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Nickel-induced Synthesis of Castasterone and 28-Homocastasterone in *Brassica juncea* L.

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ABSTRACT

Heavy metals in the environment are increasing at an alarming rate. Metals like Cu, Zn and Ni are essential for the growth of plants, although elevated levels of these metals result in growth inhibition and toxicity symptoms. Plants possess a range of cellular mechanisms that may be involved in the detoxification of heavy metals and thus tolerance to metal stress. The concentration of these heavy metals in the environment has increased drastically, posing problems to the health of organisms. Plant growth regulators provide protection from various stresses. Brassinosteroids (BRs) are an important group of steroids hormones which are involved in the amelioration of several stresses. Aiming to understand the role of BRs under heavy metal stress, plants of *Brassica juncea* L. were grown in pots at the Botanical Garden of Guru Nanak Dev University, Amritsar. The plants were subjected to various concentrations of Nickel (0.0, 0.2, 0.4 and 0.6 mM) and harvested on the 45th day to observe the synthesis of these hormones. The GC-MS characterization of the compounds after physio-chemical analysis revealed the presence of castasterone (0.018 and 0.022 ug/Kg FW) and 28-homocastasterore (0.014 to 0.029 ug/Kg FW) in the leaves of *B. juncea* L. plants.

Keywords: *Brassica juncea*, brassinosteroids, castasterone, homocastasterone, heavy metals, stress
Abbreviations: BRs, brassinosterods; **BL**, brassinolide; **CS**, castasterone; **$CHCL_3$**, chloroform; **GC-MS**, gas chromatography-mass spectrophotometry; **HPLC**, high pressure liquid chromatography; **MDA**, malondialdehyde; **mM**, mili molar; **MeOH**, methanol; **Ni**, nickel; **ROS**, reactive oxygen species; **SE**, standard error; **28-HCS**, 28-homocastasterone

INTRODUCTION

Universal contamination of soils with metals has posed immense threat to the human health as the majority of them have been proven to be carcinogenic (Krishna and Govil 2004; Kamaludeen and Ramasamy 2008). In plants, metals like cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), lead (Pb) and nickel (Ni) are engrossed by roots and then translocated to shoots, leading to impaired metabolism and reduced growth (Foy *et al*. 1978; Bingham *et al*. 1986). Metal toxicity leads to alterations of physiological processes at cellular and molecular level by inactivating enzymes, blocking functional groups of metabolically important molecules, displacing or substituting for essential elements and disrupting membrane integrity. The most frequent consequence of heavy metal toxicity is enhanced production of reactive oxygen species (ROS) (Pagliano *et al.* 2006; Rocca *et al.* 2009). This increase in ROS level exposes cells to oxidative damage leading to lipid peroxidation, biological macromolecule deterioration, membrane dismantling and DNA damage etc. (Navari *et al.* 1999; Quartacci *et al.* 2001).

Ni is present in natural soils in trace amounts, however metal concentration is amplified in certain areas by anthropogenic activities such as mining works, emission of smelters, burning of coal and oil, sewage, phosphate fertilizers and pesticides (Gimeno-García *et al.* 1996). When present in higher concentration, Ni causes various physiological alterations and assorted toxicity in plants such as chlorosis and necrosis (Pandey and Sharma 2002; Rahman *et al.* 2005). Plants affected by higher levels of Ni showed impairment of nutrient balance and disruption in cell membrane functions. Gonnelli *et al.* (2001) showed an increase in MDA concentration of Ni sensitive plants compared to Ni-tolerant ones. These changes might alter the membrane functionality and ion balance in the cytoplasm. Higher uptake of Ni results in decline in water content of many dicot and monocot plant species. The decrease in water uptake is used as an indicator of Ni toxicity in plants (Pandey and Sharma 2002; Gajewska *et al.* 2006).

