BRIEF COMMUNICATION

Effect of 24-epibrassinolide on the photosynthetic activity of radish plants under cadmium stress

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Abstract

The present study was conducted to study the effect of 24-epibrassinolide (EBL) on changes of plant growth, net photosynthetic rate, carbonic anhydrase (E.C. 4.2.1.1) and nitrate reductase (E.C.1.6.6.1) activities in the leaves of *Raphanus sativus* L. under the influence of cadmium (Cd) stress. Cd reduced plant growth, photosynthetic pigment levels, net photosynthetic rate and the activities of carbonic anhydrase and nitrate reductase. However seed application of EBL reduced the toxic effect of Cd on plant growth, pigment content, photosynthesis and enzyme activities. The studies clearly demonstrated the ameliorating effect of 24-epibrassinolide in mitigating the toxicity of Cd in plants.

Additional key words: cadmiun; carbonic anhydrase; carotenoids; chlorophyll; growth; nitrate reductase; photosynthesis; radish seedlings.

Heavy metals (HM) are of great concern as soil pollutants because they can threaten the health of human beings and animals through the food chain. Several industries and agricultural activities contribute to heavy metal contamination of agricultural lands in peri- urban areas (Rattan *et al*. 2002). Cd is a non-essential heavy metal pollutant naturally present in the environment. Major anthropogenic sources of Cd are Cd-containing phosphate fertilizers, sewage sludges and industrial emissions (Adriano 1986). Mining and smelting industries also release substantial amounts of Cd into the environment (Nriagu and Pacyna 1988). Plants readily take up Cd from the soil. Exposure to high levels of Cd results in reduced rates of photosynthesis, chlorosis, growth inhibition, decrease in water and nutrient uptake and finally death (di Toppi and Gabbrielli 1999).

Plant growth regulators (PGRs) play an important role in modern agriculture and proper applications of certain PGRs improve the productivity of crop plants (Nickell 1982). PGRs could not only regulate plant growth but also enhance resistance to various environmental stresses.

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). Ameliorative influence of brassinosteroids on salt stress induced growth inhibition in rice was observed (Anuradha and Rao 2001). Mazzora *et al*. (2002) reported the ability of brassinosteroids in imparting drought tolerance in groundnut.

The present study examines the influence of 24-epibrassinolide (EBL) on plant growth, chlorophyll (Chl) content and photosynthetic rate in radish plants under Cd stress. The effect of EBL on carbonic anhydrase and nitrate reductase activities in radish plants under Cd stress was also investigated.

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Abbreviations: CA – carbonic anhydrase; Cd – cadmium; Chl – chlorophyll; DM – dry mass; EBL – 24-epibrassinolide; FM – fresh mass; g_s – stomatal conductance; HM – heavy metals; NR – nitrate reductase; PGRs – plant growth regulators; P_N – net photosynthetic rate; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; RWC – relative water content; TM – turgor mass.
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EBL employed for the present study was purchased from *M/s CID Technologies Inc*., Brampton, Ontario,Canada. Seeds of radish, *Raphanus sativus* L. Var. Pusa Rashmi were procured from the *National Seeds Corporation*, Hyderabad, India. Cd in the form of cadmium chloride ($CdCl_2 \times H_2O$) was used. Preliminary experiments were conducted employing different concentrations of Cd. 1.0 mM of Cd was chosen as metal stress concentration, which inhibited the growth considerably. From a wide range of concentrations 1 and 2 µM EBL were selected where a significant growth promotion was observed. Seeds were surface sterilized with 0.5 % (v/v) sodium hypochlorite solution and washed thoroughly with several changes of sterile distilled water. Seeds were soaked for 24 h in either (*1*) distilled water (control) (*2*) 1 µM/2 µM EBL (*3*) 1 mM Cd^{2+} (stressed control) (*4*) 1 mM Cd^{2+} supplemented with 1 µM/2 µM EBL. Twenty seeds from each treatment were sown separately into 8×7 cm PET jars containing 80 g of vermiculite. After 3 days when seeds were germinated, the PET jars were transferred to the growth chamber *(N K System BIOTRON, model LPH -200-RD, Nippon Medical and Chemical Instruments Company Ltd*., Japan). Growth conditions were: photoperiod 12 h, temperature 25±2 ºC, relative humidity 60±5 %, white light with a photon flux density of photosynthetic active radiation (PAR) of 56 µmol m^{-2} s^{-2}. 10 ml of 1 mM Cd^{2+} was poured on alternate days throughout the experiment in all the jars except in (*1*) and (*2*) treatments, where the equal quantity of distilled water was added. 30 ml of 1/5 Hoagland solution was added uniformly to all the jars at two-day intervals throughout the duration of the experiment.

