INTRODUCTION

Due to rapid industrialization, urbanization and intensive agriculture, increasing contamination of heavy metals in soil has become a major problem. Environmental deterioration has generated an increase of stress in all forms of life. With the development of industries, mining activities, application of waste water and sewage sludge on land, heavy metal pollution of soils is becoming a serious environmental problem (Jayakumar et al., 2008). Heavy metals are of great interest for research purpose with respect to toxicological importance to human health, plants and animals (Pandey, 2008; Jain et al., 2010). Currently, environmental pollution and plant exposure to heavy metals is a matter of great concern at the global level.

Toxic heavy metals are normally present as soil constituents or can also be spread out in the environment by human activity and agricultural techniques. In most terrestrial ecosystems, there are two main sources of heavy metals: natural and anthropogenic. Natural sources are volcanoes and continental dusts. Anthropogenic activities like mining, combustion of fossil fuels, metal industries, phosphate fertilizers, sewage sludge or municipal waste, emission from municipal waste incinerators, car exhaust, residues from mining and smelting industries, etc., lead to the emission of heavy metals and accumulation of these compounds in the ecosystem.

Elemental pollutants are particularly difficult to remediate from soil, water and air because unlike organic pollutants these cannot be degraded to harmless small molecules. Toxic elements such as mercury, arsenic, cadmium, lead, copper and zinc are immutable by all biochemical reactions and thus remain in ecosystem (Kramer and Chardonnens, 2001). The heavy metals remain in various ecosystems would seep into surface water, groundwater or even channel into the food chain by crops growing on such a soil. These heavy metals may adversely affect not only the soil ecosystem, agricultural product and water quality, but also the human health.

An annual worldwide release of heavy metals has reached 22,000 metric tons for cadmium, 939,000 tons for copper, 783,000 tons for lead and 1,350,000 tons for zinc (Singh et al., 2003) and these metals are present in the soils as trace metal ions,
soluble metal complexes (sequestered to ligands), exchangeable metal ions, organically bound metals, precipitated or inorganic compounds such as oxides, carbonates and hydroxides, or they may constitute a part of the structure of silicate materials (indigenous soil content). These different fractions are in dynamic equilibrium with each other.

In the soil, mobile and immobilized fractions have to be distinguished since heavy metals may chemically or physically interact with the natural compounds, which change their forms of existence in the environment. They may react with particular species, change oxidation states and precipitate. Heavy metals may be bound or sorbed by particular natural substances. Therefore, the solubility and mobility of metals are affected by adsorption, desorption, and complexation processes, which in turn are dependent on the soil type (Rai et al., 2004). While the soluble metal in the soil solution is directly available for plant uptake and other soil metal pools are less available. Therefore, factors affecting the concentration and speciation of metals in the soil solution will affect the bio-availability of metals to the plants.

Some heavy metals like copper and zinc are essential for normal plant growth and development, but other metals like cadmium and lead have no known function in plants. The generalized dose-response curves for the two kinds of metal differ in regard to their effects on plant growth. In non-essential metals these curves comprise a no-effect zone and toxicity zone, whereas for essential metals the response curve shows an additional deficiency zone preceding the no-effect zone.

There is much evidence that agricultural land adjacent to industrial areas is polluted to a varied extent by many toxic heavy metals. Metal-contaminated wastes in various parts of the world usually contain more than one metal and these may occur at toxic concentrations e.g., metalliferous mines spoil (copper, lead, zinc), smelter wastes (copper, lead, zinc), coal spoils (aluminium, copper, manganese, nickel, zinc, iron), sewage sludge and refuse compost (copper, zinc, lead, etc., depending on the source). Hence, metal pollution is a multielement problem. Environmental effects of combined heavy metals may be quite different from those of individual metal due to interactions between heavy metals (Zhou et al., 2006). Various studies revealed that the toxicity of mixtures of metal pollutants may reflect
the actual toxicity to ecosystems in a more realistic way than those in which toxicants are tested individually (Spurgeon et al., 1994).

Guo and Zhou (2003) have noticed the antagonistic effects of cadmium and lead on Chinese watermelon and also assessed the additive effect of cadmium and arsenic on *Medicago sativa* (L.). Further, An et al. (2004) have reported that copper, cadmium and lead in combination had the additive effect on *Cucumis sativus* (L.). Shute and Macfie (2006) studied the accumulation and distribution of cadmium and zinc in combination in *Glycine max* (L.) and assessed the effect of one metal on the bio-availability of the other metal across the range of concentrations added to the soil.