In the last few decades, various effects of steroid hormones have been studied in plants. Applications of these steroids to plants stimulate the growth of plants. Brassinosteroids (BRs) are a group of plant-originated steroidal lactones that regulates growth and development (Choe 2006). These were first isolated and characterized from the pollen of *Brassica napus* L. and then were subsequently reported from 44 plants (9 monocots, 28 dicots, 5 gymnosperms, 1 pteridophyte and 1 alga). BRs are considered as hormones with pleiotropic effects as they have been found to influence various developmental processes like germination of seeds, rhizogenesis, growth, flowering and senescence. These have also been reported to confer resistance to plants against various abiotic stresses (Vardhini *et al.* 2010). They play a vital role in regulating several processes which include cell division and cell elongation, vascular differentiation, reproductive development and modulation of gene expression (Bajguz 2007). They are present in very low concentrations throughout the plant kingdom and are extensively disseminated in the reproductive and vegetative tissues (Bajguz and Tryten 2003; Symons *et al.* 2007). Survey of the literature shows scanty information on the presence of these steroids in the *Brassicaceae* family. Few members of this family report the presence of these steroids include *Brassica napus* L. (Grove *et al.* 1979), *Brassica campestris* var. *pekinensis* L. (Ikekawa *et al.* 1984), *Raphanus sativus* L. (Schmidt *et al.* 1991) and *Arabiodopsis thaliana* L. (Nomura *et al.* 2001). Till now, no report is available regarding the presence of BRs in *B. juncea* plants. Keeping in mind

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the presence of steroidal compounds in other members of the *Brassicaceae* family and their stress protective role, the present work was undertaken to explore the presence of BRs in *B. juncea* under Ni stress.

MATERIALS AND METHODS

Chemicals

Sephadex LH-20 was procured from Sigma-Aldrich; Castasterone (CS) and 28-homocasatsterone (28-HCS) from SciTech Prague, Czech Republic. Analysis of bioactive fractions was carried out on Varian GC-MS (4000). All the solvents used were of HPLC grade.

Sampling

Certified seeds of *B. juncea*, var. 'PBR 91' were procured from the Department of Plant Breeding, Punjab Agriculture University, India. Seeds were surface sterilized with 0.01% sodium hypochlorite and rinsed 5 times with double distilled water. The seeds were raised in pots containing different concentrations of nickel in the form of $NiSO_4 \cdot 6H_2O$ (0, 0.2, 0.4, and 0.6 mM) in the Botanical Garden of Guru Nanak Dev University, Amritsar (India) under natural conditions. The study material for the present investigation included fresh leaves of 45-days-old *B. juncea* plants.

Extraction of brassinosteroids

Fresh leaves of 45-days-old *B. juncea* plants (0.5 kg) were homogenized and percolated with 80% methanol (3 × 1000 ml) and the combined methanol extract was dried in vacuum under reduced pressure using a rotary evaporator (Buchi R-210, Switzerland). The methanolic extract was then partitioned between chloroform and water. The $CHCl_3$ phase was dried over Na_2SO_4, filtered off and evaporated in vacuum at 45°C under reduced pressure. The residue was again partitioned with *n*-hexane and 80% MeOH. The combined methanolic fraction was concentrated in vacuum and purified.

Purification of brassinosteroids

The residue resulting from the 80% MeOH fraction was chromatographed on a silica gel column (60–120 mesh). Elution was carried out with MeOH in $CHC1_3$ i.e. 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 50, 100% (200–250 ml of each). The bioactivity of the isolated fractions was determined using intact *Raphanus sativus* seedlings according to the method described by Takatsuto *et al*. (1983) with slight modifications. 7 days-old seedlings were placed into the test solutions and kept in the dark for 24 h at 25 ± 2°C. After 24 h, the length of hypocotyls was measured and compared to the control. The percentage increase over the control was calculated. The bioactive fractions were pooled and subjected to a second silica gel column to obtain the bioactive fraction using the same procedure describe earlier. The biologically active fractions were further chromatographed on a Sephadex LH-20 column using methanol: chloroform (4: 1) as the eluent and 20 fractions of 10 ml each were collected. The biologically active fractions were pooled, concentrated and subjected to HPLC for purification. The samples were fractionated by reverse phase (RP) HPLC (Kartion system, 450) using an RP18 column (250 × 8 mm i.d.) at a flow rate of 2 ml min^{-1}. The mobile phase used was pure acetonitrile for the first 5 min and 80% acetonitrile for the next 25 min. The solvent was degassed before use. 70 fractions of 2 ml each were collected.