On the 20th day, leaf area (using *Leaf Area Meter Model CI-203- CID Inc*., Vancouver, Washington, USA), and plant fresh and dry mass of shoot system above the vermiculite surface were recorded. Total Chl pigments and carotenoid content were estimated adopting the procedure given by Arnon (1949). Leaf discs were homogenized with 80 % acetone and centrifuged, the optical density of the acetone extract was measured at 663, 645 and 470 nm using a *UV-160A UV Visible Recording Spectrometer*, Shimadzu, Japan.

The relative water content (RWC) was calculated by putting the values in the formula proposed by Jones and Turner (1978):

$$RWC = [(FM - DM)/(TM - DM)] \times 100,$$

where the turgor mass (TM) was obtained by dipping the leaf samples in the double distilled water (DDW) for 8 h, thereby saturating them.

Net photosynthetic rate (P_N) and stomatal conductance (g_s) in fully expanded leaves of intact plants under natural light were measured by *LI-COR 6400* portable photosynthesis system (*LI-COR,* Lincoln, NE, USA). The atmospheric conditions during the experiment were: PAR, 1500 ± 30; C_i, 280 ±12 µmol mol^{-1}; atmospheric CO_2, 350 µmol mol^{-1}; relative humidity, 60±5 %; atmospheric temperature 30±2 ºC. Carbonic anhydrase (CA, E.C. 4.2.1.1) was assayed according to Dwivedi and Randhawa (1974). Fresh leaf samples (200 mg) were cut into small pieces in 0.2 M cysteine hydrochloride solution. These pieces were transferred to test tubes containing phosphate buffer (pH 6.8). Solutions of sodium bicarbonate (0.2 M) and bromothymol blue were added to the reaction mixture. CO_2 liberated during catalytic action of CA on $NaHCO_3$ was estimated by titrating the reaction mixture against 0.05 N HCl using methyl red as an indicator. Nitrate reductase activity (NR, E.C. 1.6.6.1) was determined in fresh leaf samples using the procedure described by Jaworski (1971). This method is based on the reduction of nitrate to nitrite, whose values were estimated calorimetrically.

The data were analyzed by one-way *ANOVA*, followed by *Post Hoc* test to determine which specific group pair(s) is statistically different from each other. The differences were considered significant if p was at least ≤0.05. The mean values have been compared and alphabets are used in the table to highlight the significant differences between the treatments.

Cd substantially reduced the growth of the radish plants as compared with that of unstressed plants (Table 1). However, supplementation of Cd with EBL considerably removed the inhibitory effect of Cd. EBL at 2 µM was found to be highly effective in not only removing the inhibitory effect of Cd stress but also further improved the growth in terms of leaf area, fresh and dry mass by 18, 45, and 38 %, respectively, over unstressed control. The application of EBL alone also had a significant effect and increased all the growth parameters over control. The ability of pea plant to tolerate toxic levels of zinc was found increased due to the application of succinate (Zlatimira and Doncheva 2002). Similarly, salicylic acid also increased the tolerance of maize plants against boron toxicity (Gune *et al*. 2005).

Plants treated with Cd showed a decrease in total Chl level by 48 % and carotenoid level by 41 % as compared to the control. However treatment of radish plants under stress with EBL mitigated the inhibitory effect of metal stress and restored the pigment level in plants. Aldesuquy *et al*. (2004) reported that pretreatment of grains with kinetin enhanced synthesis of photosynthetic pigments in leaves of Cd treated sorghum plants. Similarly application of brassinosteroids resulted in restoration of Chl levels in rice plants challenged with salinity stress (Anuradha and Rao 2003).

RWC in leaves is considered as a measure of plant water status, reflecting the metabolic activity in plant tissues (Flower and Ludlow 1986). Cd treatment significantly decreased RWC and the loss was in proportion to the concentration of the metal. However, EBL treatment elevated RWC in Cd-stressed plants (Table 1). Similar increase in RWC by brassinosteroids application was reported for *Brassica juncea* (Hayat *et al*. 2007).

Table 1. Effect of 24-epibrassinolide (EBL) on the plant growth in terms of leaf area, fresh mass (FM), dry mass (DM) of the shoot, total leaf chlorophyll content (Chl), carotenoid content, relative water content (RWC), net photosynthetic rate (P_N), stomatal conductance (g_s), carbonic anhydrase (CA) activity and nitrate reductase activity (NR) in radish plants under Cd (1.0 mM) stress. The values are means ±SD ($n = 3$); mean followed by the same alphabet in a row is not significantly different at p=0.05.

