Indian J. Plant Physiol., Vol. 12, No. 4, (N.S.) pp. 396-400 (Oct.-Dec., 2007)

SHORT COMMUNICATION

EFFECT OF 24-EPIBRASSINOLIDE ON THE GROWTH AND ANTIOXIDANT ENZYME ACTIVITIES IN RADISH SEEDLINGS UNDER LEAD TOXICITY

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Received on 27 July, 2007, Revised on 18 Dec., 2007

The supplementation of 24-epibrassinolide reduced lead (Pb) toxicity and enhanced the growth in radish (*Raphanus sativus* L.) seedlings. The activity of antioxidant enzymes like catalase, ascorbate peroxidase, guiacol peroxidase, superoxide dismutase showed an increase in brassinosteroid treated Pb-stressed seedlings when compared to control as well as lead alone treated seedlings. Supplementation of 24-epibrassinolide to Pb stress treatments was also associated with reduced peroxidase activity and increase in the total glutathione content. The studies demonstrated the ameliorating ability of 24-epibrassinolide in scavenging the reactive oxygen species thereby reducing the oxidative stress induced by Pb in radish seedlings.

***Key words*: Antioxidant enzymes, brassinosteroids, lead, radish, seedling growth.**

Heavy metals constitute the major pollutants that are accumulated in environment. Heavy metals induced stress causes various direct and indirect effects on all physiological processes in plants (Woolhouse 1983). Toxic effects of heavy metals on plant physiology and metabolism are very complex and they depend on plant species, nature of heavy metal and its concentration. Metals like lead, mercury, cadmium, arsenic and chromium have no biological function and are toxic to life even at very low concentrations (Salt *et al*. 1995). Lead (Pb) is one of the major heavy metals of the antiquity and is considered as a potent environmental pollutant. Though Pb is not an essential element for plants, it gets easily absorbed and accumulated in different plant parts. Excess Pb causes a number of toxicity symptoms in plants like stunted growth, chlorosis and blackening of root system. Pb also inhibits photosynthesis, upsets mineral nutrition, water balance, hormonal status and affects membrane structure and permeability (Sharma and Dubey 2005). The influence of heavy metals on growth of the plants can be minimized by employing growth regulators.

Brassinosteroids, a new group of phytohormones with significant growth promoting activity are essential for many processes in plant growth and development (Rao *et al*. 2002, Sasse 2003). Brassinosteroids have demonstrated a wide spectrum of physiological roles in plants that include stem elongation, pollen tube growth, leaf bending, xylem differentiation and regulation of gene expression (Khripach *et al*. 2000) Apart from growth stimulation, brassinosteroids have the ability to confer resistance to plants against various abiotic stresses (Vardhini *et al*. 2006). The ability of brassinosteroids to alleviate drought stress in sorghum seedlings has been reported by Vardhini and Rao (2003). Ameliorative influence of brassinosteroids on salt stress induced growth inhibition in rice was observed (Anuradha and Rao 2001). Mazorra *et al*. (2002) observed the effect of brassinosteroids on antioxidant enzyme activity,

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thereby imparting drought tolerance in groundnut. In the present study the effect of 24-epibrassinolide on growth and the activity of some key antioxidant enzymes in radish seedlings under Pb stress was investigated.

24-epibrassinolide was procured from CID Technologies Inc., Brampton, Ontario Canada. Seeds of radish (*Raphanus sativus* L. var. Pusa chetki long) were purchased from National Seeds Corporation Hyderabad. Preliminary experiments were conducted employing different concentrations of Pb and 2 mM conc. of Pb was selected as metal stress concentration where growth was considerably inhibited. Similarly, from a wide range of concentrations 1µM, 2µM conc. of 24-epibrassinolide were selected where significant growth stimulation was observed.