Characterization of brassinosteroids

1. Thin layer chromatography

The bioactive fractions obtained after ODS-HPLC were spotted – along with the standard – on TLC plates coated with 60 F_{254} silica gel, and developed with $CHCl_3$: CH_3OH (88: 12) as the mobile phase. The spots were detected by spraying Liebermann–Burchard reagent. *Rf* values for the standard and samples were recorded.

2. GC-MS analysis

GC-MS analysis was carried out with the gas chromatograph connected with a mass spectrometer (Varian, 4000) for the analysis of BRs under the following conditions: Source temperature 250°C, column CP Sil 8CB (30 m length, 0.25 mm diameter, 0.25 µm thickness), injection temperature 280°C, column temperature programmed 200°C for 5 min, then raised to 280°C at a rate of 20°C min^{-1} and held at this temperature for 40 min; carrier gas He, flow rate 1.0 mL min^{-1} with a 20 split ratio.

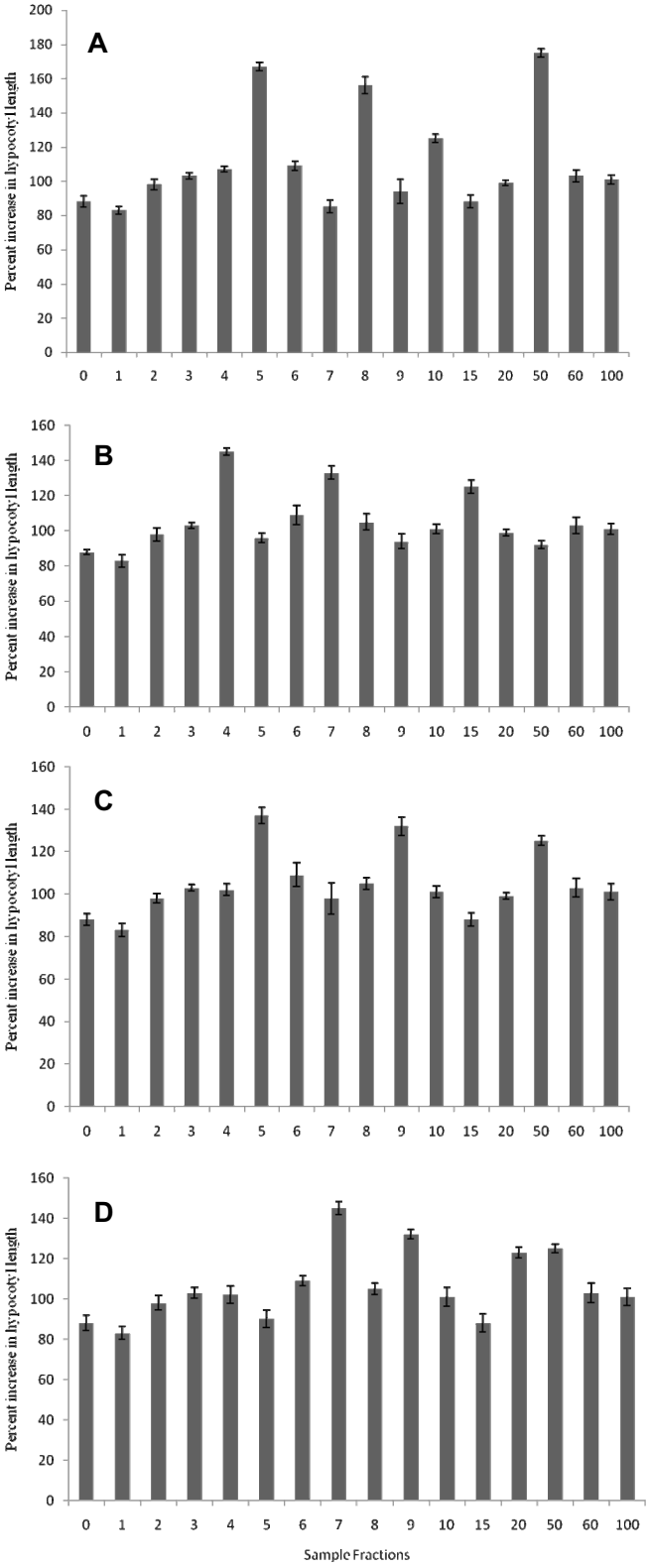

Fig. 1 Biological active fractions of *B. juncea* L. treated with 0 mM (**A**), 0.2 mM (**B**), 0.4 mM (**C**) or 0.6 mM (**D**) of nickel after silica gel chromatography employing the radish hypocotyl bioassay.

