1. INTRODUCTION

Industrial revolution is leading towards the global climate change as well as heavy metal pollution of soil, water and air. It leaves various fatal effects on human health and stability of the ecosystem. These heavy metals like cadmium (Cd), copper (Cu), mercury (Hg), zinc (Zn), cobalt (Co) etc. are non-biodegradable and persist in the nature for long time period. In some soils, Cu is present in toxic levels but in others it is added by various anthropogenic activities like mining, smelting, manufacturing, automobile exhaust, ore-refining and waste disposal technologies. Cu is used in agriculture as fertilizer (copper sulfate pentahydrate, copper chloride, copper sulfate monohydrate, and copper oxide) and fungicide (copper (II) acetate, copper(II) carbonate, copper fungicides, copper hydroxide, copper naphthenate, copper oxychloride, copper oleatecopper (II) sulfate, copper sulfate (basic), copper zinc chromate). Due to overuse of fertilizers and fungicides, level of copper is increasing in the agricultural land. It is a trace element (micro nutrient) required for the normal growth and metabolism of plants. Cu is a redox active metal which acts as a cofactor for many enzymes of photosynthesis, respiration (Cu/Zn SOD, PPO, ascorbate oxidase, cytochrome c oxidase, laccase, plastocyanin), ethylene sensing and lignification processes (Yruela, 2009). It contributes to various activities like photosynthetic electron transport, oxidative stress responses, mitochondrial respiration, cell wall metabolism, nitrogen fixation and hormone signaling (Raven *et al.,* 1999). Excess levels of Cu in soils cause various damaging effects on plants such as inhibition in growth and interference in many important cellular processes such as photosynthesis and respiration (Marschner, 1995; Prasad and Strazalka, 1999). The excess amounts of Cu show visible symptoms like decreased root growth, reduced biomass, discoloration of leaves, necrosis and chlorotic symptoms (Van Assche and Clijsters, 1990).

Plants exposed to higher Cu show toxicity symptoms by the production of reactive oxygen species (ROS) through Fenton-Haber-Weiss reactions. Reactive oxygen species or free radicals such as O_2^-, H_2O_2, and OH^- cause oxidative burst at molecular level (Van Assche and Clijsters, 1990; Babu *et al.,* 2001). These free radicals cause damage to the cellular membranes, damage various essential biomolecules like DNA, lipids, proteins and deplete important nutrients like potassium from vascular and nonvascular plants (Halliwell and Gutteridge, 1984) and thus, ultimately affect the plant metabolism. Plasma membranes are the primary target of metal toxicity. Free radicals disrupt the normal structure of bio-membranes by disintegrating the unsaturated fatty acids into smaller hydrocarbon chains (Kappus, 1985). Malondialdehyde (MDA) is one of the major products formed during the disintegration of bio-membranes and serve as biomarker of oxidative stress. Cu like other heavy metals obstructs uptake of minerals and their utilization in plant system. Nitrogen is a major plant nutrient and takes part in the formation of amino acids and nucleotides. Nitrogen deficiency by Cu stress has been reported in *Triticum aestivum* plants (Athar and Ahmad, 2002). Potassium is a cofactor of various enzymes and takes part in various plant metabolic processes like osmotic balance, photosynthesis and opening and closing of stomata. Decrease in potassium content under different heavy metals including Cu has been reported (Alaoui-Sosse *et al.,* 2004; Ahmad *et al.,* 2011). Magnesium (Mg) is central unit of chlorophyll and help in photosynthesis. Cu has been reported to replace the Mg in chlorophyll molecule when present in excess amounts. Calcium (Ca) as calcium pectate forms the middle lamella and also activates the enzymes related to the formation of chromosomes. It provides cell stability, controls permeability of cell membranes, meristematic activity and mitotic spindle organization. Alaoui-Sosse *et al.,* (2004) reported decrease in Mg and Ca content under Cu stress. Other than these, Cu also affects the uptake of other micronutrients like Fe, Zn and Mn. Decreased uptake of these mineral nutrients has been reported under Cu stress in different plant species (Lin *et al.,* 1994; Martins and Mourato, 2006; Azeez *et al.,* 2015). Excess levels of Cu lead to the reduced photosynthetic activity by lowering chlorophyll synthesis and reduced gas exchange parameters like transpiration rate, stomatal conductance, net photosynthetic rate and intercellular CO_2 concentration. Reduced chlorophyll biosynthesis is associated either with structural damage of photosynthetic machinery or interference of copper with chlorophyll molecule (Wodala *et al.,* 2012). Increased levels of Cu decrease the activity of photosystem II causing lowered photosynthetic electron transport activities (Yruela, 2009). The decreased photosynthesis directly affects the growth and productivity of plants, thus lowering crop yield.

