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Arsenic induced modulation of antioxidative defense system and brassinosteroids in *Brassica juncea* L.

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ABSTRACT

Brassica juncea (Indian mustard) L. plants were exposed to different concentrations (0.0, 0.1, 0.2 and 0.3 mM) of arsenic (V) and harvested after 30 and 60 days of sowing for the analysis of growth parameters, metal uptake, brassinosteroids (BRs) synthesis and oxidative stress markers. As (V) significantly hampered the growth of *B. juncea* plants and triggered the modulations of various stress markers like proteins, antioxidative enzymes (SOD, CAT, POD, APX, GR, MDHAR and DHAR) and MDA content. Furthermore, As (V) induced the synthesis of 4 BRs, castasterone, teasterone, 24-epibrassinolide, and typhasterol, which were isolated and characterized by gas chromatography–mass spectrometry (GC–MS). The study further highlig5895hted the significant uptake of arsenic ions by mustard plants.

1. Introduction

During the last century, the biosphere has been contaminated with heavy metals due to smelting, mining and waste disposal practices (Diwan et al., 2010). Amendment of agricultural soils with municipal sewage sludge is another important activity contributing to the load of these metals. The excess amount of these hazardous metals in the environment is reported to be dangerous to human health (Olowoyo et al., 2012). Metals like mercury (Hg), cadmium (Cd), lead (Pb), arsenic (As), copper (Cu), zinc (Zn), tin (Sn), and chromium (Cr) are of prime concern owing to their toxicities (Wright, 2007; Ghosh, 2010). These metals and metalloids are fatal to plants and can cause adverse effects even in trace amounts (Kanwar et al., 2012).

Contamination of the environment with the metalloid As has grasped a worldwide interest. It is released by geological activities, smelting operations, fossil fuel combustion and by the use of pesticides and herbicides (Gupta et al., 2011; Selvaraj et al., 2013). Arsenic exists in −3, 0, +3 and +5 oxidation states in nature and its known forms are arsenious acids (H_3AsO_4, $H_3AsO_4^-$, $H_3AsO_4^{2-}$) and arsenic acids (H_3AsO_4, $H_2AsO_4^-$, $HAsO_4^{2-}$), arsenates, arsenites, methylarsenic acid, dimethylarsinic acid and arsine etc. in environment (Mohan and Pittman, 2007). Arsenic toxicity in plants leads to oxidative stress, resulting in the formation of free radicals (Flora, 2011). These free radicals damage cell constituents causing cell damage or death (Chardi et al., 2009). Plants cope up the deleterious effects of metals, through activation of the antioxidative defense system comprising of enzymatic and non-enzymatic components.

In addition to these, phytohormones also provide stress tolerance to plants under various stresses (Peleg and Blumwald, 2011). Brassinosteroids (BRs), a group of polyhydroxylated steroidal hormones have been reported to provide stress protection to plants. Recent studies revealed stress protective properties of BRs in plants under various stresses like heavy metals, drought, salt, high and low temperature and pathogen attack (Bajguz and Hayat, 2009; Kanwar et al., 2013). BRs provide tolerance to plants by interacting with other hormones. Their crosstalks with auxins, cytokinins, jasmonic acid, salicylic acid etc. play a significant role in triggering defense mechanisms (Bajguz and Hayat, 2009). Reports have confirmed the potential of plant hormones to improve crop performance synergistically under abnormal environmental conditions (Bajguz and Hayat, 2009). *Brassica juncea* (*B. juncea*) is raised as an oilseed crop in India. It is also known as hyper-accumulator of heavy metals (Kanwar et al., 2013). Metal induced antioxidative defense system and BRs synthesis needed to be investigated in *B. juncea* plants. Keeping this in mind, the present study was framed to study modulation of antioxidative defense system and BRs in *B. juncea* plants under As (V) stress. The effect of increased concentrations of As on growth, antioxidative capacity (as SOD, POD, APX, CAT, GR, DHAR and MDHAR), lipid peroxidation

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and synthesis of BRs in 30 and 60 days old plants of *Brassica* was investigated to have a better understanding of mechanism of defense against As stress.

2. Material and methods

2.1. Standards and Chemicals used

24-epibrassinolide (24-EBL), sephadex LH-20 and methane boronic acid were procured from Sigma-Aldrich, India Pvt. Ltd., New Delhi. Castasterone (CS), typhasterol (TY) and teasterone (TE) were procured from Chemical Clones Pvt. Ltd. Canada. Silica gel (60–120 mesh size) was obtained from Qualigens fine chemicals, Glaxo India Ltd, Mumbai. For TLC analysis pre-coated ALUGRAM SIL G/UV 254 plates were used and bought from Macherey–Nagel, Germany. All the solvents used in the extraction process were of HPLC grade.