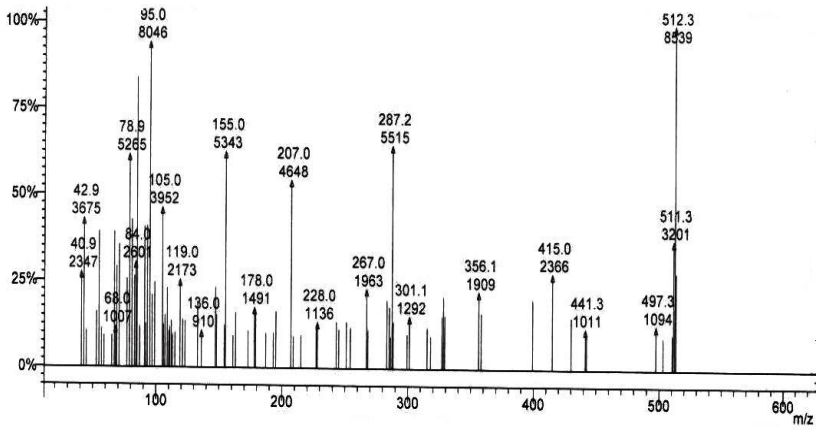

Fig. 3 GC-MS chromatograms of endogenous castasterone isolated from *B. juncea* L. plants grown under nickel stress.

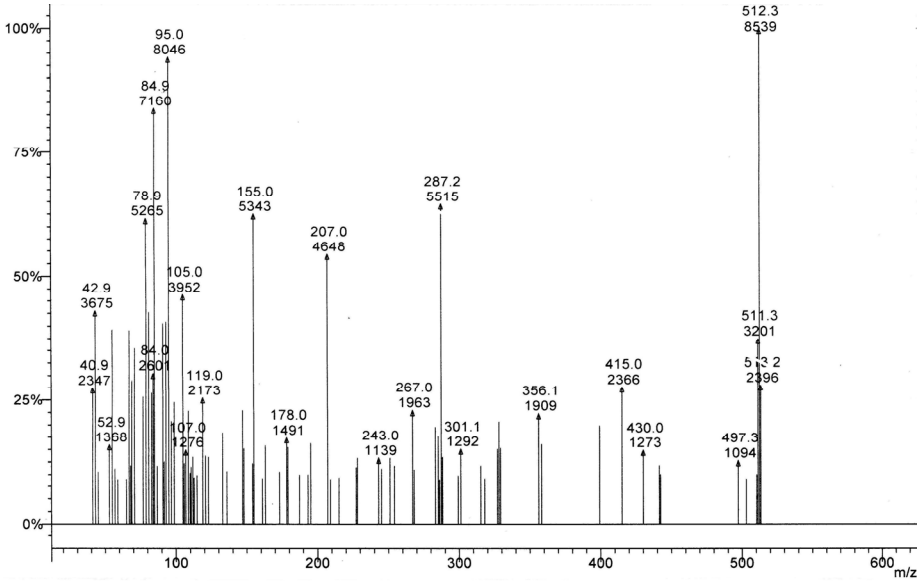

Fig. 2 GC-MS chromatograms of standard castasterone.

Preparation of boronates

Methaneboronic acid (100 µg) and dry pyridine (60 µl) were mixed for a few seconds and 20 µl of this mixture was added to the active fractions. These were heated to 80°C for 25–30 min. 3 µl of this solution was injected into GC–MS. The standard BRs were also derivatized and subjected to GC–MS.

Statistical analysis

The samples for each concentration of Ni were collected in triplicate. The radish hypocotyl bioassay was also carried out in triplicate for each fraction and data presented here is in the form of mean ± SE.

