

5. DISCUSSION

5.1 Introduction

Plants are exposed to various unfavourable edaphic and environmental conditions and adapt to these changes during evolution. Industrial revolution and increasing urbanization during past few decades is leading towards global climate change as well pollution of soil, water and air. The increasing world population is also contributing towards a global challenge and increasing use of various chemicals in agricultural practices pose threat towards environment. Soil is also getting seriously damaged due to increasing industrial, agricultural and civil activities. Various organic contaminants (explosives, petroleum products, combustible hazardous wastes) and inorganic pollutants (heavy metals) are discharged continuously. Out of these organic and inorganic contaminants, heavy metals are of major concern worldwide. Among these heavy metals, copper (Cu) as well as zinc (Zn), cadmium (Cd), lead (Pb) etc. are widely toxic for humans, animals and plants. The level of Cu is increasing in soils and waters by inappropriate agricultural practices like, overuse of fungicides with high Cu concentrations, by release of industrial wastewater and mining activities. Cu being an essential element plays an important role in various physiological processes, but it acts as toxic metal when present in amounts above than threshold level.

Copper is an active participant in various physiological activities like photosynthetic electron transport, cell wall metabolism, oxidative stress, mitochondrial respiration and hormonal signalling. Cu ions also behave as a cofactor for many enzymes (Cu/Zn SOD, PPO, ascorbate oxidase, cytochrome c oxidase, laccase, plastocyanin) (Yruela, 2009). However, plants exposed to higher Cu concentration show toxicity symptoms. At concentrations higher than the normal levels required for optimal growth, Cu has negative effect on developmental activities of plants. It has been reported that excess Cu reduces growth and interferes with cellular processes like photosynthesis and respiration (Prasad and Strzalka, 1999; Choudhary *et al.*, 2012). The decrease in chlorophyll content and changes in chloroplast structure was observed under higher copper concentrations. It has been hypothesized that Cu interferes with photosynthetic machinery and modifies the protein and pigment composition of photosynthetic membranes (Maksymiec *et al.*, 1994). This metal is redox active and

redox cycling between Cu^{2+} and Cu^+ leads to the production of reactive oxygen species (ROS) through Fenton-Haber-Weiss reactions, and thus cause oxidative stress. However, higher contents of Cu are toxic for plants and can adversely affect growth, nutrient uptake, photosynthesis, enzyme activity and biomass production (Cuypers *et al.*, 2005; Khurana *et al.*, 2006; Choudhary *et al.*, 2010; Bajguz 2011; Choudhary *et al.*, 2012).

Higher levels of Cu added to soil through anthropogenic activities become phytotoxic and produce oxidative stress in plants by enhancing ROS production. Like other heavy metals, toxicity of Cu might be due to the replacement of various enzyme cofactors, protein oxidation, lipid oxidation, inhibition of pigments and photosynthetic processes, DNA damage and cellular redox imbalance (Nagajyoti *et al.*, 2010). In addition, change in hormones is also linked with the heavy metal stress and salt stress leading to the reduced photosynthesis and growth (Masood *et al.*, 2012; Khan *et al.*, 2014). ROS or free radicals are continuously produced in plant cells under normal growth conditions but their concentration remains low due to the activity of protective enzymatic antioxidants (SOD, APX, POD, CAT, GR, GST etc.) and non-enzymatic antioxidants (ascorbic acid and glutathione). These antioxidants scavenge and detoxify excess ROS and provide protection. Other than these, accumulation of various metabolites like proline, sugars, amino acids, thiols etc. provide protection against any harm produced by ROS and help in maintaining cellular osmotic adjustment, stabilize enzymes/proteins and protect biological membranes (Kavi Kishor *et al.*, 2005; Rejeb *et al.*, 2014) (Fig. 5.1).

Primary response of plants is production of excessive ROS to heavy metal stress. The free radicals are produced under Cu metal ions directly through Haber-Weiss reactions (Yruela *et al.*, 2005). The production of free radicals directly affects the membrane lipids and leads to lipid peroxidation. Thereby, leads to imbalance in pro-oxidant and antioxidative defence system causing oxidative stress. However, plants have developed complex mechanisms to fight with this metal induced oxidative burst. The activity of enzymatic and non-enzymatic antioxidants increases under metal stress and acts as one of the chief ROS scavenging mechanism (Sharma *et al.*, 2011). SOD acts as the first line of defence against ROS production and it catalyse the superoxide radicals (O_2^-) to O_2 and H_2O_2 . Other enzymes like POD, APOX, CAT, GPOX, PPO etc.

scavenge free radicals. Further, GR catalyse the GSSG to GSH and help in maintaining the GSH pools and dehydroascorbate is reduced to ascorbate (Noctor and Foyer, 1998). Dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) use NADPH as reducing power and maintains the levels of ascorbic acid in its reduced form (Mittler, 2002).

