are new kind of growth regulator, able to influence different physiological plant processes at very low concentrations [6, 7]. Analysis of brassinosteroids- (BR) deficient mutants from *Arabidopsis* [8], pea [9] and tomato [10] has not only established BRs are essential endogenous growth regulators but also essential for plant growth and development. Apart from plant growth stimulation, BRs have the ability to confer tolerance in plants against biotic and abiotic stresses [11, 12]. BRs strengthen drought resistance and show favorable effects on plant growth and yield under soil water deficient conditions [13]. They exhibit salt tolerance [14] and disease resistance [15] in a number of plants. Due to multiple effects and ecofriendly nature BRs are considered plant hormones with pleiotropic effects [16].

In the present study the effect of brassinolide (BL) on growth, metabolite content and activities of certain antioxidant enzymes that have crucial bearing on the growth of the radish plants under Pb stress is being investigated.

MATERIALS AND METHODS

Brassinolide (BL) was purchased from CID Tech. Research Inc., Mississauga, Ontario Canada. Seeds of radish (*Raphanus sativus* L) procured from National Seed Corporation; Hyderabad, India were employed for the study.

Seeds of radish were surface sterilized with 0.5% (v/v) sodium hypochlorite and washed thoroughly with several changes of sterile distilled water. Surface sterilized seeds were soaked for 24 hours in either: 1) distilled water (control) 2) 2.5mM Pb solution (stressed control) 3) 2.5mM Pb solution supplemented with 1µM / 2µM brassinolide solutions. After 24 hours of soaking, twenty seeds from each treatment were sown into 8 (height) x 7 (upper diameter) x 4 (base diameter) cm. PET (polyethylene terepthalate) jars containing eighty grams of vermiculite. For each treatment five replicates were maintained. The seeds were allowed to germinate in dark at $20^0 \pm 1^0$C. After three days when the seedlings were emerging from vermiculite the PET jars were shifted to growth chamber [N K System BIOTRON (Model:LPH -200-RD), Nippon Medical and Chemical Instruments Company Limited, Japan]. The plants were allowed to grow in growth chamber under conditions of 25°C temperature, 60% relative humidity and 4000-lux light intensity from fluorescent tubes with a photoperiodic cycle of 12 hours light and 12 hours dark. 30 ml of 2.5mM Pb solution was poured on alternate days throughout the experiments in all the jars except in control, in which equal quantity of distilled water was added. 30ml of 1/5 Hoagland solution was added uniformly to all the jars at two days interval throughout the duration of the experiments. On 20th day, growth of the plant was recorded in terms of height, fresh weight and dry weight. On the same day leaf material was homogenized with 70% (v/v) ethyl alcohol. This ethanol homogenate was stored in the deep freezer and used for further biochemical analysis. Fresh leaf material was employed for chlorophyll estimation and for assay of enzymes.

For estimation of metal content plants were oven dried and digested in 1:5:1 (v/v/v) H_2SO_4:HNO_3:$HClO_4$ and estimated by following the method of Allen et.al [17]. Pb content in the plants was estimated by Atomic Absorption Spectrophotometer (Perkin Elmer 2380).

The chlorophyll pigments were extracted and estimated adopting the procedure by [18]. Photosynthetic rate (P_N) in fully expanded leaves of intact plants under natural light was measured using a portable photosynthesis system LI COR 6400 (PAR 1500; block temperature 31ºC, flow-500). The ethyl homogenate was heated and centrifuged. The supernatant was used for the estimation of total sugars [19] and reducing sugars [20]. Activity of enzyme nitrate reductase (1.6.6.1.) was determined following the in vivo method [21] and enzyme activity is expressed as µ moles of NO_2 liberated per hour / gram fresh weight.

For catalase (CAT; EC 1.11.1.6) and peroxidase (POD; EC 1.11.1.7) enzyme studies, fresh leaf material was homogenized in a prechilled mortar with chilled phosphate buffer (pH=7). The reaction mixture for the assay of catalase contained enzyme extract, hydrogen peroxide and phosphate buffer [22]. The reaction was stopped by adding conc. sulphuric acid and the residual hydrogen peroxide was titrated with potassium permanganate.