Auda and Ali (2010) analysed the combined effect of cadmium and zinc in *Daucus carota* (L.) and pointed out that zinc application can overcome the toxic effect of cadmium in plants indicating some kind of interaction between these metals. Therefore, it is more appropriate to study the combined effect of sub-toxic and toxic concentration of heavy metals because the effect of combined heavy metals on plants may be quite different from those of individual pollutants due to interaction between heavy metals.

On the other hand, zinc metal is a non-redox micronutrient element which has a key structural and catalytic role in many proteins and enzymes involved in energy metabolism (Hall and Williams, 2003). Zinc plays an important role in several plants metabolic processes, activation of enzymes and is also involved in protein synthesis, carbohydrate, nucleic acid, lipid metabolism, nitrogen metabolism, photosynthesis and auxin synthesis (Broadley et al., 2007; Ozdener and Aydin, 2010). Moreover, it has also key structural roles such as its use in the zinc finger family of transcription factors for the formation of the DNA binding domain that interacts with the major groove of DNA (Ciftci-Yilmaz and Mittler, 2008).

The primary input of zinc metal to soil is from the chemical and physical weathering of parent rocks. Other anthropogenic inputs of zinc to soil include fossil fuel combustion, mine waste, phosphatic fertilizers (typically 50-1450 µg Zn g⁻¹), limestone (10-450 µg Zn g⁻¹), manure (15-250 µg Zn g⁻¹), sewage sludge (91-49000 µg Zn g⁻¹), other agrochemicals, particles from galvanized (zinc plated) surfaces and rubber mulches. Zinc metal toxicity occurs in soil contaminated by mining and smelting activities, in agricultural soil treated with sewage sludge, and in urban and
peri-urban soil enriched by anthropogenic input of zinc, especially in low-pH soil. Zinc metal ion toxicity cause symptoms like reduced yields and stunted growth, Fe-deficiency-induced chlorosis through reductions in chlorophyll synthesis and chloroplast degradation, and interference with phosphorous uptake (Sagardoy et al., 2009).

Copper is one of the most important micro-nutrient essential for plant growth. Copper functions as the co-factor within plant cells in a variety of physiological processes, including electron transfer in photosynthesis, mitochondrial respiration, superoxide scavenging, lignifications of cell walls, ethylene sensing, structural element in regulatory proteins and hormone signaling (Epstein and Bloom, 2005). Various sources of copper are mining and metal production, electric goods, kitchenware, alloys, pesticides and pigments, insecticides, fungicides, ice-makers, industrial emissions, copper IUCDs, phosphate fertilizer production and find its way to agricultural soils and water bodies through the disposal of copper containing waste water, sludge and combustion of fossil fuel. Copper metal is also incorporating by natural agencies like wind blown dust, decaying vegetation, forest fire and sea spray.

A very common practice of sewage sludge amendment to agricultural soil about 1:20 ratio can expose more than 100 ppm of copper to the plants (Singh et al., 2007b). The redox properties that make copper an essential element also contributes to its inherent toxicity. Redox cycling between Cu$^{2+}$ and Cu$^{+}$ can catalyze the production of highly toxic hydroxyl radicals with subsequent damage to DNA, lipids, proteins and other bio-molecules. At high concentration copper metal ions can become extremely toxic, causing symptoms such as chlorosis, necrosis, stunting, leaf discoloration and inhibition of root growth (Kopittke and Menzies, 2006).

Different plant absorbs toxic and non-toxic metals from soil and water to a varied extent and accumulates in different body parts (Umebese and Motajo, 2008). The transfer of heavy metals from soil to plant is dependent on the following three factors: quantity factor (the total amount of potentially available elements), intensity factor (the activity as well as the ionic ratios of elements in the soil solution) and reaction kinetics (the rate of element transfer from solid to liquid phases and to plant roots) (Brummer et al., 1986). Heavy metals adversely affect the seed germination, plant growth, alter the levels of bio-molecules in the cell and interfere with the
activities of many key enzymes related to normal metabolic and developmental processes. So, heavy metal stress is a serious intimidation to agriculture. The plants are endowed with an inherent capability of tolerating toxic metals to some extent. Metal ions turn toxic as soon as their concentration exceeds a metal specific threshold which varies strongly among plant species, ecotypes and also with metal properties. Tolerance to heavy metals in plant is defined as the ability to survive in a soil that is toxic to other plants and is manifested by an interaction between a genotype and its environment (McNair et al., 2000).

