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# Modulation of antioxidative defense expression and osmolyte content by co-application of 24-epibrassinolide and salicylic acid in Pb exposed Indian mustard plants

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ABSTRACT

The study focuses on potential of combined pre-soaking treatment of 24-Epibrassinolide (EBL) and Salicylic acid (SA) in alleviating Pb phytotoxicity in *Brassica juncea* L. plants. The seeds after treatment with combination of both the hormones were sown in mixture of soil, sand and manure (3:1:1) and were exposed to Pb concentrations (0.25 mM, 0.50 mM and 0.75 mM). After 30 days of growth, the plants were harvested and processed, for quantification of various metabolites. It was found that pre-sowing of seeds in combination of EBL and SA, mitigated the adverse effects of metal stress by modulating antioxidative defense response and enhanced osmolyte contents. Dry matter content and heavy metal tolerance index were enhanced in response to co-application of EBL and SA. The levels of superoxide anions, hydrogen peroxide and malondialdehyde were lowered by the combined treatment of hormones. Enhancement in activities of guaiacol peroxidase, catalase, glutathione reductase and glutathione-s-transferase were recorded. Contents of glutathione, tocopherol and ascorbic acid were also enhanced in response to co-application of both hormones. Expression of *POD, CAT, GR* and *GST1* genes were up-regulated whereas *SOD* gene was observed to be down-regulated. Contents of proline, trehalose and glycine betaine were also reported to be elevated as a result of treatment with EBL + SA. The results suggest that co-application of EBL + SA may play an imperative role in improving the antioxidative defense expression of *B. juncea* plants to combat the oxidative stress generated by Pb toxicity.

## 1. Introduction

*Brassica juncea* L. is a known hyperaccumulator of metals and posses strong antioxidative defense system (Ariyakanon and Winaipanich, 2006; Jagtap et al., 2013). During its life time, it comes across a number of abiotic stresses out of which metal stress leads to significant loss of productivity (Yusuf et al., 2010; Shekhawat et al., 2012). Lead (Pb) is a highly toxic metal of huge environmental concern. Among various metals ions, Pb has been documented as most hazardous along with arsenic (As), cadmium (Cd) and chromium (Cr) (ATSDR, 2007). Pb is being added to the agricultural soil via i) breakdown of old rocks, ii) industrial activities like manufacturing of paints, petroleum, batteries and explosive, iii) exhaust clouds from industries and automobiles and iv) fertilizers and pesticides (Sharma and Dubey, 2005). It has been reported to disturb several physiological processes in plants by enhancing synthesis of ROS (Reactive Oxygen Species), leading to oxidative stress and suppression of photosynthetic machinery (Yang et al., 2015). These responses result in retarded growth and germination. At cellular level, Pb also disrupts membrane stability, imbalance in nutrient uptake and disturbed water balance, alteration in endogenous level of hormones, various enzymes and mitotic divisions (Jili et al., 2009; Kaur et al., 2012). The toxic metal ions bind to protein sites by displacing original essential metal ions eventually causing disruption of cellular functions and consequently resulting in phytotoxicity (Nagajyoti et al., 2008; Jaishankar et al., 2014).

Plants adopt several stress protection strategies to counteract Pb ion toxicity, out of which the endogenous synthesis and exogenous supplementation of phytohormones is of primary concern. Phytohormones are reported to enhance productivity and quality of crops in terms of growth, differentiation and stress management (Choudhary et al., 2010). Brassinosteroids (BRs) are a nonpareil or a distinct class of plant hormones which are reported to modulate wide array of biochemical and physiological processes in plants under varied stresses including water deficit, heavy metal, salt and temperature (Gupta et al., 2004;

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Sharma et al., 2011). Exogenous application of 24-epibrassinolide (EBL) counteracts various metal stresses including Zn stress in radish (Ramakrishna and Rao, 2015), Cr stress in radish (Sharma et al., 2011, 2016a, 2016b) and Al stress in common bean (Ali et al., 2008) by boosting growth and photosynthetic efficiency. Major adaptive mechanisms by which EBL combat oxidative stress include increased membrane stability, photosynthetic efficiency, antioxidative defense responses and osmotic adjustments (Houimli et al., 2010; Bajguz, 2010; Kocova et al., 2010). Another important class of plant growth regulators is salicylic acid (SA), a phenolic phytohormone which plays an imperative role in modulating various physiological and metabolic processes (Popova et al., 2009; Dong et al., 2015). Additionally, SA has been elucidated to play an important role in anti-stress activities under variable stress conditions. Exogenous supplementation with SA has been widely studied to counters Pb metal stress for eg. Pb stress in rice (Chen et al., 2007), Cd stress in maize (Krantev et al., 2008) and Cu stress in common bean (Zengin, 2014) by boosting growth and photosynthetic efficiency.

Several phytohormone signaling cascades are reported to interplay during abiotic stresses which include gibberellins (GB), abscisic acid (ABA), SA, BRs, jasmonic acid (JA) and ethylene (Vlot et al., 2009; Kanwar et al., 2015). Moreover, Divi et al. (2010) pointed out that application of combination of BRs and SA, resulted in increase in the expression of two important components of SA biosynthetic pathway i.e. *NPR-1* (Nonexpression of pathogenesis-related genes 1) and *WRKY70* transcription factor. These two components enhance tolerance of *Arabidopsis thaliana* plants to heat and salinity stress. A recent observation by Deng et al. (2016) demonstrates involvement of BRs in enhancing of MAPK (Mitogen Activated Protein Kinase) activity. In plants MAPK pathway plays a key role in plant acquired tolerance to pathogen attack. In tobacco (*Nicotiana benthamiana*) plants, two imperative MAPKs have been found including wound-stimulated protein kinases (WIPKs) and Salicylic acid-stimulated protein kinases (SIPKs) (Jin et al., 2003; Kobayashi et al., 2010). The SIPKs pathway is also induced in response to SA application. According to Deng et al. (2016), the NbMEPK2-NbSIPK pathway has an imperative role in BRs induced tolerance to various stresses.