For the assay of POD, the reaction mixture contained phosphate buffer (pH=7), pyragallol, hydrogen peroxide and enzyme extract [23]. After incubation, the reaction was stopped by adding conc. sulphuric acid. The amount of purpurogallin formed was estimated by measuring the absorbance at 420 nm.

For ascorbate peroxidase (APOX; EC 1.11.1.11), the reaction mixture contained 50mM phosphate buffer (pH 7.0), 0.2mM EDTA, 0.5mM ascorbic acid, 250mM H_2O_2 and 50 µg of protein. The activity of APOX was measured spectrophotometrically by measuring the rate of ascorbate oxidation at 290 nm for 1 min. The amount of ascorbate was calculated from the extinction coefficient of $2.6mM^{-1}$ cm^{-1} [24].

The assay of glutathione reductase (GR; EC 1.6.4.2) was performed according to [25]. The reaction mixture contained 500µL of sodium phosphate buffer pH 7.0, 100µL each of 10mM GSSG, 1mM NADPH and 180 µL of distilled water. The reaction was started by addition of protein and NADPH oxidation was recorded as the decrease in absorbance at 340nm for1 min. The activity was calculated using the extinction coefficient for NADPH ε= 6.22 $mM^{-1}cm^{-1}$.

The activity of superoxide dismutase (SOD; EC 1.15.1.1) was determined based on inhibition of the photochemical reduction of nitroblue tetrazolium(NBT) [26]. One unit of activity is the amount of protein required to inhibit 50% initial reduction of NBT under light. The reaction mixture contained 50mM sodium phosphate buffer (pH7.8), 1.5ml methionine, 1ml of NBT, 0.75ml triton-X-100, 2mM EDTA, 10 µl of riboflavin and 50 µg of protein.

Lipid peroxidation was determined by estimating the malondialdehyde content [27]. One gram of material was macerated in 5 ml of 0.1% (w/v) tricholroacetic acid. The homogenate was centrifuged at 10,000 X g for 5 minutes. For 1 ml of the aliquot of the supernatant 4 ml of 20 % TCA containing 0.5% thiobarbutric acid was added. The mixture was heated at 95^0 C for 30 minutes and cooled quickly in ice bath. The absorbance was measured at 532 nm and 600 nm. The concentration of malondialdehyde (MDA) was calculated by using extinction coefficient of 155 $mM^{-1}cm^{-1}$.

The data were analyzed by one-way ANOVA, followed by Post Hoc Test (Multiple Comparisons). The differences were considered significant if P was at least ≤0.05. The mean values have been compared and alphabets are used in the tables to highlight the significant differences between the treatments

RESULTS AND DISCUSSION

The observations made on morphological parameters indicated that the treatment of Pb significantly affected plant growth. Pb (2.5mM) hampered the growth of radish plants, and caused maximum decline in plant height, however the impact of metal stress on plant was reduced by BL thereby revealing improved growth [Table 1]. Gradual improvement in growth parameter was observed with the increase in BL. Earlier it was reported that plant growth in maize pretreated with salicylic acid alleviated the deleterious effects of salinity, drought and boron toxicity [28]. Similarly, enhanced growth in sorghum drought stress by the application of epibrassinolide was observed [29]. The results of the present study clearly indicate the ameliorative influence of BL on the Pb stress induced growth inhibition in case of radish.

Seed application of BRs reduced the uptake of metal by the plants (Table 2). Significant decrease in Pb uptake in BL (2µM) supplemented treatments was observed. Similarly [30]

Table.1. Effect of brassinolide on the growth of radish plants subjected to Pb stress.

Treatment	Height (cm)	Fresh weight (mg)	Dry weight (mg)
Control	12.06 ± 0.21c	253.4 ± 3.98b	15.50 ± 0.46b
Pb	5.38 ± 0.50d	202.0 ± 1.26c	10.24 ± 0.50c
Pb + 1.0 µM BL	15.60 ± 0.42b	293.0 ± 15.4b	22.19 ± 0.73a
Pb + 2.0 µM BL	18.10 ± 0.27a	332.3 ± 1.05a	27.55 ±1.15a

The values are mean ± S.E (n=5); Pb: 2.5 mM; BL: brassinolide. Mean followed by the same alphabet in a column is not significantly different at P= 0.05 level.

Table.2. Effect of brassinolide on metal content in radish plants under Pb stress.

