EFFECT OF 24-EPIBRASSINOLIDE ON SEED GERMINATION, SEEDLING GROWTH AND HEAVY METAL UPTAKE IN *BRASSICA JUNCEA* L.

P. Sharma and R. Bhardwaj*

Department of Botanical and Environmental Sciences,Guru Nanak Dev University, Amritsar 143005 Punjab, India

Received 22 May 2006

Summary. The effect of 24-epibrassinolide (24-epiBL) on seedling growth, metal uptake and accumulation were investigated in seedlings of *Brassica juncea* L. cv. PBR 91. Seeds of *B. juncea* treated with different concentrations (0, 10^{-7}, 10^{-9} and 10^{-11} M) of 24-epiBL for 8 h were subjected to various concentrations (0, 25, 50 and 100 mg l^{-1}) of heavy metals (Zn, Mn, Co and Ni). Seedling growth was improved by 24-epiBL treatments under heavy metal stress. In addition, 24-epiBL applied at concentrations of 10^{-9} and 10^{-11} M blocked heavy metal uptake and accumulation.

***Key words*:** Brassinosteroids, heavy metal stress, Indian mustard, hormone.

***Abbreviations*:** 24-epiBL - 24-epibrassionolide; BCF - bio-concentration factor; BRs - brassinosteroids; ROS - reactive oxygen species.

*Corresponding author, e-mail: renu_bhardwaj@rediffmail.com

INTRODUCTION

Brassinosteroids are phytohormones with pleiotropic effects. They influence growth, seed germination, cell elongation, photomorphogenesis and senescence. They also help to overcome stress provoked by low and high temperature (Dhaubhadel et al., 1999), drought (Li and Van Staden, 1998), salt (Sasse et al., 1995), infection and pesticides (Sasse, 1999) and heavy metals (Bajguz, 2000b; Janeczko et al., 2005).

Heavy metal pollution has developed as a horrible ecological crisis. Industrial pollution by heavy metals such as Cr, Mn, Co, Ni, Cu, Zn and Hg is harmful not only to human beings, but also to all living organisms. Brassinosteroids have been proposed to reduce the absorption and accumulation of heavy metals and radioactive elements in plants (Bajguz, 2000b; Kaur and Bhardwaj, 2004). It was observed that the low concentrations of brassinosteroids applied at a certain stage of development reduced the metal absorption in barley, tomatoes and sugar beet significantly (Volynets et al., 1997).

The present study was designed to determine the 24-epiBL - regulated effects on growth parameters, metal uptake and accumulation (Zn, Mn, Co and Ni) in *Brassica juncea* L. seedlings.

MATERIAL AND METHODS

Treatments

Certified seeds of *Brassica juncea* L. used in the present investigation were procured from the Department of Plant Breeding, Punjab Agriculture University, Ludhiana. The seeds were surface sterilized with 0.01% $HgCl_2$ and rinsed with double distilled water 3-4 times. The sterilized seeds were soaked for 8 h in different concentrations of 24-epiBL (0, 10^{-7}, 10^{-9} and 10^{-11} M), which was purchased from Sigma Aldrich Ltd. (USA). 10^{-3} M stock solution of this brassinolide was prepared by dissolving it in methanol and further serial dilutions were made in distilled water to prepare 10^{-7}, 10^{-9} and 10^{-11} M concentrations. In control (distilled water), an appropriate amount of methanol was added.

The 24-epiBL-treated seeds were then transferred to Petri dishes lined with Whatman No 1 filter paper and containing different concentrations (0, 25, 50 and 100 mg l^{-1}) of heavy metal solutions (Zn, Mn, Co and Ni), which were chosen after determining the LD_{50} of all these metals. The experiment was conducted in a germinator at 25 ºC and 16 h light/8 h dark in three replicates.

Seed germination percentage, root and shoot lengths and fresh weight of 7-day-old seedlings were measured.

