Chapter 1

INTRODUCTION
Steroidal hormones are essential for growth and development of both plants and animals. Numerous animal sterols and steroidal sex hormones viz. corticosteroids, estrone, testosterone, progesterone and analogues of these compounds had been reported from plants (Geuns, 1978; Jones and Roddick, 1988; Bishop and Koncz, 2002; Janeczko and Skoczowski, 2005; Yang et al., 2005; Iino et al., 2007; Janeczko et al., 2008; Simersky et al., 2009; Erdal and Dumlupinar, 2011). These animal steroid sex hormones play an imperative role in sex determination, stress protection against overgrazing, wounding etc. in many plants. Besides this, exogenous applications of animal sex hormones have significant effects on plant physiology and morphology (Dogra and Thukral, 1991a, 1991b, 1994a and 1994b; Bhardwaj and Thukral, 2000a; Czerpak and Szamrez 2003; Szamrez and Czerpak 2004;). Brassinosteroids (BRs) are well-documented as an emerging group of crucial plant steroid hormones that integrate diverse growth and developmental processes in addition to five classical phytohormones (Piñol and Simón, 2009; Bhardwaj et al., 2010). These are hydroxylated derivatives of cholestan e and structural deviations of BRs are attributed to varied substitutions at the C-17 side-chain, rings A and B. On the basis of length of the side-chain, these compounds are classified as C\textsubscript{27}, C\textsubscript{28}, or C\textsubscript{29} BRs (Bajguz, 2007). More than 70 analogs of BRs have been identified, and among these, brassinolide, 24-epibrassinolide (EBL) and 28-homobrassinolide (HBL) are known to have economic impact on various agronomic traits of plants (Bhardwaj et al., 2006, 2007a; Hayat et al., 2010).

Recently, several BR-regulated genes associated with diverse physiological responses, such as cell division and expansion, differentiation, programmed cell death, stomatal development and functions, homeostasis and gene expression have been identified by genome-wide microarray analysis (Divi and Krishna, 2009; Tanaka et al., 2009). In addition to their growth regulatory activities, BRs have also been reported to play pivotal role for their implication in both stress-protection and stress-amelioration (Krishna, 2003; Bajguz and Hayat, 2009; Bari and Jones, 2009; Divi et al., 2010; Bajguz, 2010). Exogenous application of BRs stimulated inner potentials of plants that are helpful not only in better survival in stress conditions, but also in receding biotic...
stress caused by pathogens like viruses, fungi and bacteria (Nakashita et al., 2003; Wachsman et al., 2004a; Swaczynova et al., 2006; Romanutti et al., 2007; Ohri et al., 2008). Recent studies on biological activities of BRs in various animal test systems depicted their antibacterial, anticancerous or antiproliferative, antifungal, antigenotoxic, antiviral and ecdysteroidal properties strengthening their prospects as a potential future medicine (Volynets et al., 1997a, 1997b; Franěk et al., 2003; Nakashita et al., 2003; Wachsman et al., 2004a, 2004b; Malíková et al., 2008; Romanutti et al., 2007; Ohri et al., 2008). Due to active involvement of BRs in multifarious biological processes, these are anticipated as hormones of the 21st century (Khripach et al., 2000). Besides this, EBL was tested for antigenotoxic activity by employing Allium cepa chromosomal aberration bioassay, thereby, supporting its safe use in agricultural practices (Sondhi et al., 2008). Further, an investigation on the developmental toxicity of a commercially available HBL also concluded that it is non-teratogenic at doses as high as up to 1000 mg/kg body weight in wistar rats (Murkunde and Murthy, 2010). In Arabidopsis thaliana, Li et al. (2007) isolated a gene HSD1 encoding a protein with homology to animal 11-b-hydroxysteroid dehydrogenase (HSD). Divi and Krishna (2009) observed that overexpression of AtHSD1 in Arabidopsis led to BR-responsive gene expression and in Brassica napus enhanced stress tolerance. Divi and Krishna (2009) suggested that crop yields and stress tolerance in plants could be achieved by manipulating the genes involved in the BRs biosynthetic and signaling pathways.