RESULTS AND DISCUSSION

The leaves of 45-days-old plants (0.5 kg in each concentration) of *B. juncea* L. exposed to Ni stress (0.0, 0.2, 0.4 and 0.6 mM) were harvested and extracted with 80% MeOH. The extract (20 g) was partitioned with chloroform-water (1: 1) and hexane-80% MeOH (1: 1). 80% MeOH extract thus obtained was passed through silica gel-I column for purification. The fractions obtained by the elution of 5, 8, 10 and 50% MeOH in $CHC1_3$ revealed biological activity when employing the radish hypocotyl bioassay in control plants (**Fig. 1A**). Similarly, fractions obtained by the elution of 4, 7 and 15% MeOH in $CHCl_3$ showed biological activity in 0.2 mM Ni-treated plants (**Fig. 1B**). In plants treated with 0.4 and 0.6 mM Ni, the bioactive fractions were 5, 9 and 50% (**Fig. 1C**) and 7, 9, 20 and 50%, repectively (**Fig. 1D**). These bioactive fractions were pooled and again subjected to purification in a silica gel-II column. The fractions obtained with maximum biological activity were again pooled and passed through a Sephadex LH-20 column. The fractions obtained by the elution of methanol: chloroform (4: 1) were pooled on the basis of their activities in the bioassay. These biologically active fractions were further subjected to ODS-HPLC using an acetonitrile–water system to obtain 70 fractions of 2 ml each. The fractions showing bioactivities were also analyzed by TLC and compared with authentic standard after spraying with Liebermann–Burchard reagent. These were then derivatized with methaneboronic acid and pyridine to form their bismethaneborates (BMBs) and subjected to GC-MS for characterization.

The identification of derivatized pure and isolated BRs was made on the basis of their mass spectra and retention times. For CS and 28-HCS the retention times were 34.129 and 35.20 min, respectively (**Fig. 2-5; Table 1**). Isolated BMB-CS showed a molecular peak at 512 (M, 100) and other fragmented *m/z* were 415, 356, 287 and 155. On the other hand, BMB-28-HCS showed its molecular peak at 526 (M, 100) and other *m/z* were 441, 399, 358, 287 and 169. These BMBs of the bioactive compounds, were found to be identical with CS and 28-HCS standards. On the basis of GC retention time, mass spectral data and TLC with known standard, the isolated active fractions were determined as CS {(22R,23R,24S)-2α,3α,22,23-tetrahydroxy-2-methyl-5α-cholestane-6-one} and 28-HCS {(22R,23R,24S)-2α,3α,22,23-tetrahydroxy-2-ethyl-5α-cholestane-6-one}. The findings of the present study are the first report regarding the presence of CS and 28-HCS in *B. juncea* under

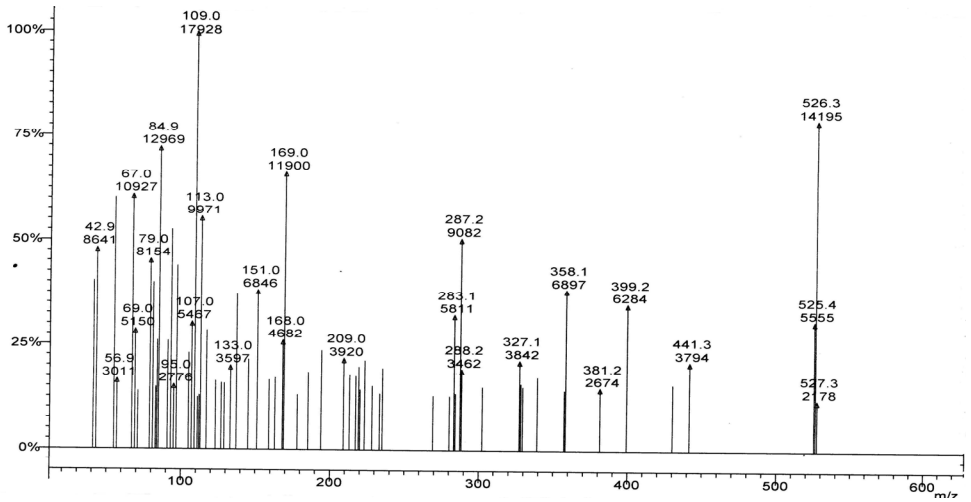

Fig. 4 GC-MS chromatograms of standard 28-homocastasterone.