Heavy Metal Analysis

Samples of ground, dried plant material were digested using digestion mixture (H_2SO_4: HNO_3: $HClO_4$ in 1:5:1 ratio) by following the method of Allen et al. (1976). The digested samples were fine filtered through Whatman No 1 filter paper by using double distilled water. The analyses of heavy metals were done using an atomic absorption spectrophotometer (AA-6200 Shimadzu, Japan). The bioconcentration factor (BCF) was calculated as follows:

$$BCF = \frac{\text{Trace element concentration in plant tissues (mg kg}^{-1}\text{) at harvest}}{\text{Initial concentration of the element in the external nutrient solution (mg l}^{-1}\text{)}}$$

Statistical Analysis

Morphological parameters, heavy metal uptake and the bioconcentration factor (BCF) were statistically analyzed by calculating the mean value, standard deviation, standard error, Student's t-test and Z–test using the methodology proposed by Bailey (1995). Significant values were calculated at $P \leq 0.05$ compared to the seedlings grown in heavy metals alone.

Variants			Control	Zn	Mn	Co	Ni
Control	dH_2O	Concentr.	92.66±0.66				
	24-epiBL (M)	10^{-7}	98.00±1.15				
		10^{-9}	96.66±0.66				
		10^{-11}	96.00±0.66				
	Heavy metal (mg l^{-1})	25	-	94.1±1.21	82.2±1.31	74.1±1.23	89.3±1.63
		50	-	90.2±0.87	75.5±1.47	70.5±0.94	86.6±0.45
		100	-	90.8±1.43	73.3±0.86	68.5±0.56	83.3±1.12
24-epiBL (M)+25 mg l^{-1}		10^{-7}		95.0±4.67	90.5±1.53*	84.5±1.90	94.6±0.62
		10^{-9}		95.5±1.39	88.7±0.52	95.2±1.61*	92.6±0.55
		10^{-11}		94.9±0.95	87.3±1.40	90.5±2.94*	92.2±0.58
24-epiBL (M)+50 mg l^{-1}		10^{-7}		94.4±1.62	85.5±1.67	91.1±2.08	96.6±0.70*
		10^{-9}		96.1±1.87*	83.8±2.32	75.5±2.46	87.6±0.72
		10^{-11}		95.5±0.58	78.3±1.02	92.5±0.95	94.4±0.69
24-epiBL (M)+100 mg l^{-1}		10^{-7}		95.5±0.70	85.5±1.67*	73.3±1.27	95.3±0.63*
		10^{-9}		95.5±1.02*	79.4±2.05	80.5±0.95	92.2±0.40
		10^{-11}		91.6±1.30	78.8±2.81	83.3±1.84	95.3±1.15*

Table 1. Effect of 24-epibrassinolide (24-epiBL) upon presowing treatment (8 h) on germination of *B. juncea* seeds under different concentrations (0, 25, 50 and 100 mg l^{-1}) of heavy metals. Means±SE (n=3), * - values significantly different from control at $P \leq 0.05$ (Z-test).

RESULTS

Seed Germination

There was a reduction in seed germination percentage with increasing metal concentration in various treatments. However, when 24-epibrassinolide presowing treatments (10^{-7}, 10^{-9} and 10^{-11}M) were applied, the percent germination increased as compared to the control. Out of different concentrations of 24-epiBL applied, 10^{-7} M caused the maximum increase. It was observed that presowing treatment for 8 h reduced considerably the inhibitory effect of heavy metals, especially of Co on seed germination. The application of 10^{-9} M 24-epiBL promoted seed germination in seeds