Several other reports also reveal significant involvement of BRs and SA in defense responsive mechanisms in plants under different stresses. However, there is need to work out the interactive effect of EBL and SA on Pb stressed *B. juncea* plants. Kohli et al. (2017) determined the effect of combined treatment with EBL and SA on some of the metabolites including phenolic compounds, metal chelating compounds, organic acids and antioxidative capacity of *B. juncea* seedlings grown hydroponically. The present work is further extension to observe ameliorative effect of EBL and SA treatment to 30 days old plants of *B. juncea* plants raised under natural conditions in field.

## 2. Materials and methods

### 2.1. Plant material and treatments

The seeds of Indian mustard (var. RLC 1) were sterilized using 0.01% $HgCl_2$ followed by washing with double distilled water. Seeds were then pre-sown in combined solution of 24- EBL, $10^{-7}$ M and SA, 1 mM for 8hr. The treatments of EBL and SA were decided on the basis of growth promotion. The field trial was conducted in earthen pots of uniform sizes (10 × 12 in.) which contained 5 kg of the soil mixture comprising of soil, sand and manure in ratio 3:1:1. Soaked seeds of B. juncea were then sown in respective pots. The pots were filled with soil supplemented with three Pb $(NO_3)_2$ concentrations (0.25 mM, 0.50 mM and 0.75 mM) which were selected on the basis of IC 50 (50% inhibiting concentration). The plants were then grown under natural conditions and were irrigated with ground water as per need. The plants were harvested after 30 days of sowing, followed by performing independent experiments (each with three replicates of each treatments) in order to analyze statistically.

### 2.2. Evaluation of dry matter content (DMC) and metal tolerance index

DMC of 30 days old plant samples were estimated by methods proposed by Wolf et al. (1995). For determination of DMC, the weights of empty dishes were recorded. Then fresh plant samples were kept in evaporating dishes and their weights were again measured. Then these samples were dried in oven for 16 h at 105 °C, after which it was cooled and weighed again.

$$\% \text{ Dry matter content} = \frac{(\text{weight of dry sample} + \text{evap. dish}) - \text{weight of evap. dish}}{(\text{weight of fresh sample} + \text{evap. dish}) - \text{weight of evap. dish}} \times 100$$

Method for estimation of heavy metal tolerance index was given by Bálint et al. (2002).

$$\% \text{ Heavy metal tolerance index} = \frac{\text{Dry weight of Treatment Plants}}{\text{Dry weights of Untreated plants (CN)}} \times 100$$

### 2.3. Metal accumulation

Pb metal accumulation in root and shoot was determined using flame atomic absorption spectrophotometer (flame AAS AA240 FS, Agilent Technologies) with VGA 77 vapor generation assembly. The lamp used for Pb estimation was Single Element Ultra AA Hollow Cathode Lamp. The digestion of dried plant samples were done by following the method given by Allen et al. (1976). After harvesting of the plants, shoots and roots were washed with distilled water and then dried at 80 °C. The dried plant samples were finely powdered and 0.5 g of dried plant material was then digested by wet digestion method using aqua regia in ratio 1:5:1 ($H_2SO_4$: $HNO_3$: $HClO_4$, v/v) in glass beakers using a hot induction plate. The digested samples were cooled and filtered and the final volume was made upto 50 ml using double distilled water. The digested samples were stored at room temperature until further analysis.

### 2.4. Oxidative damage

**Superoxide anion content** was determined by method proposed by Wu et al. (2010). 1 g sample of seedlings was homogenized in 50 mM phosphate buffer (pH- 7.8) (consisting of 2% PVP-30% and 0.50% Triton X-100). 500 µl of supernatant was mixed with 100 µl of hydroxylamine hydrochloride and 500 µl of 50 mM phosphate buffer (pH-7.8). Mixture was incubated at 25 °C for 30 min. 1 ml of above solution was taken for further estimations. 1.0 ml of 58 mM 3-aminobenzene-sulphonic acid and 1 ml of 1-napthylamine followed by incubation at 25 °C for 20 min. Absorbance was read at 530 nm. **Hydrogen peroxide ($H_2O_2$) Content** was determined by method proposed by Velikova et al. (2000). 500 mg of seedling sample was homogenized in 2 ml of TCA (trichloroacetic acid), followed by centrifugation at 12000 rpm for 15 min. To 500 µl of supernatant, 500 µl of potassium phosphate buffer and 1.0 ml of PI (potassium iodide) were added. Absorbance was read at 390 nm. **Lipid Peroxidation** was estimated in terms of content of MDA (malondialdehyde), which was determined by following method given by Heath and Packer (1968). 1.0 g of seedling sample was homogenized in 5 ml of TCA (0.1%, w/v), followed y centrifugation at 5000 rpm for 15 min at 4 °C. This mixture was heated at 95 °C for 30 min and immediately cooled in ice bath. The absorbance of sample was read at 532 nm.