2.2. Plant material

Certified seeds of *B. juncea* L. variety PBR 91 were arranged from Department of plant breeding, Punjab Agriculture University (Punjab) India. Seeds were surface sterilized with 0.01% sodium hypochlorite and rinsed five times with double distilled water. Then rose in earthen pots containing different concentrations of Arsenic metal stress in the form of $Na_2HAsO_4.7H_2O$ (0, 0.1, 0.3, and 0.5 mM) in the Botanical garden of Guru Nanak Dev University, Amritsar, Punjab, India under natural conditions.

2.3. Studies on morphological parameters

After 30 and 60 days of sowing, plants were harvested and analyzed for morphological parameters like shoot length and number of leaves per plant. Fifteen plants from three replicates of same concentration were selected for analysis.

2.4. Heavy metal analysis

The leaves and shoots of 30 and 60 days old *B. juncea* L. plants were harvested. The collected samples were oven dried at 80 °C for 24 h. The dried samples were digested in a mixture of concentrated nitric acid (HNO_3) and perchloric acid ($HClO_4$) (V/V 3:1). The solution was mixed well and kept on a hot water bath at 60–70 °C for about 20 min until clear solutions were left. The solutions were supplemented with double distilled water after cooling, filtered through Whatman filter paper. The samples were then analyzed in triplicate for arsenic content by atomic absorption spectroscopy (AAS) through SHIMADZU AAS-6300 attached with HVG.

2.5. Analysis of biochemical parameters

2.5.1. Antioxidative enzymes

For estimation of antioxidative enzyme activities and protein, content 0.5 g of 30 and 60 days old *B. juncea* L. plants were homogenized in 5.0 ml of 100 mM potassium phosphate buffer (pH-7.0). The homogenate was centrifuged at 4 °C for 20 min at 15,000*g*.

2.5.1.1. Ascorbate peroxidase (APX, EC 1.11.1.11). Ascorbate peroxidase activity was determined following the method proposed by Nakano and Asada (1981).

2.5.1.2. Catalase (CAT, EC 1.11.1.6). Catalase activity was calculated by the method suggested by Aebi (1984).

2.5.1.3. Guaiacol peroxidase (POD, EC 1.11.1.7). The activity of peroxidase was measured according to the method proposed by Putter (1974).

2.5.1.4. Superoxide dismutase (SOD, EC 1.15.1.1). SOD activity was estimated according to Kono (1978) by noting its potential to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) dye by superoxide radicals, which are produced by the auto-oxidation of hydroxylamine hydrochloride.

2.5.1.5. Glutathione reductase (GR, EC 1.6.4.2). Glutathione reductase was measured by the method proposed by Carlberg and Mannervik (1975).

2.5.1.6. Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4). Monodehydroascorbate reductase activity was determined according to the method proposed by Hossain et al., (1984).

2.5.1.7. Dehydroascorbate reductase (DHAR, EC 1.8.5.1). Dehydroascorbate reductase activity was measured by the method given by Dalton et al., (1986).

2.5.2. Protein estimation

Protein content was determined following the method of Lowry et al., (1951).

2.5.3. Malondialdehyde content

The malondialdehyde (MDA) content was measured using the method described by Heath and Packer (1968).

2.6. Analysis of BRs

2.6.1. Extraction and purification of BRs

30 and 60 days old plants *B. juncea* plants exposed to As (V) stress (0.0, 0.1, 0.2 and 0.3 mM) were harvested and processed for extraction and purification of BRs as per the method described earlier by Kanwar et al., (2013). The biologically active fractions obtained after extraction and purification were subjected to TLC and GC–MS analysis for their characterization.

2.6.2. Radish hypocotyl bioassay

The bioactivity of isolated fractions was determined using intact seedlings of *Raphanus sativus* L. with minor modifications (Takatsuto et al., 1983). Three days old seedlings of radish were transferred to test solutions. After incubation at 25°C in the darkness for 24 h, the elongation percentage of the hypocotyls with respect to the control, determined the biological activity.

2.6.3. Characterization of brassinosteroids

2.6.3.1. Thin layer chromatography. The bioactive fractions and the standards were spotted on TLC plates coated with silica gel 60 F_{254}, and developed with $CHCl_3$: MeOH (8:2) as mobile phase. The spots were detected by spraying Liebermann–Burchard. R_f values for the standard and samples were recorded.

2.6.3.2. Derivatization of purified fraction. Methaneboronic acid (100 μg) and dry pyridine (60 μL) were mixed and 20 μL of this mixture was added to the active fractions. These were heated to 80 °C for 25–30 min. Further trimethyl silylation of methaneboronates was conducted by reacting with N-methyl-N-trimethylsilyltriflouroacetamide (MSTFA). 3 μL of this solution was injected into GC–MS. The standard BRs were also derivatized and subjected to GC–MS analysis.

2.6.3.3. GC–MS analysis. The GC–MS (Shimadzu, GC–MS, QP 2010) analysis of BRs was carried out with the following conditions: EI