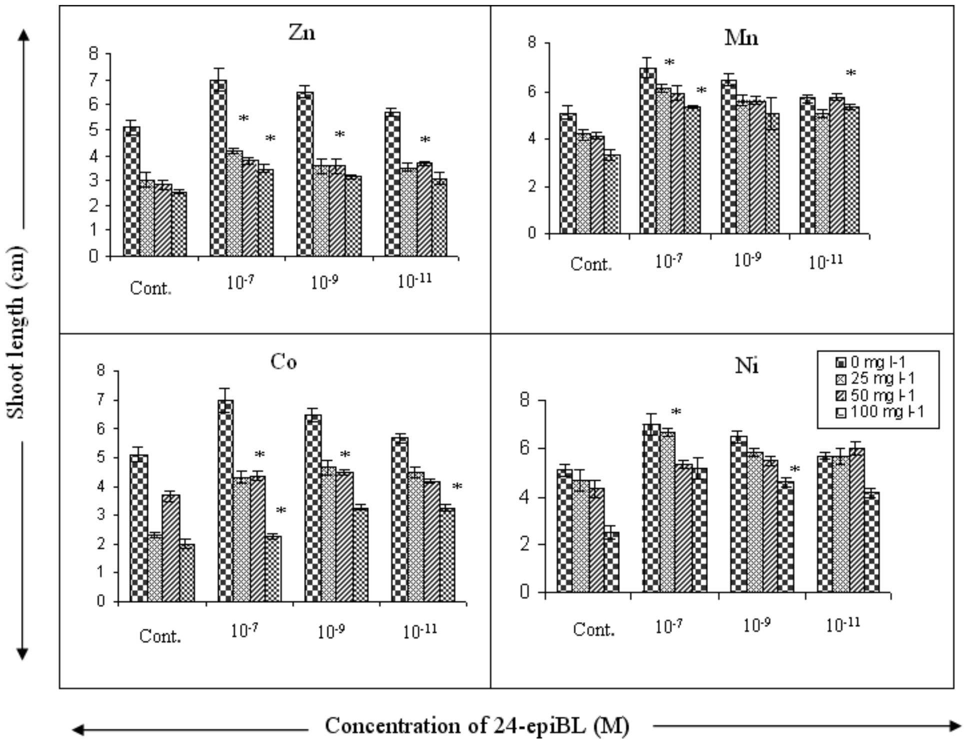

Fig 1. Shoot length of 7-day-old seedlings of *B. juncea* after treatment with 24-epibrassinolide. Bars represent the SE; * indicates statistically significant difference from control at $P \leq 0.05$.

treated with 25 mg l^{-1} Co and 50 mg l^{-1} Zn. In the seeds treated with Mn and Ni, the most effective concentration of 24-epiBL was found to be 10^{-7} M (Table 1).

Shoot length

Similar to germination, shoot length progressively decreased (2.55 cm) with increasing the concentrations of all the heavy metals tested (5.1 cm). When brassinolide treatments alone were given, there was a maximum increase in the shoot length of the seedlings as compared to the control, 10^{-7} M concentration being the most effective (Fig. 1). 24-epiBL applied at 10^{-7} M increased significantly shoot length at all concentrations (25, 50 and 100 mg l^{-1}) of Zn. Similar effects were obtained with Mn treatment. It was recorded that 10^{-7} M significantly reduced toxicity of Mn applied at 50 and 100 mg l^{-1}. At 50 mg l^{-1} Mn shoot length was increased (5.88 cm) due to the effect of 10^{-7} M 24-epiBL (4.11 cm) (Fig. 1) while 10^{-9} M 24-epiBL was found to be the most effective concentration in lowering the toxicity of Co. In addition, the concentrations 10^{-7} and 10^{-11} M 24-epiBL reduced Ni toxicity to the highest degree (Fig. 1).

Root Length

Presowing treatments with 24-epiBL increased root length (Fig. 2). The application of 10^{-7} and 10^{-9} M 24-epiBL led to a maximum reduction in the toxicity of the heavy metals tested. Treatment with 10^{-7} M of 24-epiBL promoted the root lengths in Zn, Mn, Co and Ni-treated seedlings as compared to the control. At lower concentrations (25 mg l^{-1}) of Zn, Mn, and Ni, the most effective concentration of 24-epiBL was 10^{-7} M. On the other hand, 10^{-9} M was found to be the most effective concentration for promoting root length in the presence of Co (Fig. 2). It was observed that presowing treatment with 24-epiBL reduced also the toxicity of the higher concentrations (50 and 100 mg l^{-1}) of the heavy metals tested (Fig. 2).