Radish seeds were surface sterilized with 0.5 % (v/v) sodium hypochlorite solution and thoroughly washed with sterile distilled water. Seeds were soaked for 24h in either (i) distilled water (control), (ii) 2.0 mM Pb solution (stress control) and (iii) 2.0mM Pb supplemented with 1mM or 2mM 24-epibrassinolide solution. For each treatment 20 seeds were placed per sterile petri plates of 15 cm diameter, layered with whatman No.1 filter paper with 5ml test solution. The plates were kept at 25±1ºC. On 6th day various antioxidant enzymes such as catalase, peroxidase, ascorbic peroxidase, guaiacol peroxidase, superoxide dismutase and total glutathione contents were assayed. Seedling growth in terms of seed length, seed fresh and dry weights, was recorded on the 7th day.

For extraction of antioxidant enzymes, the seedlings were homogenized with sodium phosphate buffer at pH 7.0 with a chilled pestle and mortar (Kar and Mishra 1976). The homogenate was centrifuged at 12,000g for 20 min and the resulting supernatant was used to determine antioxidant enzyme activity. The whole procedure was carried out at 4ºC and protein content of the supernatant was determined according to Lowry *et al.* (1951).

The catalase activity was measured according to the method of Barber (1980). The assay mixture was composed of 3.5ml of phosphate buffer (pH 6.8), 2.0 ml of hydrogen peroxide and 0.5 ml of enzyme extract. The reaction was stopped by adding 10 ml of 2% (v/v) conc. sulphuric acid, the residual hydrogen peroxide was titrated against 0.01M potassium permanganate and catalase is expressed as unit g^{-1} fw. The peroxidase activity was assayed as per Kar and Mishra (1976). The reaction mixture contained 2.5 ml of phosphate buffer (pH 7), 1.0 ml of 0.01mM pyragallol, 1.0 ml of 5mM hydrogen peroxide and 0.5 ml enzyme extract. After 5min the reaction was stopped by adding 1.0 ml of 2.5N sulphuric acid. The amount of purpurogallin formed was estimated by measuring the absorbance at 420nm. The peroxidase activity is expressed in absorbance units. For ascorbate peroxidase (APOX), the reaction mixture contained 50mM phosphate buffer (pH 7.0), 0.2mM EDTA, 0.5 mM ascorbic acid, 250mM H_2O_2 and 50µg of protein. The activity of APOX was measured spectophotometrically by measuring the rate of ascorbate oxidation at 290 nm for 1min. The amount of ascorbate was calculated from the extinction coefficient of 2.6 mM^{-1} cm^{-1} by the method of Nakano and Asada (1981). Guaiacol peroxidase (GPX) was measured by the method of Mazhoudi *et al*. (1997). The reaction mixture contained 50 mM phosphate buffer, 0.2 mM guaiacol, 10 mM H_2O_2 and distilled water in a total volume of 3 ml. The reaction was started by adding 50µg of protein. The change in absorbance of one unit per min at 470nm (extinction coefficient of 26.6 mM^{-1} cm^{-1}) gave the activity of GPX. The activity was expressed in enzyme units.

The activity of superoxide dismutase was determined based on inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) (Beauchamp and Fridovich 1971). One unit of activity is the amount of protein required to inhibit 50% initial reduction of NBT under light. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 1.5 ml methionine, 1 ml of NBT, 0.75 ml triton-X-100, 2 mM EDTA, 10µl of riboflavin and 50µg of protein.

Total glutathione [GSH (reduced) +GSSG (oxidized)] content was estimated according to the method of Hissin and Hilf (1976).One gram of seedlings were homogenized with 10 ml Tris EDTA (pH=8.2) and centrifuged at 25,000 g for 30 minutes at 4^0 C. From the homogenate 300µl was pipetted into 1 ml of tube to which 60 µl of 25% phosphoric acid is added and kept