Heavy metals are non-biodegradable and change the biological structure and system into flexible and irreversible conformation leading to deformity in the body or finally death (Kumar & Kesarwani, 2004). The effect of their toxic influence on plants is largely a strong and fast inhibition of growth processes of the above and underground parts as well as the decreased activity of the photosynthetic apparatus, often correlated with progressing senescence processes (Palmer and Guerinot, 2009). Visible symptoms of metal toxicity stress in plants are an expression of preceding metal induced alterations at the structural and ultra structural level. These changes at the cell, tissue and organ level in turn are either the result of a direct interaction of the toxic metals with structural components at these sites or a more direct consequence of changes in signal transduction and/or metabolism.

Seed germination is the first interface of material exchange between plant development cycle and environment. Germination of seed is a complex process involving series of events such as activation of respiration, repair of macromolecules, reserve mobilization, etc. Breakdown of storage reserves and its mobilization are among the crucial events that govern seed germination following imbibition. The subsequent growth of embryonic axis is a key step of the plant life, which is highly sensitive to the surrounding medium fluctuation (Ernst, 1998). Thus, alterations in this system would impair seed germination as well as seedling growth. Metal induced changes in the development of plants are the result of either a direct and immediate impairment of metabolism or signaling processes that initiate adaptive or toxicity responses that need to be considered (Liu et al., 2009). Excessive level of heavy metals in the soil environment adversely affects the germination of seeds, plant growth, alter the level of bio-molecules in the cells and
interfere with the activities of many key enzymes related to normal metabolic and developmental processes (Luo et al., 2010).

According to Li et al. (2005) seed is well protected against various stresses, but after imbibition and subsequent vegetative developmental processes, they become stress sensitive. Especially if the crop is grown in the vicinity of heavy industries, particularly in developing countries (Bi et al., 2006). One of the most fascinating and yet least understood aspects of seed physiology is the extensive reversals in metabolism, which ultimately takes place in the nutrient storage tissue after development has been completed. Cells have been factories for carbohydrates, lipid and protein production, but during embryo-genesis cells become the sites of hydrolysis of complex biomolecules, which is followed by translocation of food reserves to the growing embryonic axis. This is achieved through the hormonal control mechanism which increases the activity of proteolytic enzymes, starch degrading enzymes, nuclease, phytase, etc. at the appropriate time (Mayer & Polijakoff-Mayber, 1975).

Hydrolyzing enzymes play a major role in the mobilization of food reserves by hydrolyzing carbohydrates, protein and fats. The elevated level of heavy metals in plants meddle the metabolism and trans-location of reserve material to the growing regions and their subsequent utilization. Thus, heavy metals at supra-optimal concentrations affect the agronomic traits of plants (Mohamed et al., 2009; Posmyk et al., 2009a). Decreased hydrolysis of endospermic reserves due to decline in the activities of hydrolytic enzymes followed by delayed transportation of mobilized reserves from endosperm to embryonic axes are the key factors responsible for decreased germination of seeds and decreased seedling growth under various stressful conditions, including metal toxicity (Mihoub et al., 2005; Kuriakose & Prasad, 2008). Heavy metal generally causes severe health hazards in human beings as the metals may be transferred and accumulated in the bodies of animals or human beings through the food chain. So, it is important to ascertain the possible effects of heavy metal stress on biochemical reserves and mechanisms of tolerance and detoxification of heavy metals in plants.

Nitrogen is a constituent of various metabolites synthesized by plants upon exposure to heavy metals. Hajiboland et al. (2006) reported that plants often synthesize a set of diverse metabolites that accumulate to concentrations in the
millimolar range, particularly specific amino acids such as proline and histidine, peptides such as glutathione and phytochelatins and the amines. Thus, nitrogen metabolism is central to the response of plants to heavy metals. Nitrate is the primary form of nitrogen available to cereal crops and the enzyme nitrate reductase initiates the reduction process, and this enzyme has a major role in regulating nitrogen metabolism (Beevers and Hageman, 1969). Its regulatory nature is evident in the sense that it is the first enzyme in the pathway of nitrate reduction and index of total protein production potential of the grains. Panda and Choudhury (2005) have revealed that heavy metals induce alterations in nitrate reductase activity in plants.