Fresh weight

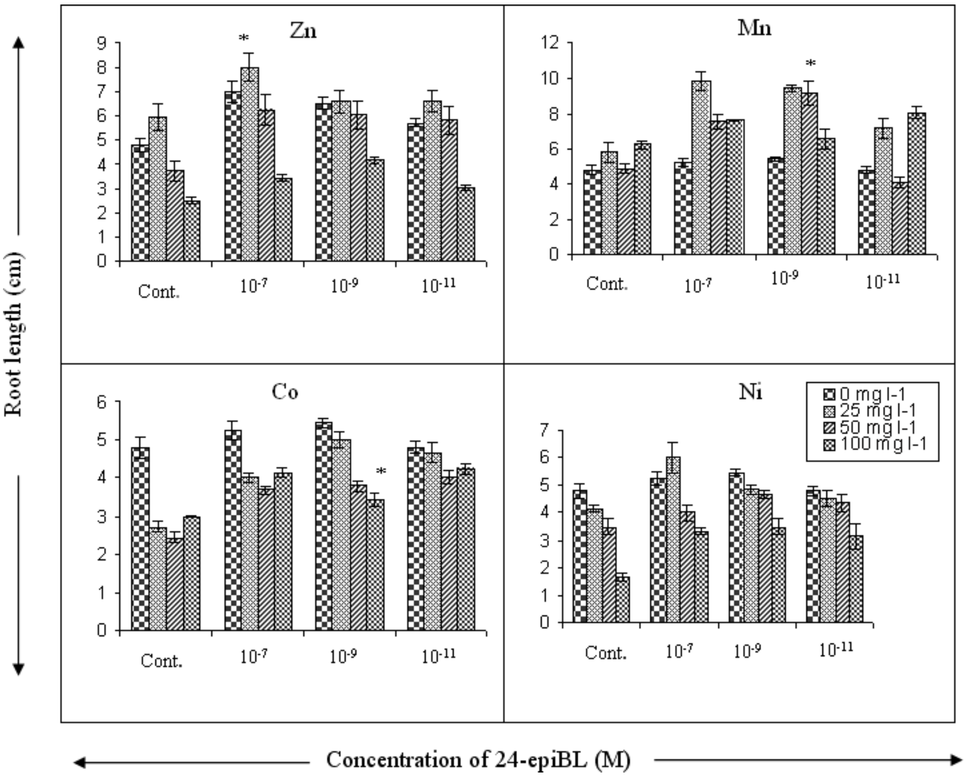

Fig 2. Root length of 7-day-old seedlings of *B. juncea* after treatment with 24-epibrassinolide. Bars represent the SE; * indicates statistically significant difference from control at $P \leq 0.05$.

The high concentration (100 mg l^{-1}) of heavy metals alone decreased the fresh weight of the seedlings (Table 2). Our results showed that 10^{-7} M brassinolide alone (2.77 gm) increased the fresh weight of the seedlings when compared with the control (1.68 gm). 24-epiBL applied at 10^{-11} M could effectively overcome the effect on seedling fresh weight due to 25 mg l^{-1} of the heavy metals tested (Zn, Mn and Co) (Table 2). The toxicity caused by 25 mg l^{-1} Ni was significantly reduced by 10^{-7} M 24-epiBL as compared to the control. Similarly, at a heavy metal concentration of 50 mg l^{-1} (Zn, Mn, Co and Ni) the most effective concentration of 24-epiBL was10^{-9} M. Almost similar results were obtained for 100 mg l^{-1} concentration of heavy