To reduce the effect of free radicals, plants have evolved different mechanisms which assist the plants to combat toxicity of metal abundance (Kováĉik *et al.,* 2010).

Primary mechanisms for free radicals scavenging include the involvement of antioxidative enzymes (peroxidases, reductases, catalases, superoxide dismutase, transferases, polyphenol oxidase) and non-enzymatic antioxidants (glutathione, ascorbic acid, anthocyanin etc.) (Skorzynska-Polit *et al.,* 2010; Sharma *et al.,* 2011). Superoxide dismutase disintegrates superoxide anion radical into O_2 and H_2O_2 and acts as first line of defense. Either guaiacol peroxidase (membrane bound enzyme) removes the H_2O_2 or it is removed by APOX in chloroplasts or by CAT in peroxisomes (Foyer *et al.,* 1997). Glutathione reductase helps in maintaining glutathione pool in reduced form which is required for the biosynthesis of other metabolites. Dehydroascorbate reducatse (DHAR) and monodehydroascorbate reducatse (MDHAR) helps in the maintenance of ascorbic acid in reduced form which is used by APOX. Other than it, ascorbic acid also acts as antioxidant and help in ROS scavenging (Mittler, 2002). Glutathione peroxidase (GPOX) consumes glutathione and reduces H_2O_2 and lipid and organic hydroperoxides (Noctor *et al.,* 2002). Glutathione transferase catalyzes the conjugation of xenobiotics and heavy metals with glutathione. Polyphenol oxidase, a copper-containing enzyme, catalyzes the oxidation of phenols to quinones. It is synthesized in cytoplasm and found in the chloroplast of healthy plant cells (Lax *et al.,* 1984). The role of PPO as antioxidative enzyme under heavy metal stress has been established (Sharma *et al.*, 2014; Devi Chinmayee *et al.*, 2014). It has been observed that short term and long term exposure of Cu has led to significant up-gradation of various enzymes (Srivastva *et al.*, 2006; Karimi *et al.,* 2012; Brahim *et al.,* 2011; Fariduddin *et al.,* 2013; Han *et al.,* 2013). Non-enzymatic system comprises ascorbic acid and glutathione which are important antioxidants produced in cell (Aly and Mohamed 2012). Ascorbic acid has ability to transfer electrons to various enzymatic and non-enzymatic reactions which makes it powerful ROS scavenger. Similarly, glutathione also plays an important role in antioxidative defense system by taking part in regeneration of ascorbic acid by participating in ascorbic acid-glutathione cycle. Glutathione in reduced form takes part in several physiological processes such as conjugation of metabolites, regulation of sulfate transport, detoxification of xenobiotics etc. (Xiang *et al.,* 2001).

