Chapter 6

SUMMARY
Agricultural soils have been contaminated with heavy metals such as Cd, Cu, Zn, Ni, Co, Cr, Pb, and As due to the prolonged use of fertilizers, sewage sludge applications, dust from smelters and use of industrial waste for watering (Schwartz et al., 2001; Passariello et al., 2002; Yadav et al., 2010). As a primary response, reactive oxygen species (ROS) are generated on exposure to high levels of heavy metals in plants. Heavy metals induced generation of ROS can either be directly through Haber-Weiss reactions or could be an indirect consequence of heavy metal stress in plants (Wojtaszek, 1997; Mithofer et al., 2004; Yadav et al., 2010). The ROS are highly toxic and can oxidize biological macromolecules such as nucleic acids, proteins and lipids, thereby disturbing the membrane permeability (Schutzendubel and Polle, 2002; Gajewska and Skłodowska, 2008; Sudo et al., 2008). These ROS can interact with the antioxidant system thereby, disrupt the electron transport chain or interfere with various metabolic pathways through indirect mechanisms (Yadav et al., 2010). Thus, accumulation of free radicals and ROS leads to imbalance in pro-oxidant and antioxidant defence system resulting in oxidative stress. The metal-induced phytotoxicity consequences in altered plant metabolic activities such as water relations, gas exchange, respiration and photosynthesis via modulating the activities of antioxidant enzymes, accumulations of antioxidants and osmoprotectants (Ünyayar et al., 2006; Drazkiewicz et al., 2007; Monteiro et al., 2009).

However, plants have adopted several stress protective strategies to combat such adverse stressed conditions. ROS are also scavenged via antioxidant defence system comprising enzymatic (superoxide dismutase, catalase, peroxidases, reductases etc.) and non-enzymatic components (glutathione, tocopherols, ascorbate, carotenoids etc.) (Mittler, 2002; Skórzyska-Polit et al., 2010; Sharma et al., 2011a). Plants also produce low molecular weight thiols like glutathione (GSH) and cysteine that possess high affinity for metals (Bricker et al., 2001). GSH acts as a substrate for phytochelatin synthesis which further synthesis phytochelatins that can bind or form complexes with metals (Freeman et al., 2004). The levels of GSH are maintained by the antioxidant enzymes through Asada-Halliwell pathway. Among antioxidant enzymes, superoxide
dismutase (SOD) acts as first line of defence and catalyzes the dismutation of superoxide radicals (O\text{2•−}) to O\text{2} and H\text{2}O\text{2}. The H\text{2}O\text{2} is further removed by catalase (CAT) or by ascorbate peroxidase (APOX) or by guaiacol peroxidase (POD) (Foyer et al., 1997). Further, glutathione reductase (GR) catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH) dependent reduction of glutathione disulphide (GSSG) to reduced glutathione (GSH) and maintains glutathione in the reduced state, which in turn reduces dehydroscorbate to ascorbate (Noctor and Foyer, 1998). Besides, ascorbic acid in its reduced form is maintained by dehydroscorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) using NADPH as reducing power (Mittler, 2002). Apart from regulation of several antioxidant genes, the oxidative stress also consequences in the evolution of new metabolic pathways, the accumulation of low molecular weight metaboliteus, the synthesis of special proteins and changes in phytohormone levels (Banu et al., 2009; Bari and Jones, 2009).

Several plant hormones like abscisic acid (ABA), ethylene, jasmonates and BRs play a determinant role in mitigation of oxidative stress (Bari and Jones 2009; Depuydt and Harptke, 2011). Recent studies had indicated that a class of plant steroid hormones known as brassinosteroids (BRs) have promising role in reducing the effects of various biotic and abiotic stresses (Dhaubhadel et al., 2002; Krishna, 2003; Kagale et al., 2007; Ali et al., 2008; Arora et al., 2008; Bari and Jones, 2009). BRs are steroidal group of plant hormones that are distributed throughout the plant kingdom and till date more than 70 analogs of BRs have been identified (Bajguz and Tretyn, 2003). Among these compounds, mainly two BRs i.e., 28-homobrassinolide (HBL) and 24-epibrassinolide (EBL) at 10⁻⁶ to 10⁻¹¹ M doses have been reported to combat drought, salt, temperature, and heavy metals stress in Brassica juncea, Raphanus sativus, Triticum aestivum and Zea mays (Bhardwaj et al., 2007; Hayat et al., 2007; Sharma et al., 2007, 2010; Yusuf et al., 2010). Since cultivation practices of R. sativus face the challenges of startlingly high levels of toxic HMs in groundwater and agricultural soils throughout the world including Punjab state of India (Zahir et al., 2005), it becomes warranted to explore effects of these steroidal phytohormones in heavy metals stress mitigation in a
commonly edible *R. sativus* L. Burgeoning studies also revealed that radish plants as potent hyperaccumulators of heavy metals (Máthé-Gáspár *et al.*, 2002; Vamerali *et al.*, 2010). Thus, the present investigation has been planned to meet the following objectives:

1. To study the effects of 24-epibrassinolide (EBL) and 28-homobrassinolide (HBL) (0 M, $10^{-11}$ M, $10^{-9}$ and $10^{-7}$ M) on root/shoot length, fresh and dry biomass of *Raphanus* seedlings under different concentrations of Cd, Cr, Ni and Hg.