Treatment	Pb metal content ($\mu g\ gm^{-1}$ d.wt)
Control	105.03±1.05c
Pb	136.16±1.08a
Pb + 1.0 µM BL	124.12±1.18a
Pb + 2.0 µM BL	117.25±0.16b

The values are mean ± S.E (n=5); Pb: 2.5 mM; BL: brassinolide. Mean followed by the same alphabet in a column is not significantly different at P= 0.05 level.

Table.3. Effect of brassinolide on total chlorophylls, photosynthetic rate (P_N) and levels of carbohydrate fractions in the leaves of radish plants subjected to Pb stress.

Treatment	Total Chlorophylls mg g^{-1}fr.wt	P_N (µmol CO_2 $m^{-2}s^{-1}$)	Reducing sugars mg g^{-1}fr.wt	Total sugars mg g^{-1}fr.wt
Control	2.57±0.01b	16.69±0.25b	2.18±0.08c	5.44± 0.12c
Pb	1.48±0.10c	10.02±0.46c	1.86±0.12d	3.86 ± 0.14d
Pb + 1.0µM BL	2.68 ± 0.14b	20.01±0.07a	4.48 ± 0.18b	7.06± 0.11ab
Pb + 2.0µM BL	3.46±0.04a	23.04±0.16a	5.02±0.09a	7.95± 0.08a

The values are mean ± S.E (n=5); Pb: 2.5 mM; BL: brassinolide.
Mean followed by the same alphabet in a column is not significantly different at P= 0.05 level.

observed reduced uptake of Cd and Zn in tomato and Pb in sugar beet due to application of brassinosteroids. Further [31] observed that BRs in combination with Pb caused stimulation of phytochelatin synthesis in *Chlorella vulgaris* and reduced the metal toxicity.

Pb stress resulted in remarkable lowering in chlorophyll levels and net photosynthetic rate (P_N) in radish plants [Table 3]. However application of BL minimized the inhibitory effect of metal stress and restored the pigment level in plants. Pretreatment with kinetin increased photosynthetic pigments, photosynthetic activity, and Hill reaction in leaves of Cd treated sorghum plants [32]. Similarly brassinosteroids restored pigment levels in rice plants under salinity stress [33]. There was a drop in the levels of total sugars and reducing sugars in radish plants under Pb toxicity [Table 3]. Seed treatment with BL increased the levels of total sugars and reducing sugars in radish plants under Pb-stress. It has been suggested that the reduction in photosynthesis during stress is mainly due to reduction in leaf area, chlorophyll content and key enzyme RUBP carboxylase/ oxygenase [34]. Further increase in CO_2 fixation and levels of reducing sugars were reported in rice [35] and wheat and mustard [36] by the application of brassinolide. Epibrassinolide enhanced the activity of RUBISCO and elevated the levels of soluble sugars in *Cucumis sativum* [37].

Table.4. Effect of brassinolide on the activity of nitrate reductase, catalase,peroxidase in the leaves of radish plant subjected to Pb stress.

Treatment	Nitrate reductase activity[a]	Catalase[b]	Peroxidase[c]
Control	3.68±0.32c	57.23±1.05c	0.585±0.05d
Pb	1.94±0.10d	28.34±1.94d	0.775±0.03a
Pb + 1.0μM BL	4.20±0.22b	66.35±2.16b	0.720±0.07b
Pb + 2.0μM BL	4.78±0.03a	72.56±2.10a	0.678±0.02bc

The values are mean ± S.E (n=5); Pb: 2.5 mM; BL: brassinolide.
Mean followed by the same alphabet in a column is not significantly different at P= 0.05 level.
[a] Nitrate reductase activity expressed as μ Mol of NO_2 h^{-1} g^{-1} fr.wt.
[b] Catalase activity is expressed in terms of units mg^{-1}protein.
[c] Peroxidase activity is expressed in absorbance units which indicated the amount of purpurogallin formed.

Table.5. Effect of brassinolide on antioxidant enzymes activity in the leaves of radish plant subjected to Pb stress.