Heavy metal inhibits the normal uptake and utilization of mineral nutrients (Dong et al., 2006). Phosphorus plays a vital functional role in energy transfer and acts as modulators of enzyme activity and gene transcription; hence its assimilation, storage and metabolism are of major importance to plant growth and development. Organic phosphorous is present in the soil; it may be utilized and results in subsequent release of inorganic phosphorus. Acid phosphatase catalyses the hydrolytic cleavage of the C-O-P ester bond of the organic phosphorus present in soil and releases phosphorus as plant-available inorganic forms (H$_2$PO$_4^-$, HPO$_4^{2-}$), may originate from plant and soil micro-organisms (Tarafdar, 1989). In plants, this enzyme is mainly found in the nuclei, the cell wall and intracellular spaces and, to a lesser extent, in amyloplasts, mitochondria, Golgi bodies and the endoplasmic reticulum (Chen et al., 1992). Hydrolytic breakdown of phosphate esters is brought about by phosphatases is a critical process in energy metabolism, metabolic regulation and a wide range of signal transduction pathways in plant. Heavy metals are known to affect phosphorus nutrition and metabolism in plants due to their inhibitory effect on phosphorolytic enzymes (Mishra and Dubey, 2008).

Robert and Ellis (1982) reported that decline in seed viability, whether by natural or artificial aging has long been associated with the damage to nucleic acids and membranes. Damage of the cell membrane system, especially the plasma membrane, is one of the primary events in heavy metal toxic action in plants. Disruption of membrane integrity is thought to be an effect of a complex interaction between heavy metals and functional groups of membranes. It is well-known that metal ions are easily bound to both the sulphhydryl groups of proteins and the hydroxyl part of phospholipids (Devi and Prasad, 1999). All these events result in an
increase of a non-specific membrane permeability and the parallel decrease of specific transporting activities, which disrupt the ionic homeostasis and subsequently, the activities of many enzymes crucial for basic cell metabolism (Janicka-Russak et al., 2008).

Plasma membrane functions are rapidly altered by heavy metals present in the environment at high concentrations. The first diagnostic symptom of membrane damage by heavy metals is an increase in its permeability with a subsequent disturbance in the ionic balance of the cell. It is mainly caused by metal-induced changes in the composition of the membrane lipids and the saturation of fatty acids (De Vos et al., 1993). The enzymatic activities controlling free radical reactions rapidly decline and therefore, random transformations of antioxidant systems as well as lipids and nucleic acids will be increased as part of the inexorable positive changes in entropy that follow death of the cell. To understand the mode of action leading to heavy metal toxicity in living cells their chemical properties have to be considered.

Based on the chemical and physical properties of the metals three different molecular mechanisms of heavy metal toxicity in plants can be distinguished- (a) blocking of essential functional groups in bio-molecules (b) displacement of essential metal ions from bio-molecules (c) production of Reactive oxygen species by auto-oxidation and Fenton reaction (Schützendübel & Polle, 2002). Heavy metals have a high affinity to sulphhydryl groups and disulfide bonds, which cause damage to secondary structure of proteins and alter the enzymatic activities (Siedlecka and Krupa, 2002). The mechanism of their action lies in their ability to form strong bonds with bases and phosphates of nucleic acids. This binding affinity is related to free enthalpy of the formation of the product of metal and ligand because of these features heavy metals can inactivate enzymes by binding to cysteine residues. They compete with other divalent cations and replace them in their physiological roles (Tabaldi et al., 2007).

Many enzymes contain heavy metals either as co-factors or co-enzymes and the displacement of one metal by another will lead to inhibition or loss of enzymatic activity. Divalent cations such as Ni$^{2+}$, Zn$^{2+}$, Co$^{2+}$ was found to displace Mg$^{2+}$ in ribulose-1,5-biphosphate-carboxylase/oxygenase and resulted in loss of activity (Van Assche and Clijsters, 1986). Quzoundou et al. (1995) stated that copper
accumulation influence the tissue distribution of Ca, Mg, Fe and K. Jensén and Adalsteinsson (1989) indicated that copper tends to displace Ca\(^{2+}\) ions from exchange sites. The integrity of the nucleolus depends on the existence of Ca\(^{2+}\). Because of low level of free Ca\(^{2+}\) in the cells calmodulin does not activate Ca-ATPase, which leads to failure in regulation of calcium concentration and disturbance or inhibition of various cellular metabolic processes (Liu et al., 2009).

Further action of heavy metals is due to generation of reactive oxygen species and induction of oxidative stress (Schützendübel and Polle, 2002). When plants are subjected to any biotic or abiotic stress, it results in production of reactive oxygen species such as superoxide anion radical (O\(_2^-\)), hydrogen peroxide (H\(_2\)O\(_2\)), hydroxyl radical (OH\(^-\)) and singlet oxygen (Malekzadeh et al., 2007a). To counter the deleterious effect of reactive oxygen species plants have evolved various enzymatic (CAT, APX, GPX, SOD, GR, etc.) and non-enzymatic (ascorbate, glutathione, α-tocopherol) antioxidant systems which protect the plants from their toxic action (Ahmed et al., 2010). Antioxidant pathways are usually sufficient to protect them from oxidative stress during periods of normal growth and moderate stress, but when severely stressed the production of reactive oxygen species (ROS) can exceed the capacity of the antioxidant system to neutralize them and oxidative damage can occur.