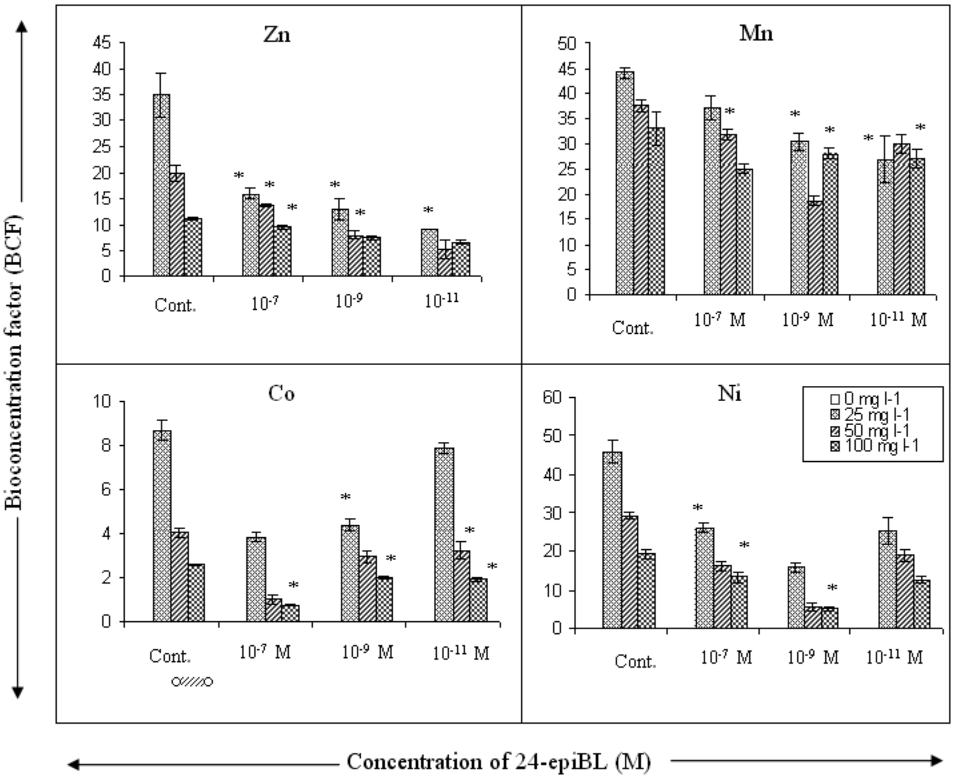

Fig 3. Bioconcentration factor (BCF) of 7-day-old seedlings of *B. juncea* after treatment with 24-epibrassinolide. Bars represent the SE; * indicates statistically significant difference from control at $P \leq 0.05$.

metals in combination with 10^{-7} M and 10^{-9} M 24-epiBL (Table 2).

Heavy metal uptake and bioconcentration factor (BCF)

The uptake of heavy metals and BCF content were significantly lowered under the influence of 24-epibrassinolide treatments. Heavy metal uptake was increased with increasing the metal concentration alone when compared to untreated plants (Table 3). The presowing treatment with different concentrations (10^{-7}, 10^{-9} and 10^{-11} M) of 24-epiBL led to lesser accumulation of heavy metals in the seedlings than the control plants treated with heavy metals alone. A maximum reduction in the uptake and BCF was

Variants			Control	Zn	Mn	Co	Ni
Control	dH_2O	Concentr.	1.68±0.19				
	24-epiBL (M)	10^{-7}	2.77±0.24				
		10^{-9}	2.08±0.18				
		10^{-11}	2.50±0.16				
	Heavy metal (mg l^{-1})	25	-	1.83±0.18	1.06±0.15	1.17±0.07	1.68±0.08
		50	-	1.72±0.09	1.58±0.02	1.05±0.14	1.24±0.02
		100	-	1.14±0.22	1.93±0.12	0.90±0.08	0.82±0.02
24-epiBL (M)+25 mg l^{-1}		10^{-7}		1.87±0.16	2.09±0.04	1.26±0.07	2.01±0.09*
		10^{-9}		1.90±0.20*	1.74±0.21	1.56±0.08	1.37±0.10
		10^{-11}		2.60±0.32	2.49±0.13	1.82±0.03*	1.66±0.08*
24-epiBL (M)+50 mg l^{-1}		10^{-7}		1.91±0.05	2.42±0.14	1.05±0.04	1.84±0.13
		10^{-9}		2.69±0.39	2.51±0.15	1.82±0.02*	1.87±0.06
		10^{-11}		2.10±0.08	2.06±0.39	1.02±0.01	1.15±0.02
24-epiBL (M)+100 mg l^{-1}		10^{-7}		1.56±0.20	1.84±0.09	1.82±0.08	1.15±0.13
		10^{-9}		1.61±0.26	2.13±0.10	1.57±0.07	0.79±0.20
		10^{-11}		1.44±0.18	2.09±0.04	1.28±0.01	1.07±0.15

Table 2. Effect of 24-epibrassinolide (24-epiBL) upon presowing treatment (8 h) on fresh weight of *B. juncea* seeds under different concentrations (0, 25, 50 and 100 mg l^{-1}) of heavy metals. Means±SE (n=3), * - values significantly different from control at $P \leq 0.05$ (T-test).