in ice for 5 minutes, centrifuged at 25,000g for 30 minutes at 4^0 C. Supernatant was collected for the estimation of GSH and GSSG. For reduced glutathione (GSH) estimation, 50 µl of supernatant was added to 450µl of cold phosphate EDTA buffer (pH=8) and mixed thoroughly. Aliquots (25-50 µl) of this was taken into 5 ml test tubes and made up to 100 µl with cold glass distilled water to which 1.8 ml of phosphate EDTA buffer and 100 µl of OPT (*o*-phthalaldehyde) solution was then added and incubated at 25^0C for 15 minutes. Fluorescence was measured in spectrofluorometer at 350nm and 420 nm. For oxidized glutathione (GSSG) assay, aliquot (50 µl) was incubated with 20 µl of NEM (N-ethyl malcimide) reagent for 30 minutes, then 430µl of 0.1 N NaOH was added. From this 50 and 100 µl aliquots were taken into 5 ml test tubes and made up to 100 µl with glass distilled water, 100 µl of OPT and 1.8 ml of 0.1 N NAOH was added and incubated at room temperature for 15 minutes. After incubation, fluorescence was measured in spectrofluorometer at 350 nm and 420 nm.

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 lower case alphabets are used in the figures to highlight the significant differences between the treatments.

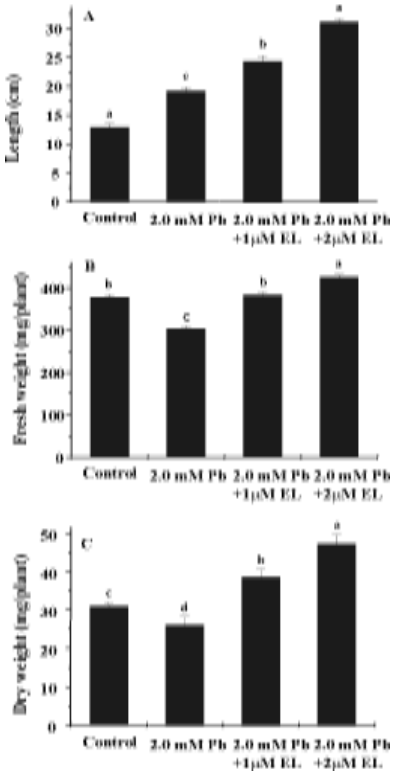

Fig. 1. Effect of 24-epibrassinolide (EL) on the seedling growth (A-C) of radish seedlings under Pb stress. The values are means of three individual experiments. *N*=3 for ±SD. The differences were considered significant if *P* was at least <0.05. The mean values have been compared and lower case alphabets are used in the figures to highlight the significant differences between the treatments.

There was a considerable decrease in fresh and dry weight of radish seedlings when subjected to Pb stress as compared to unstressed radish seedlings (Fig.1A-C.). Similarly decrease in seedling growth of rice due to Pb was reported by Verma and Dubey (2003). The application of brassinosteroids resulted in stimulation of seedling growth under metal stress, thus alleviating the suppression of growth caused by metal stress as reflected by the increase in seedling length, seedling fresh weight and dry weight. Metwally *et al.* (2003) reported that salicylic acid alleviates the Cd toxicity in barley seedlings. Similarly brassinosteroids were found to ameliorate the impact of salinity stress on growth in rice (Anuradha and Rao 2003).

The catalase activity was declined in radish seedlings under Pb stress. Brassinosteroid treatment enhanced the activity of catalase (Fig.2A). The enzyme activity extracted from radish seedlings in metal plus brassinosteroid supplemented Pb media was more when compared to the enzyme extracted from only metal stressed radish seedlings. Catalase is an important scavenging enzyme which helps in break-down of H_2O_2 induced by stress (Foyer *et al.* 1994). Enhanced catalase activity in radish seedling treated with 24-epibrassinolide under metal stress might have resulted in scavenging reactive oxygen species (ROS) leading to restoration of growth. Cao *et al.* (2005) suggested that brassinosteroids enhance the oxidative stress resistance in *Arabidopsis* by increasing the transcript levels of defense gene catalase.