Nascent studies have revealed the future prospective of BRs in phytoremediation technology to remediate soil, water and air contaminated with organic or inorganic wastes (Barbafieri and Tassi, 2011; Sharma et al., 2010, 2011a, 2011b). Organic and inorganic contaminants comprise heavy metals, radio nuclides, chlorinated solvents, petroleum hydrocarbons, organophosphate insecticides, explosives, and surfactants etc. Burgeoning evidences revealed that industrial revolution and anthropogenic activities have aggravated the heavy metals (HMs) pollution of the biosphere at terrifying pace and resulted in the degradation of ecosystem (Chary et al., 2008; Chen et al., 2009).
Also, being persistent in nature these HMs get accumulated in soils and plants and it has posed a serious threat to mankind by its incorporation to the food chain (Chary et al., 2008; Sharma et al., 2011b). Regular use of cosmetics, fungicides, pesticides, soil fertilizers as well as revolutionary processes like metal mining, smelting, electroplating, drilling muds and refractory steel industries have magnified the levels of Hg, Cd, Ni, Pb, Cr, Zn in soil (Benavides et al., 2005; Shanker et al., 2005; Chen et al., 2009). Mismanaged dumping of wastes from paper and leather tanning industries, sewage sludge, effluent disposal or wastewater treatment plants; disposal of nickel-cadmium batteries and thermometers to municipal landfills, are the critical sources of Hg, Cd, Cr and Ni in the environment (Arabi, 2005; Benavides et al., 2005; Shanker et al., 2005; Chen et al., 2009).

Though plants are concurrently exposed to a range of abiotic and biotic stresses yet one of the major abiotic stress faced by the agricultural crops, is heavy metal (HM) stress (Mittler, 2006). Some metals like Fe, Se, Mn, Co, Zn, Mo and Ni, are essential micronutrient for most of the redox reactions which are fundamental for cellular functions (Brown et al., 1987; Gerendás et al., 1999). However, their concentrations beyond “certain tolerable limits” are highly toxic to plants that lead to over-production of reactive oxygen species (ROS) and free radicals through the catalysis of the Haber-Weiss reaction (Eskew et al., 1983; Hall and Williams, 2003; Seregin and Kozhevnikova, 2006; Halliwell and Gutteridge, 2007; Llamas and Sanz, 2008). 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 Sklodowska, 2008; Sudo et al., 2008). 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 stunted growth, leaf chlorosis, mitotic inhibition, vein necrosis, poorer seed/pollen germination, inhibition of the development of roots and leaves, fruit quality and quantity (Patra and Sharma, 2000; Shanker et al., 2005; Chen et al. 2009; Elbaz et al.,...
2010). Also, HM-induced oxidative stress alters other 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).

Plants have adopted several stress protective strategies such as chelation, compartmentalization, detoxification, immobilization and exclusion of metal ions through phytoremediation to overcome an array of environmental cues (Vamerali et al., 2010). The ROS are also scavenged via antioxidant defence system comprising enzymatic (superoxide dismutase, catalase, peroxidaes, reductases etc.) and non-enzymatic components (glutathione, tocopherols, ascorbate, carotenoids etc.) (Mittler, 2002; Skórzyńska-Polit et al., 2010; Sharma et al., 2011a). Among antioxidant enzymes, superoxide dismutase (SOD) acts as first line of defence and catalyzes the disproportionation of superoxide radicals ($\text{O}_2^{-}$) to $\text{O}_2$ and $\text{H}_2\text{O}_2$. The $\text{H}_2\text{O}_2$ is further removed by catalase (CAT) in the peroxisomes or by ascorbate peroxidase (APOX) in the chloroplast or by membrane bounded guaiacol peroxidase (POD) (Foyer et al., 1997). Further, glutathione reductase (GR) catalyzes the nicotinamideadenine dinucleotidephosphate (NADPH) dependent reduction of glutathione disulphide (GSSG) to reduced glutathione (GSH) and maintains glutathione in the reduced state, which in turn reduces dehydroascorbate to ascorbate (Noctor and Foyer, 1998). Besides, ascorbic acid in its reduced form is essential for ROS scavenging and its cellular pool is maintained by dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) using NADPH as reducing power (Mittler, 2002). Thus, antioxidant enzymes play an efficient role in ROS scavenging thereby maintaining the normal redox potential of cells and their effectiveness against ROS varies with type of plant and heavy metal, responsible for ROS production (Bhattacharjee, 2005; Scandalios, 2005; Hayat et al., 2010; Sharma et al., 2010, 2011a, 2011b).

The extremely polluted sites have higher levels of HMs and generally not suitable for the growth and development of normal plants. Although some plant genera
are identified to possess higher capacity to extract and accumulate metal ions in their above-ground plant parts to levels far exceeding than in soil, are well known hyperaccumulators (Memon et al., 2001). More than 400 hyperaccumulator plant species belonging to 45 plant families have been identified (Ghosh and Singh, 2005; Malik et al., 2010). About 87 plant species of hyperaccumulators distributed in 11 plant genera belong to only Brassicaceae or Crucifereae family (Baker and Brooks, 1989). Some plants of Brassicaceae family viz., Thlaspi caerulescens, Raphanus sativus, Brassica napus and Brassica juncea are explored for their hyperaccumulation and phytoremediation potential against Cu, Zn, Ni, Cd and Pb metals (Salt et al., 1995; Marchiol et al., 2004; Kapourchal et al., 2009; Vamerali et al., 2010).