2. To study the effects of EBL and HBL (0 M, $10^{-11}$ M, $10^{-9}$ and $10^{-7}$ M) on root length, shoot length and number of leaves in radish plants at three different developmental stages (30, 60 and 90 days) under Cd, Cr, Ni and Hg metals stress.

3. To study the effects of EBL and HBL (0 M, $10^{-11}$ M, $10^{-9}$ and $10^{-7}$ M) in *Raphanus* seedlings and field grown plants (both roots and shoots) at different developmental stages under heavy metals (Hg, Cr, Ni, Cd) stress on osmolality, reducing sugars, contents of chlorophyll A, B and total chlorophyll, proteins, prolines, malondialdehyde (MDA) content and activities of antioxidant enzymes. The activities of enzymes namely catalase (CAT), guaiacol peroxidase (POD), superoxide dismutase (SOD), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and ascorbate peroxidase (APOX) were determined.

4. To analyze the influence of EBL and HBL on sugars through analytical HPLC in 7-days old radish seedlings grown under Cd, Hg, Ni and Cr metals.

5. To study the effects of EBL and HBL in radish seedlings on the expression of SOD and CAT using semi-quantitative RT-PCR in radish seedlings under Cd, Hg, Ni and Cr metals toxicity.

In order to meet these objectives, certified and disease-free seeds of *Raphanus sativus* L. (Pusa Chetki) or radish were procured from Punjab Agricultural University,
Ludhiana, India. These seeds were surface sterilized with 0.4 % sodium hypochlorite for 15 minutes followed by repeated rinses in sterile distilled water. The surface sterilized seeds were given 8-hour presoaking treatment of different concentrations \((0, 10^{-11}, 10^{-9} \text{ and } 10^{-7} \text{ M})\) of either HBL or EBL. These pre-treated seeds were then sown either in glass Petri dishes or in soil beds i.e., laboratory or field conditions. In laboratory, BRs treated seeds were germinated on *Whatman* No. 1 filter paper lined autoclaved glass Petri dishes (10 cm diameter, 20 seeds/dish) containing different concentrations \((0, 0.5, 1.0 \text{ and } 1.5 \text{ mM})\) of heavy metals (Cd, Cr, Ni and Hg). The experiment was conducted under controlled conditions \((25 ± 0.5°C, 16 \text{ h photoperiod, } 175 \mu\text{mol m}^{-2} \text{ s}^{-1} \text{ light intensity})\). Seedlings were harvested on the 7\text{th} day and observations were made on morphological and biochemical parameters.

A seasonal field experiment was also conducted to study the effects of seed-pre-soaking treatment of BRs on morphological and biochemical parameters of radish plants grown under above mentioned heavy metals stress. A randomized blocks design was used to prepare two fields (Field Plan-1 and Field Plan-2). The soil beds were prepared that contained ridges and troughs. The water holding capacity of 10 kg soil was estimated and each ridge contained 10 kg soil that was having clay: sand: manure in the proportion of 2:1:1. The soil of ridge was pretreated with different concentrations of four (Cr, Cd, Ni and Hg) heavy metals \((0, 0.5, 1.0 \text{ and } 1.5 \text{ mM})\). Then, the seeds pre-soaked in either HBL or EBL radish seeds were sown on metal treated ridges in soil beds that contained different concentrations of metals. Plants were allowed to grow under natural conditions with regular water supply for 90 days. Field management followed normal agronomic practices. Plant sample was harvested on 30\text{th}, 60\text{th} and 90\text{th} day.

In shoots of 7-days old radish seedlings, the total dissolved solutes in terms of osmolalities were measured through vapor pressure osmometer (VPO) and levels of reducing sugars were measured by following dinitrosalicylic acid (DNSA) method as described by Miller (1972). Also, rate of superoxide anion \((O_2^-)\) generation was
determined spectrophotometrically by monitoring the nitrite formation from hydroxylamine in the presence of $O_2^{* -}$ as described by Wu et al. (2010). Further, contents of proteins (Bradford, 1976), prolines (Bates et al., 1973), MDA (Hodges et al., 1999) and chlorophylls (Arnon, 1949) were also analyzed. The activity of SOD was determined by monitoring its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) at 540 nm (Kono, 1978). Activity of DHAR and POD was determined according to Dalton et al. (1986) and Sánchez (1995) respectively. CAT activity was determined by following the initial rate of disappearance of $H_2O_2$ at 240 nm (Aebi, 1984). The activities of APOX, GR and MDHAR were measured by the methods of Nakano and Asada (1981); Carlberg and Mannervik (1975); and Hossain et al. (1984) respectively. In roots and shoots of plants activities of antioxidant enzymes and contents of MDA, prolines and total proteins were analyzed. In 7-days old seedlings, the expression studies of antioxidant enzyme using semi-quantitative Reverse Transcriptase-Polymerase Chain Reaction (semi-qRT-PCR) were carried out. In present investigation, two most important antioxidant enzymes namely CAT ($Cat1$, $Cat2$ and $Cat3$) and SOD ($Cu/ZnSod$, $FeSod$ and $MnSod$) were analyzed by semi-qRT-PCR taking $26S-rRNA$ as housekeeping/constitutive gene. RNA was isolated by TRIzol method and then cDNA was prepared. This cDNA was further used as template to amplify the product with specific primers for above mentioned genes. The bands were visualized on 1% agarose gels and densitometry of bands was carried out to check the relative abundance of transcripts so as to determine the relative expression of particular gene.