Plants show a great ability to adapt their metabolism to rapid changes in the environment and for this purpose they are equipped with complex processes such as perception, transduction and transmission of stress stimuli (Kopyra and Gwo’z’dz’, 2004). In their adaptation to the environment, plants have a specific mechanism of increasing their reaction to the action of a stress stimulus. It induces their proper defense even when the initial strength of the stress is small. Thus, molecular, biochemical and physiological processes are set in motion by a specific stress condition. Contamination of soil with heavy metals enhances plant uptake causing their accumulation in different plant organs (Mathe-Gasper et al., 2005). Proteomics is one of the most recent biotechnological approaches that are being used to address the biological function of plant proteins in different biotic and abiotic responses (Kim et al., 2004). Proteomics or the systematic analysis of proteins expressed by the genome is not only a powerful molecular tool for describing complete proteomes at the organelle, cell, organ or tissue levels, but, also for comparing proteomes under
various physiological conditions, such as those resulting from the exposure to heavy metals or other stressful environmental factors (Yuan et al., 2009).

*Vigna mungo* (L.) is also known as Black gram, urdbean, mash, black maple, etc. an important short-duration pulse crop. This crop is grown in cropping systems as a mixed crop, catch crop, sequential crop besides growing as sole crop under residual moisture conditions after the harvest of rice and also before and after the harvest of other summer crops under semi-irrigated and dry land conditions. Its seeds are highly nutritious with protein (25-26%), carbohydrates (60%), fat (1.5%), minerals, amino acids and vitamins. It is one of the most important and highly prized pulses in India. Due to the fermenting capability of the pulse, it is used widely in fermented foods that are the speciality of South Indian Cuisine.

Black gram has been distributed mainly in tropical to sub-tropical countries where it is grown mainly in summer season. Besides India, it is also grown in Pakistan, Sri Lanka, Burma and some countries of south East Asia. India is the largest producer of black gram in the world followed by Myanmar and Thailand. India has been the largest producer, consumer and importer of pulses because pulses dominantly constitute the staple diet of the people in India. In India, black gram is popularly grown in Andhra Pradesh, Bihar, Madhya Pradesh, Maharashtra, Uttar Pradesh, West Bengal, Punjab, Haryana, Tamil Nadu and Karnataka with an area of about 3.29 million ha with a total production of 1.6 million tones with an average productivity 485 Kg/ha.

Though it is grown on a variety of soils ranging from sandy to black cotton soils, but the most ideal one is well drained loam soil. It can also be grown on saline and alkali soils as it tolerates slight alkalinity. Major soil type in Rohtak district of Haryana is sandy loam and it is saline too. The increasing demand for food production to feed geometrically growing population is of serious concern. Efforts have been directed by agricultural and plant scientists towards modernizing agriculture for higher food production.

Recently, the molecular and physiological basis for plant interaction with heavy metals has attracted considerable interest. It is very important to know which heavy metal and in what concentration, they will be toxic to the plants in order to assess optimal growth on more or less contaminated soils. This can be achieved by comparative investigations of the effect of heavy metals at the biochemical,
physiological and molecular levels. Thus, the present study is an attempt to explore a possible relationship between zinc and copper metal induced changes at the biochemical, physiological and molecular level in *Vigna mungo* (L.). Various objectives of the study are-

1) To evaluate the effect of heavy metal interaction on germination behaviour of *Vigna mungo* (L.) seeds.

2) To determine the proportion of major biochemical food reserves in seeds.

3) To measure the amount of nucleic acids in germinating seeds.

4) To study the effect of heavy metals and their interaction on trans-location of major biochemical reserves during seed germination (Starch, Sugars, Proteins, free Amino acids, inorganic phosphates).

5) To estimate the activity of hydrolytic enzymes (amylase, protease and acid phosphatase).

6) To assess the effect of heavy metals & their interaction on nitrogen metabolism in germinating seeds by measuring the activity of Nitrate reductase.

7) To investigate the response of antioxidant enzymes (Catalase and Peroxidase) under heavy metal stress.

8) To study total seed protein profile under various heavy metal concentrations and combinations.