Radish (Raphanus sativus L.; 2n=18) is an important vegetable crop with diverse culinary and medicinal features and a long cultivation history (Wang et al., 2008). In European countries small-rooted and short-season radishes are cultivated whereas in Asian countries, large-rooted and long-season radishes are preferred (Wang et al., 2008). It is widely used agro-economic crop with protective role against environmental mutagens and their eventual use as therapeutics (Ghayura and Gilani, 2007; Alquasoumi et al., 2008). Radish plant extracts have been reported for their anti-diabetic, anti-hepatotoxic, anti-oxidant, choleretic and multi-potent chemo-preventive potential (Barillari et al., 2006, 2008; Baek et al., 2008). Although entire radish is edible but mostly it’s napiform large taproot is favored in salads in comparison to leaves. Radish roots stimulate the appetite and digestion because they have a tonic and laxative effect upon the intestine and indirectly stimulating the flow of bile (Chevallier, 1996). Leaves, seeds and old roots of this plant are very useful in the treatment of asthma and other chest complaints (Duke et al., 1985). Radish is a rich source of carotenoids, vitamins, magnesium, copper, calcium and carbohydrates (Sgherii et al., 2002; Villaluenga et al., 2007). Its principal constituents also encompasses isothiocyanates, anthocyanins, catechols, flavanols, raphanusanol, glucosinolates and brassinosteroids (BRs), alongwith other minerals (Romero-Puertas et
al., 2006; Csiszar et al., 2008). Four analogs of BRs i.e. Castasterone, Brassinolide, 28-homoteasterone and teasterone are identified from seeds of *R. sativus* (Schmidt et al., 1991; 1993). However, there is no information available linking BRs and diminution of heavy metals induced oxidative stress in *R. sativus* seedlings or plants.

Since cultivation practices of *R. sativus* face the challenges of startlingly high levels of toxic HMs in groundwater and agricultural soils throughout the world (Zahir et al., 2005), it becomes warranted to explore effect of plant hormones in HM stress mitigation in a commonly edible *R. sativus*. Radish plants are also known as hyperaccumulators of heavy metals (Máthé-Gáspár et al., 2002; Vamerali et al., 2010). In view of the above as well as the wide occurrence and economic importance of *R. sativus* (radish), the present piece of work is carried out to study the effect of BRs on antioxidant defence system of *R. sativus* L. (cv. Pusa Chetki) under heavy metals stress employing biochemical and molecular approaches. Besides, prior reports revealed that $10^{-6}$ to $10^{-11}$ M concentrations of both HBL and EBL are highly effective in combating salt and heavy metals stress in *B. napus*, *B. juncea*, *Triticum aestivum*, *Zea mays* and *Oryza sativa* (Sasse, 1999; Bhardwaj et al., 2007; Hayat et al., 2007; Sharma et al., 2007; Arora et al., 2008a; Bajguz and Hayat 2009; Yusuf et al., 2010). Further, acquisition of knowledge at both biochemical and molecular levels might give an insight into understanding the anti-stress effects of HBL and EBL elaboratively for radish seedlings or plants protection against heavy metals stresses. Thus, the present investigation has been planned to meet the following objectives:

- To study the toxic effects of Cd, Cr, Ni and Hg (IC-50) in *Raphanus* seedlings
- To study the effects of 24-epibrassinolide (EBL) and 28-homobrassinolide (HBL) ($0$ M, $10^{-11}$ M, $10^{-9}$ and $10^{-7}$ M) on seedlings and field grown plants (both roots and shoots) of *R. sativus* at different developmental stages under heavy metals (Hg, Cr, Ni, Cd) stress on:
  - Protein content
  - Proline content
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➢ Malondialdehyde (MDA) content

➢ Activities of Antioxidant Enzymes:
  • Catalase (CAT)
  • Guaiacol Peroxidase (POD)
  • Superoxide Dismutase (SOD)
  • Glutathione Reductase (GR)
  • Monodehydroascorbate reductase (MDHAR)
  • Dehydroascorbate reductase (DHAR)
  • Ascorbate Peroxidase (APOX)

➢ To study the effects of EBL and HBL on chlorophylls (Chlorophyll A, B and total), reducing sugars, osmolalities and rate of superoxide anion production in shoots of radish seedlings under heavy metals stress.

➢ To study the effects of EBL and HBL on the carbohydrates analyzed through HPLC in shoots of radish seedlings under heavy metals stress.

➢ To study the effects of EBL and HBL on the expression of some key antioxidant enzyme(s) using semiquantitative RT-PCR in radish seedlings.