The present study on effect of these BRs in $R. sativus$ seedlings or plants under Cr, Hg, Cd and Ni stress had revealed the following important observations:

1. The heavy metals were found to be toxic in order: $Cr < Ni < Cd < Hg$, after calculating their IC-50 values. However, same concentrations i.e., 0, 0.5, 1.0, 1.5 mM of each metal was used to analyze their effects on various parameters.
2. Treatments of metals (Cd, Cr, Hg and Ni) at different concentrations showed reduced growth of both radish seedlings and plants. This was reflected in
significant reductions in root shoot length of seedlings and plants at different stages. Further, lowered fresh and dry biomass was observed in radish seedlings under metal toxicity and maximum decrease was observed at 1.5 mM treatments of heavy metals. Also, metal toxicity resulted in remarkable change in the number of leaves on 60\textsuperscript{th} and 90\textsuperscript{th} days old radish plants.

3. A significant increase in the levels free prolines, osmolalities, and enhanced rate of generation of O$_2$•$^-$ anion was also recorded in radish seedlings and plants subjected to various concentrations of heavy metals.

4. Also, heavy metals stress showed significant alterations in contents of total soluble proteins, chlorophylls, reducing sugars and on the activities of antioxidant enzyme.

5. With increase in the concentrations of heavy metals, lipid peroxidation rate measured by determining the content of malondialdehyde (MDA) was observed to enhance significantly in roots/shoots of radish plants and the seedlings.

6. Pre-sowing treatments of EBL and HBL remarkably lowered the levels of MDA thereby mitigating the peroxidation of membrane lipids under Cd, Cr, Hg and Ni stress in both radish seedlings and plants. Hence, the extent of metals toxicity in radish plants and seedlings was reduced.

7. Supplementation of different doses of EBL and HBL was effective in stress amelioration by enhancing osmolalities, the contents of free prolines, reducing sugars, chlorophylls and soluble proteins under Cd, Cr, Hg and Ni stress in radish.

8. Seed pre-soaking treatments of EBL and HBL also showed enhanced rate of O$_2$•$^-$ anion production in radish seedlings subjected to heavy metals (Cd, Cr, Hg and Ni) toxicity.

9. Furthermore, pre-treatments of EBL and HBL helped in stress alleviation by modifying the activities of stress protective antioxidant enzymes (SOD, POD, CAT, APOX, DHAR, MDHAR and GR) under Cd, Cr, Hg and Ni stress in both radish seedlings and plants.
10. Results indicated that there were less activities of antioxidant enzyme in roots as compared to the shoots of radish plants on 30th, 60th and 90th day grown under heavy metals stress. The radish plants raised from seeds pre-soaked in either EBL or HBL also confirmed the same pattern in roots when compared with shoots in both metal-stressed and un-stressed plants.

11. Overall, pre-sowing with HBL was observed to be more successful in comparison to EBL in heavy metals stress mitigation.

12. Also, 8-h seed pre-soaking treatments of EBL and HBL were found to be more effective at $10^{-7}$ and $10^{-9}$ M concentrations than at $10^{-11}$ M.

13. HPLC analysis of sugars revealed an increase in levels of two sugars i.e., sorbitol and raffinose under the influence of BRs in radish seedlings during metals stress.

14. Qualitative analysis of CAT and SOD genes through qRT-PCR analysis revealed altered expression of Cat1, Cat2, Cat3, FeSod, MnSod and Cu/ZnSod under Cr, Ni, Hg and Cd stressed radish seedlings which was improved remarkably in seedlings pre-treated with EBL and HBL at $10^{-7}$ M dose.

Further, insight into underlying molecular mechanism of involvement of some key enzymes, transcription factors and specific genes regulated by exogenous application of BRs and their role in providing defense against heavy metals stress might be analysed. Also, cross-talk of BRs with other phyto-hormones or factors in plant stress-protection would also be an imperative aspect. The role of BRs in protecting plants against environmental stresses will be an important research theme for clarifying the mode of action of BRs and may contribute greatly to their implications in agricultural production. Also, in future, the synthesis of low-cost and economic eco-friendly BRs might be very helpful in agronomy.