Peroxidase is another antioxidant enzyme involved in scavenging H_2O_2. Radish seedlings grown under Pb stress exhibited elevated peroxidase activity (Fig.2B). However, in 2 µM brassinosteroid treated radish

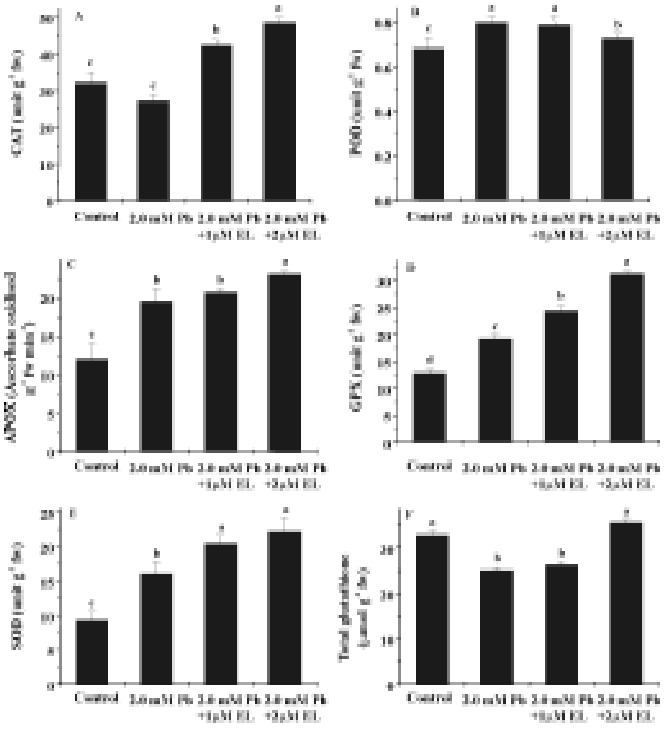

Fig. 2. Effect of 24-epibrassinolide (EL) on antioxidant enzyme activities A (Catalase), B (Peroxidase), C (Ascorbate peroxidase), D (Guaiacol peroxidase), E (Superoxide dismutase) and F (Total glutathione) of radish seedlings under Pb stress. The values are means of three individual experiments. *N*=3 for ±SD. The differences were considered significant if *P* was at least <0.05. The mean values have been compared and lower case alphabets are used in the figures to highlight the significant differences between the treatments

seedlings under Pb stress, the activity of peroxidase was lower as compared to only metal treated radish seedlings. APOX and GPX activity also showed an increase with heavy metal stress (Fig. 2C-D). However, brassinosteroid treatment further enhanced the activity in radish seedlings. Like catalase, APOX and GPX break down H_2O_2 to H_2O and O_2. Spraying with spermidine also increased the activity of APX and GPX in *Typha latifolia* under Cd stress (Tang *et al.* 2005).

The activity of superoxide dismutase in radish seedlings under Pb stress increased as compared to the control plants. 24-epibrassinolide treatment further enhanced the activity of superoxide dismutase (Fig. 2E). Higher superoxide dismutase activity in brassinosteroid supplemented treatments might have offsetted the Pb stress leading to the restoration of growth of radish seedlings. Increase of superoxide dismutase activity in *Arabidopsis* under heavy metal toxicity by brassinosteroid application has been also reported by Cao *et al.* (2005).

Under Pb stress the level of total glutathione was reduced in radish seedlings (Fig. 2F). Glutathione is the most abundant thiol (SH) compound in plant tissues, bacteria and yeast. GSH is the dominant form in organisms and plays many different roles such as protection against reactive oxygen species and maintenance of protein 'SH' groups. Under heavy metal stress GSH is converted to its oxidized form GSSG. Schutzendubel and Polle (2002) suggested that the depletion of GSH was critical step in Cd induced ROS generation. GSH is regenerated by the activity of glutathione reductase (GR). Application of brassinosteroids to radish seedlings under Pb stress enhanced the level of glutathione reductase (GR) which maintains glutathione homeostasis by reducing GSSG to GSH.

The present investigation demonstrated the ameliorative effects of 24-epibrassinolide on the Pb toxicity induced growth inhibition in radish seedlings. Metal stress alleviation by brassinosteroids was also reflected in the activities of several free oxy radical scavenging enzymes.

ACKNOWLEDGEMENTS

The financial support to S. Anuradha by Council of Scientific and Industrial Research (CSIR), New Delhi, India in the form of RA is gratefully acknowledged.

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