Treatment	APOX mg g^{-1} FW protein	GR Units min^{-1}g FW	SOD Unit g^{-1} FW	MDA mg g^{-1}FW protein
Control	16.50±1.08d	9.8±1.52a	10.34±0.52d	45.03±0.20d
Pb	23.10±0.62c	3.5±1.20d	19.25±1.02c	73.54±0.16a
Pb + 1.0μM BL	25.34±0.42b	6.4±1.09c	22.65±1.25b	68.56±0.24b
Pb + 1.0μM BL	28.60±0.73a	8.2±1.05b	25.40±1.15a	63.56±0.26c

The values are mean ± S.E (n=5); Pb: 2.5mM; BL: brassinolide. Mean followed by the same alphabet in a column is not significantly different at P= 0.05 level.

Metal stress decreased the nitrate reductase activity in radish plants. Pretreatment with various concentrations of brassinolide (BL) resulted gradual increase in nitrate reductase activity [Table 4]. The treatment of 2.0μM of BL resulted in maximum increase of nitrate reductase activity. Nitrate reductase plays a pivotal role in the supply of nitrogen and in the growth and productivity of plants [38]. The activity of nitrate reductase is considered as a measure of the habitat dependent nitrate utilization of a plant [39]. Increase in nitrate reductase activity of rice plants under salt stress by application of brassinosteroids has been reported earlier [40]. The inhibitory effect of Cd on nitrate reductase activity of *Erythrina variegata* could be effectively counter balanced by application of triacontanol [41]. The results obtained in the present study clearly demonstrated improved nitrate reductase activity in Pb challenged radish plants due to brassinolide treatment.

Pd toxicity decreased the CAT activity in radish plants (Table 4). In case of Pb treatments supplemented with BL there was a gradual increase in catalase activity. CAT is an important oxidizing enzyme; its activity appears to be positively correlated with increase in growth. Similar increase in catalase activity of sorghum seedling under water stress by application of brassinosteroids has been reported earlier [29].

In contrast to the CAT activity, metal stress increased the POD activity in radish plants [Table 4]. An increase in POD activity is a common response to oxidative and abiotic stress. However reduced POD activity was observed in BRs treated stressed radish plants. Similar reduction in POD activity in BRs-alleviated Cd stress in radish seedlings was reported [42].

Pb stress significantly increased the activity of APOX. Further APOX activity was observed to increase with increasing concentration of BL [Table 5]. The activity of APOX was observed maximum at both 1.0μMand 2.0μM when compared with untreated plants. Like CAT, APOX break down H_2O_2 to H_2O and O_2in brassinosteroid regulated Asada-Halliwell pathway in plants. The results obtained are in consistence with the studies [43] reporting heavy metal stress amelioration earlier by BRs in Cicer arietinum by increasing the activity of CAT and APOX.

Pb significantly reduced the activity of GR [Table 5], supplementation of BL to stressed plants elevated GR activity. Maximum GR activity was observed at 2.0μM compared to control plants. Pre-treatment with BL might help in maintaining the novel pool of glutathione in the reduced state by enhancing GR activity under stressed condition.

The activity of SOD under stress has increased [Table 5]. The activity of SOD which acts as first line of defense against ROS, dismutating O_2^- to H_2O_2 is further enhanced with BL. The study clearly showed that application of BRs to stressed plants further enhanced the antioxidative defense system of the plants by stimulating the activities of various antioxidant enzymes CAT, APOX, GR and SOD. Our findings are supported by observations [44], that BRs application could alleviate Cu stress in radish seedlings by improving the activities of antioxidant enzymes.

Significant production of MDA under Pd stress was observed when compared with control plants [Table 5]. Enhanced MDA levels recorded in stressed plants than control indicate severe damage to the lipid membrane of the radish cells caused by ROS. However supplementation of BL reduced membrane damage by reducing MDA levels. BL at 2.0µM caused maximum downfall in MDA level. Similar observations were also recorded by [45], which showed significant reduction in MDA production n chick pea under salt stress when treated with putriscine.

CONCLUSIONS

The present investigation revealed the ability of brassinolide to alleviate Pb stress. The stress alleviation was associated with increase in chlorophyll and P_N rate. Nitrate reductase, which improves the habitat fitness, was found increased. The results further reveal that BRs strongly protect radish plants by increasing antioxidant enzyme activities, limiting ROS levels, and improving tolerance. Thus it can be inferred that brassinosteroids can act both as growth promoter and inhibition of heavy metal absorption by alleviating plant growth in heavy metal challenged environs.

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