Plants also activate sulfur assimilation pathway to fulfill the requirement of glutathione for the synthesis of phytochelatins, which further helps in the sequestration of heavy metals (Ederli *et al.,* 2004). Amassing of various organic molecules such as proline, sugars, free amino acids (osmoprotectants) protect the enzymes from degradation and stabilize protein synthesis mechanism. Proline formerly was known as osmoprotectant to regulate the osmotic balance under salinity stress but now it is regarded as efficient antioxidant and inhibits programmed cell death (PCD) and alleviates the toxic effects produced by ROS (Chen *et al.*, 2005; Moussa and El-Gamal, 2010). Flavonoids are also known to behave as ROS scavengers by neutralizing them under abiotic stress conditions. The ability of flavonoids as scavengers is due to the arrangement and number of hydroxyl groups attached to the rings. The antioxidant potential of flavonoids depends on the accessibility of radicals. Sugars maintain osmotic potential of cells by acting as osmolyte molecules. They also help in the stabilization of proteins, maintain cell integrity and detoxify ROS produced under metal stress (Dhir *et al.*, 2012). Polyphenols have been reported to accumulate under environmental stress and show metal stress tolerance (Quideau *et al.*, 2011). The studies indicate that phenols metabolism stimulate under metal stress to protect and recover the plants from metal injury (Guangqiu *et al.*, 2007). Amino acids are traditionally considered as building blocks for the protein synthesis. It has been additionally reported that various amino acids like cysteine, proline, methionine, histidine etc. play important role in enhancing heavy metal tolerance. Cysteine is produced as final product of sulfur assimilation in plants and it acts as sulfur donor for synthesis of methionine, some vitamins and protein containing thiols. Generally, cysteine content is low in the unstressed cells, but it has been reported that under heavy metal stress, its content increases along with the increase in glutathione content. Cysteine is mainly required for the biosynthesis of sulfur rich molecules with anti-stress properties like glutathione and stress related proteins (Zagorchev et al 2013).

Phytohormones act as major endogenous signals to morphological, physiological and molecular adaptive responses to soil metal toxicity. A close relationship between hormonal stimuli and improved metal tolerance, leads to the basis for current strategies of application of hormones to plants under metal stress to enhance the stress protection Brassinosteroids (BRs) are the novel class of phytohormones involved in the regulation of several developmental and physiological processes required for the vegetative and reproductive development. BRs were firstly isolated from pollen grains of *Brassica napus* in 1979. They are essential for various physiological and molecular responses like stem elongation, leaf bending and epinasty, differentiation of tracheary elements, polarization of membranes, vascular differentiation, photosynthesis, ethylene biosynthesis, activation of proton pump, gene expression, protein and nucleic acid synthesis (Sasse, 2003; Gruszka, 2013; Fridman and Savaldi-Goldstein, 2013). Besides these physiological roles, BRs are also known to enhance the tolerance against various abiotic stresses including heavy metal stress (Divi *et al.*, 2009; Qu *et al.*, 2011; Li *et al.*, 2013). On the basis of nature of alkyl groups at the C-24 position in side chain of 5α-cholestane carbon skelton, BRs are classified into three groups (C27-, C28-, and C29-). Castasterone (CS) is among C-28 group of BRs which was first isolated in 1982 by Yakota *et al*. from the insect galls of chestnut. Castasterone is naturally occurring BR with molecular weight 464.68 and molecular formula $C_{28}H_{48}O_5$. It has showed strong biological activity and plays an important role in growth and development of plants. It has shown strong biological activity to inclinate rice lamina assay (Yakota *et al.*, 1982). BRs are being studied for their role in stress management (Ye *et al.*, 2010; Clouse 2011) but the role of castasterone in this direction is yet to be explored.

The *Brassica juncea* is common oil yielding plant of Indian sub-continent. It has therapeutic potential due to the presence of various bioactive molecules like glycosides, flavonoids, phenols, triterpene alcohols etc. (Kumar *et al.*, 2011). *Brassica* has been studied widely due to its hyperaccumulator nature towards various heavy metals like Cu, Cd, Cr, Ni, Pb etc. (Lim *et al.*, 2004; Ariyakanon and Winaipanich, 2006; Jagtap *et al.*, 2013). Excess of heavy metals leads to toxicity of plants. Keeping in mind the toxicity of Cu and active biosynthesis and participation of CS under abiotic stress responses the present study was designed to study the role of CS on the physiological and biochemical parameters of the *Brassica* plants under the stress of Cu by using biochemical approaches. The present work was designed with the following objectives:

1. To observe the effect of castasterone on growth and oxidative stress in *Brassica juncea* under Cu metal stress.
2. To investigate the effect of castasterone on enzymatic and non-enzymatic antioxidants in metal treated *B. juncea* plants.

3. To study the castasterone mediated changes in the levels of osmoprotectants and other molecular players of stress management in *Brassica* plants.
4. To explore the protective effect of castasterone on photosynthetic parameters in metal treated *Brassica* plants.