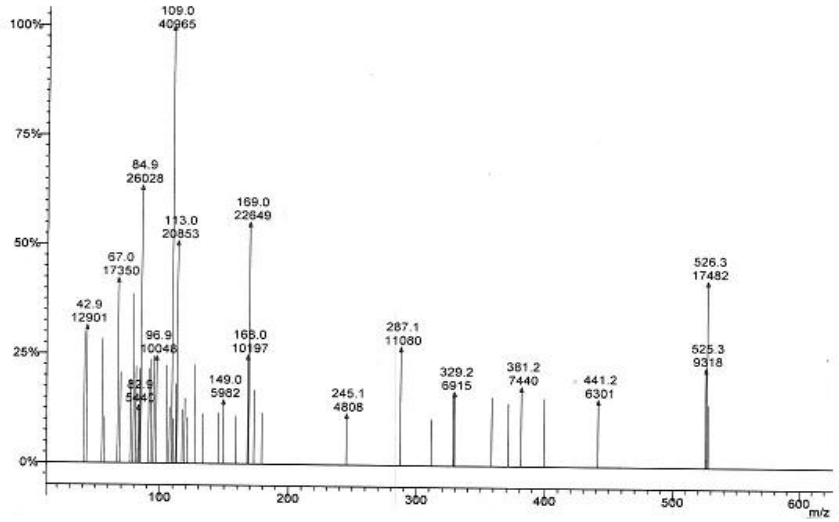

Fig. 5 GC-MS chromatograms of endogenous 28-homocastasterone isolated from *B. juncea* L. plants grown under nickel stress.

Table 1 GC-MS analysis of characteristic ions of brassinosteroids isolated from 45 days old *B. juncea* L. plants under nickel stress[a]

Compounds	Treatments (nickel) mM				Rt (min)	Derivative[b]	Prominent ion *m/z* [relative intensity (%)]
	0	0.2	0.4	0.6			
Standard castasterone					34.12	BMB	512 (M^+, 100), 415 (28), 356 (24), 287 (62), 155 (60), 95 (95).
Standard 28-homocastasterone					33.29	BMB	526 (M^+, 77), 441 (20), 381 (15), 287 (48), 169 (65).
Endogenous castasterone	+	-	+	-	34.129	BMB	512 (M^+, 95), 415 (25), 356 (20), 287 (65), 155 (60), 95 (95).
Endogenous 28-homocastssterone	-	+	+	+	35.20	BMB	526 (M^+, 45), 441 (15), 381 (20), 287 (27), 169 (55).

[a]Compounds identified on basis of their retention times of corresponding, authentic samples.
[b]BMB = bismethaneboronate. + shows presence of compound; - shows absence of compound; Rt-Retention time

heavy metal stress. The concentrations of 28-HCS were detected in the range of 0.014 to 0.029 µg/Kg fresh weight (FW) in the treated plants whereas the concentrations of CS were found at 0.018 and 0.022 µg/Kg FW in the control and 0.4 mM Ni-treated plants, respectively. The identified BRs with retention time and their respective *m/z* are given in **Table 1**.

In our earlier studies, BRs were isolated and characterized from leaves and seeds of *Camellia sinensis* (L.) O. Kuntze. (Gupta *et al.* 2004; Bhardwaj *et al.* 2007), and leaves of *Aegle marmelos* Corr. (Rutaceae) and *Centella asiatica* (Sondhi *et al.* 2008, 2010). Naturally occurring BRs have been classified as C_{27}, C_{28}, and C_{29}-steroids (Fujioka 1999). Similarly, the BRs isolated and characterized from leaves of *B. juncea* plants belong to C_{28} (CS) and C_{29} (28-HCS) group of BRs and are of 6-ketone type. The carbon skeleton of the side chain in plant sterols signifies that BRs are biosynthesized from plant sterols. The C_{28} BRs carry an α-methyl, β-methyl or methylene group that may be derived from campesterol, dihydrobrassicasterol or 24-methylenecholesterol, respectively and C_{29} BRs having an α-ethyl group that may came from sitosterol. Furthermore, the C_{29} BRs carrying a methylene at C-24 and an additional methyl group at C-25 might be derived from 24-methylene-25-methylcholesterol (Yokota 1995, 1999b).