Variants			Control	Zn	Mn	Co	Ni
Control	dH_2O	Concentr.	120.78±8.90				
	24-epiBL (M)	10^{-7}	98.50±6.70				
		10^{-9}	82.42±5.60				
		10^{-11}	80.64±7.10				
	Heavy metal (mg l^{-1})	25	-	873.4±103.70	1104.0±35.92	217.8±11.27	1150.0±77.93
		50	-	993.3±69.95	1182.0±54.10	202.2±10.60	1462.0±41.29
		100	-	1109.0±41.19	3302.0±334.90	259.70±6.30	1953.0±119.80
24-epiBL (M)+25 mg l^{-1}		10^{-7}		396.4±26.39	926.4±56.83	96.70±5.29	648.5±30.80
		10^{-9}		320.6±51.95	763.1±44.78*	110.00±7.20*	395.3±28.08
		10^{-11}		225.5±11.74	671.4±116.80	197.70±9.10	633.1±92.48*
24-epiBL (M)+50 mg l^{-1}		10^{-7}		685.7±13.54	1591.0±57.29	52.30±9.20	809.8±62.91
		10^{-9}		401.4±32.10	934.4±51.56	148.30±15.22	278.7±53.24
		10^{-11}		262.1±89.49*	1501.4±101.80	163.90±18.50	946.3±78.50
24-epiBL (M)+100 mg l^{-1}		10^{-7}		955.6±52.83	2504.0±91.68	79.40±6.60*	1331.0±145.10
		10^{-9}		793.1±44.10	2813.0±116.00	203.30±7.92	502.9±45.20
		10^{-11}		655.3±49.30	2716.0±177.50	192.50±8.83	1280.0±71.90

Table 3. Metal uptake (μg g^{-1} DW) of 7-day-old seedlings of *B. juncea* as affected by 24-epibrassinolide (24-epiBL). Means±SE (n=3), * - values significantly different from control at $P \leq 0.05$ (T-test).

observed at 10^{-11} M 24-epiBL with all concentrations of Zn and Mn applied (Table 3 and Fig.3). The different concentrations (25, 50 and 100 mg l^{-1}) of Co caused a significant reduction in metal uptake and BCF content in the presence of 10^{-7} M 24-epiBL (Table 3 and Fig. 3). The concentration of 10^{-9} M 24-epiBL was found to be most effective in lowering heavy metal uptake and accumulation of Ni (Table 3 and Fig. 3).

DISCUSSION

Heavy metals have become essential environmental contaminants due to rapid industrialization and urbanization. Increasing levels of heavy metals in the environment affect various physiological and biochemical processes in plants. The toxicity symptoms observed in the presence of excess amounts of heavy metals may be due to interactions at the cellular/ molecular level. Toxicity may be due to binding of metals to sulphydryl groups in proteins, thus leading to inhibition of activity or disruption of structure (Hall, 2002).

The antistress and immunomodulatory activities of brassinosteroids have made them proper candidates for third generation chemicals, which are natural and ecofriendly. The protective effects of BRs have been reported especially under unfavorable low temperature conditions (Takeuchi et al., 1996). BRs further strengthen drought resistance and show favorable effects on plant growth and yield under soil water deficient conditions (Upreti and Murti, 2004). They exhibit salt tolerance (Ozdemir et al., 2004) and disease resistance (Nakashita et al., 2003; Wang and He, 2004) in a number of plants.