Parameters	Control	EBL (1.0 µM)	EBL (2.0 µM)	Cd (1.0 mM)	Cd+EBL (1.0 µM)	Cd+EBL (2.0 µM)
Total leaf area [cm^2 $plant^{-1}$]	155.2±7.8c	189.8±9.8b	214.2±8.4a	57.3±6.7d	148.0±8.5c	193.3±9.8b
Shoot FM [g]	2.3±0.7c	2.5±0.8b	2.6±0.8a	1.2±0.9f	1.9±0.5e	2.0±0.7d
Shoot DM [g]	1.3±0.5c	1.8±0.8b	2.4±0.6a	0.8±0.7e	1.1±0.6d	1.3±0.8c
Total Chl [mg g^{-1}(FM)]	2.6±0.7bc	2.6±0.7b	3.1±0.6a	1.3±0.8d	2.3±0.5c	2.8±0.7b
Carotenoids [mg g^{-1}(FM)]	0.22±0.1c	0.23±0.1b	0.24± 0.6a	0.13±0.8f	0.17±0.5e	0.21±0.7d
RWC [%]	78 ±6.1c	81±4.0b	86±3.5a	60±5.1f	68±4.5e	73±5.0d
P_N [µmol (CO_2) $m^{-2}s^{-1}$]	16.7±0.7c	20.7±2.3b	22.7±1.2a	8.6±0.7d	14.4±0.6c	19.1±0.5b
g_s [mol m^{-2} s^{-1}]	2.6±0.5c	2.8±0.7b	3.0±0.6a	1.8±0.8f	2.3±0.5e	2.5±0.8d
CA [units mg^{-1} (protein)]	2.3±0.6cd	2.5±0.5c	3.0±0.4a	1.2±0.6f	1.8±0.6e	2.8±0.8b
NR [µM (NO_2) h^{-1} g^{-1}(FM)	3.7±0.7d	4.1±0.4c	4.6±0.3a	1.7±0.4e	3.3±0.5d	4.3±0.4b

Plants exposed to Cd had increased stomatal resistance, structural damage to chloroplast and imbalance in water relations resulting in impairment of photosynthetic functions. P_N in radish plants subjected to Cd showed a decrease of 48 % as compared to the unstressed control plants (Table 1). The decrease in the photosynthetic rate strongly indicated stomatal closure as a factor for reduction during increased level of stress. The reduction in photosynthesis under stress is generally attributed to the reduction in Chl content and the activity of Rubisco (Pandey 2000, Adak and Das 1999). In the present study the seed treatment with EBL resulted not only in a lowering of the inhibitory effect of Cd, but also further enhanced P_N. Enhanced levels of photosynthetic pigments by EBL might have contributed to increase in P_N in radish plants even under Cd stress. Similarly, brassinosteroids enhanced P_N in jack pine seedlings under drought stress (Rajasekaran and Blake 1999) and in cucumber seedlings under chilling stress (Yu *et al*. 2004).

The results of the assay of CA revealed a drastic reduction in the activity of this enzyme in Cd treated plants when compared to the control plants (Table 1). The activity of the enzyme was not only restored by EBL in Cd-treated plants, but also further increased by 18 % over the levels observed in unstressed control plants. Plants treated with only EBL showed a slight increase in CA activity. CA catalyses the reversible interconversion of HCO_3^- and CO_2 in the leaves and regulates the availability of CO_2 to Rubisco (Badger and Price 1994). Increase in CA activity in *Brassica juncea* under Ni stress by the application of 28-homobrassinolide was reported by Alam *et al*. (2007).

The activity of NR in presence of Cd decreased in radish plants by 54 % over the control levels (Table 1). However, a pre-treatment of seeds with EBL resulted in increase in NR activity in radish plants. The activity of NR plays a pivotal role in the supply of nitrogen, and the growth and productivity of plants. Muthuchelian *et al*. (2001) found the inhibiting effect of Cd on NR activity in *Erythrina variegata* could be effectively counter balanced by application of triacontanol.

The results of the study clearly indicated that the application of brassinosteroids is beneficial in the reduction of inhibition caused by Cd. 24 h soaking of the seeds with EBL resulted in the improvement of growth and photosynthetic activity in radish plants under Cd stress. These finding clearly suggest that EBL regulates the response of plants to the Cd stress and could be used as a growth regulator to improve plant growth under stress conditions.

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