Among the naturally occurring BRs, CS and brassinolide (BL) are the most important BRs because of their extensive distribution and strong biological activity (Kim 1991; Fujioka 1999). Among the plant species investigated, CS is most extensively distributed (49 plant species) followed by BL (33), typhasterol (TY) (24), 6-deoxocastasterone (6-deoxoCS) (19), teasterone (TE) (18), and 28-norcastasterone (28-norCS) (11) (Bajguz and Tretyn 2003). The occurrence of CS in *B. juncea* indicates that the early C-6 oxidation pathway seems to be operating in this plant. The metabolic conversion of CS was studied in explants of mung beans (*Phaseolus vulgaris*) which revealed that the 6-oxo group of CS is oxidized to a lactone group of BL (Baj-

guz 2007) but no such conversion was noticed in the present study as BL was not detected. In addition to this, metabolic studies on BRs revealed that the conversions of BRs to other forms might be accomplished through the modification of the steroidal skeleton or by changes in the side-chain. Therefore, 28-HCS might have been formed due to the modification of side chain as it carries an additional ethyl group at C-24, likely to be derived from isofucosterol or sitosterol, respectively (Bajguz 2007). Earlier studies also report the presence of CS and 28-HCS in trace levels from different plants species. Yokota *et al.* (1982a) reported the isolation of 1 µg/Kg FW of CS from the galls of the *Castanea crenata* while Park *et al.* (1994b) reported CS from the seeds of *Perilla frutescens*. Similarly, Fujioka *et al.* (1995) isolated 0.6-4.5 µg/Kg FW of CS from *Catharanthus roseus* G. Don. Nomura *et al.* (2001) isolated 0.4-2.4 µg/Kg FW CS from the seeds of *Pisum sativum* L. CS was also isolated from the seeds of pumkin by Pachtong *et al.* (2006) and the amount isolated was 112 µg/Kg of dry weight. Abe *et al.* (1983) reported the isolation of 28-HCS from seeds and leaves of *Brassica campestris* (130 µg/Kg FW) and *Thea sinensis* L. (<0.001 µg/Kg FW), respectively. 28-HCS (4.0 µg/Kg FW) was also isolated from lower plants like *Hydrodictyon reticulum* L. (Yokota *et al.* 1987). The presence of 28-HCS in the gymnosperm, *Cupressus arizonica* Greene, was also reported, and when isolated from the pollen of the plant, the amount detected was 4 µg/Kg FW (Griffiths *et al*. 1995). Further, BRs are reported to assist in the combat of different types of stresses in plants by modulating the activities of antioxidative enzymes involved in the Asada-Halliwell pathway (Bajguz and Hayat 2009; Arora *et al*. 2010). The isolation of BRs from *B. juncea* plants under Ni stress in the present study clearly indicates their stress amelioration prospects. The study also shows the synthesis of CS and 28-HCS in a hyperaccumulator plant like *B. juncea*. It needs to be studied more elaborately to understand the underalying mechanism between metal accumulation and BR synthesis, if any. Furthermore, the present study highlights the interplay of expression of endogenous BRs under metal stress which opens up a new era of research.

González-García *et al.* (2011) have reported the regulatory role of BRs in controlling cell-cycle progression and differentiation in the *Arabidopsis* root meristem. In another study conducted by Hasan *et al.* (2011), two tomato cultivars, grown under cadmium metal stress, were supplied with BRs in form of foliar spray. Improvement in the activity of both photosynthetic machinery and antioxidant defence system was observed with the application of BRs in both the cultivars. In our other study, we have reported the isolation of 24-epibrassinolide, castasterone, dolicholide and typhasterol from nickel stressed 60-days-old *B. juncea* plants. The isolated 24-epibrassinolide was applied in form of foliar spray to *B. juncea* plants grown under Ni metal stress, and was found to lower metal uptake and regulates the antioxidative defence system (Kanwar *et al.* 2012).

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