The growth-promoting and other regulatory properties of BRs in plants are well known (Khripach et al., 1999). In the present investigation, 24-epiBL were found to reduce the toxicity of heavy metals in *Brassica juncea* plants. The germination percentage of 7-day-old seedlings was significantly increased due to the application of 24-epiBL especially at the lower concentrations applied (10^{-9} M and 10^{-11} M). This result may further be supported by the observation of Hayat and Ahmad (2003) that homobrassinolide increased the percent germination by 17% in wheat grains. Similarly, shoot and root lengths and their fresh weights were increased by the application of BRs. It was observed that seed pretreatment with 24-epiBL reduced significantly heavy metal toxicity. Abd El-Wahed and Gamal El Din (2004) reported stimulation of growth and biochemical constituents in *Chamomilla recutita* upon spermidine and stigmasterol (sterol compounds) application.

Plants have a range of potential mechanisms at the cellular level that might be involved in the detoxification of heavy metals, thus increasing plant tolerance to heavy metal stress. The tolerance could also involve the plasma membrane either by reducing the uptake of heavy metals or by stimulating the efflux pumping of metals that have entered the cytosol. One of the mechanisms for heavy metal detoxification in plants is the chelation of the metal ion by ligands, such as organic acids, amino acids, peptides and polypeptides. Peptide ligands include the metalothionins or small gene-encoded cysteine-rich polypeptides (Bajguz, 2002). The present study revealed the ability of 24-epiBL to reduce the toxic effect of heavy metals (Zn, Mn, Ni and Co) by lowering their uptake and accumulation (BCF). Practically promising results concerning the regulation by BRs of the cell permeability for ions have been recently obtained using different model systems in experiments on the absorption of heavy metals and radionucleides by plants. These may be due to the effect of BRs on the electrical properties of membranes, their permeability and the structure, stability and activity of membrane enzymes. The reduction of toxicity by BRs is associated with enhanced levels of soluble proteins and nucleic acids with the increasing activity of ATPase (an enzyme responsible for acid secretion and changes in membrane level) (Bajguz, 2000a). Brassinosteroids bind to the membrane proteins and scavenge the reactive oxygen species which are generated by heavy metal toxicity, thereby reducing the membrane destruction that results from AOS-induced oxidative damage (Cao et al., 2005). After binding to the membrane proteins BRs may enhance the enzyme and metabolic activities, thus detoxifying heavy metals in plants.

The ability of BRs to regulate cell membrane permeability and transport of ions has found an agricultural application in the areas polluted with heavy metals. Earlier reports showed that treatment with 24-epiBL reduced significantly the absorption of heavy metals in barley, sugarbeet, tomato and radish (Khripach et al., 1996). Bajguz (2000b) reported that 24-epiBL applied at the concentration range of 10^{-6} – 10^{-4} M in combination with heavy metals blocked metal accumulation in algal cells. The present study revealed the involvement of BRs in lowering the uptake of heavy metals, thus reducing their toxicity in plants.

References

Abd El-Wahed, M.S.A., K.M. Gamal El Din, 2004. Stimulation of growth, flowering, biochemical constituents and essential oil of Chamomile plant (*Chamomilla recutita* L., Rausch) with spermidine and stigmasterol application. Bulg. J. Plant Physiol., 30(1-2), 89-102.

Allen, S.E., H.M. Grimshaw, J.A. Parkinson, C. Quarmby, J.D. Roberts, 1976. Chemical Analysis. In: Methods in Plant Ecology. Ed. S.B. Chapman, Blackwell Scientific Publications, Oxford – London, 424-426.

Bailey, N.T.J., 1995. Statistical Methods in Biology. The English University Press, London.

Bajguz, A., 2000a. Effects of brassinosteroids on nucleic acids and protein in cultured cells in *Chlorella vulgaris*. Plant Physiol., 38, 209-215.

Bajguz, A., 2000b. Blockage of heavy metal accumulation in *Chlorella vulgaris* cells by 24-epibrassinolide. Plant Physiol. Biochem., 38(10), 797-801.

Bajguz, A., 2002. Brassinosteroids and lead as stimulators of phytochelatins synthesis in *Chlorella vulgaris*. J. Plant Physiol., 159, 321-324.

Cao, S., Q. Xu, Y. Cao, K. Qian, K. An, Y. Zhu, H. Binzeng, H. Zhao, B. Kuai, 2005. Loss-of- function mutation in DET2 gene lead to an enhanced resistance to oxidative stress in *Arabidopsis*. Physiol. Planta., 123, 57-66.

Dhaubhadel, S., S. Chaudhary, K. F. Dobinson, P. Krishna, 1999. Treatment with 24-epibrassinolide (a brassinosteroid) increases the basic thermotolerance of *Brassica napus* tomato seedlings. Plant Mol. Biol., 40, 333-342.

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. J. Exp. Bot., 53(366), 1-11.

Hayat, S., A. Ahmad, 2003. 28-Homobrassinolide induced changes favoured germinability of wheat grain. Bulg. J. Plant Physiol., 29(1-2), 55-62.

Janeczko, A., J. Koscielniak, M. Pilipowicz, G. Szarek-Lukaszewsa, A. Skoczowspi, 2005. Protection of winter rape photosystem 2 by 24-epibrassinolide under cadmium stress. Photosynthetica, 43(2), 293-298.

Kaur S., R. Bhardwaj, 2004. Blockage of Zn-accumulation in seedlings of *Brassica campestris* L. by 24-epibrassinolide. Keystone Symposium on Plant Responses to Abiotic Stresses, Abstract No. 216: 64, Feb. 19-24.

Khripach, V.A., L.V. Voronica, N.N. Malevannaya, 1996. Preparation for the diminishing of heavy metals accumulation of agricultural plants. Pat. Appl. RU, 95, 101,850.

Khripach, V.A., V.N. Zhabinskii, A.E. de Groot, 1999. Brassinosteroids: A new class of plant hormones. Academic Press, San Diego - CA.

Li, L., J. Van Staden, 1998. Effects of plant growth regulators on drought resistance of two maize cultivars. SAJB, 64(2), 116-120.

Nakashita, H., M. Yasuda, T. Nitta, T. Asami, S. Fujioka, Y. Arai, K. Seikimata, S. Takatsuto, I. Yamaguchi, S. Yoshida, 2003. Brassinosteroid functions in a broad range of disease resistance in tobacco and rice. Plant J., 33, 887-898.

Ozdemir, F., M. Bor, T. Demiral, I. Turkan, 2004. Effects of 24-epibrassinolide on seed germination, seedling growth, lipid peroxidation, proline content and antioxidative system of rice (*Oryza sativa* L.*)* under salinity stress. Plant Grow. Regul., 42, 203-211.

Sasse, J., 1999. Physiological action of brassinosteroid. In: Brassinosteroids: Steroidal Plant Hormones. Ed. A. Sakurai, T. Yokota, S. D. Clouse, Springer-Verlag, Tokyo, 137-161.

Sasse, J.M., R. Smith, R. Hudson, 1995. Effect of 24-epibrasinolide on germination of seeds of *Eucalyptus* in saline conditions. Proc. Plant Grow. Regul. Soc. Am., 136-141.

Takeuchi, Y., M. Ogasawara, M. Konnai, Y. Kamuro, 1996. Promotive effectiveness of brassinosteroid (Ts303) and jasmonoid (PDJ) on emergence and establishment of rice seedlings. Proc. Jpn. Soc. Chem. Regul. Plant, 31, 100-101.

Upreti, K.K., G.S.R. Murti, 2004. Effects of brassinosteroids on growth, nodulation, phytohormone content and nitrogenase activity in French bean under water stress. Bio. Plant., 48(3), 407-411.

Volynets, A.P., L.A. Pschenichanye, V.A. Khripach, 1997. The nature of protective action of 24-epibrassinolide on barley plants. Plant Grow. Regul. Soc. Am., 24, 133-137.

Wang, Z.Y., J.X. He, 2004. Brassinosteroid signal-transduction - choice of signals and receptors. Tends Plant Sci., 9, 91-96.