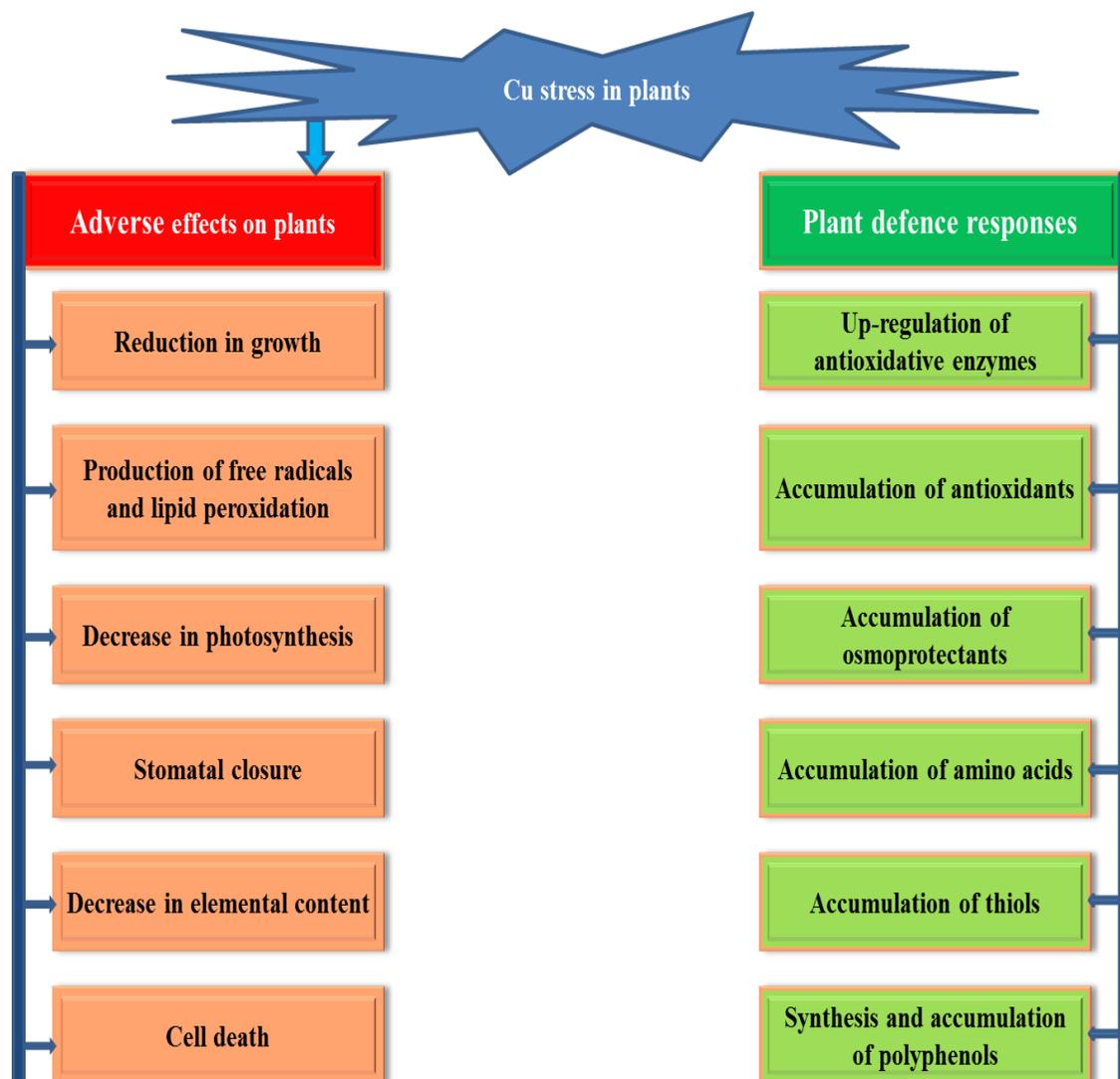


Fig. 5.1 Effect of copper on plants and plant defence responses for copper

In addition to the antioxidative enzymes, plants have also developed other stress protective mechanisms. These strategies include the accumulation of osmoprotectants like proline, amino acids, thiols etc., the accumulation of low molecular weight metabolites, the synthesis of some special proteins and variation of levels of phytohormones (Bari and Jones *et al.*, 2009). Recent studies has shown that various

plant hormones like ethylene, ABA, salicylic acid, jasmonic acids and BRs play an active role in the protection against oxidative stress (Depuydt and Hardtke, 2011). Out of these, BRs have been found to be effective against alleviation of various stresses including heavy metal stress in rice, maize, tomato, wheat, *Arabidopsis*, mustard and radish. Our previous studies have shown that two commercially available BRs (EBL and HBL) actively remove metal stress either by activating the antioxidant defence system or by decreasing its uptake in plants (Poonam et al. 2014). Very little information is available on the role of castasterone in regulation of plant metabolism and heavy metal tolerance.

The present work was undertaken to have clear understanding on the role of castasterone in metabolism and development of *Brassica* plants under Cu stress.

5.2 Effects of castasterone on metabolism of *B. juncea* plants under metal stress

The effect of different concentrations of castasterone on oxidative stress, antioxidative machinery, sulfur metabolism and amino acids was observed in the present study under influence of different concentrations of copper. The significant observations are discussed below:

5.2.1. Metal uptake

B. juncea plants/seedlings grown in growth media (soil or Hoagland medium) containing various concentrations of Cu (0.25, 0.50 and 0.75 mM) depicted increase in the uptake of Cu in various part of plants/seedlings (root, shoot and leaves). The accumulation of Cu increased with the increasing concentration of Cu in growth medium. Cu is transported across cell membrane with the help of COPT proteins and P-type ATPases (PAA1, PAA2 and HMA5). Cu chaperons also play an important role in intercellular mobilization of Cu ions. These chaperons are low molecular weight, cytosolic, soluble metal receptor proteins (Yruela, 2005). Chloroplast acts as main site of accumulation of Cu under normal environmental conditions while under Cu stress, it mainly accumulates in the cell walls. Miotto *et al.*, (2014) reported that concentration of Cu in various parts of grapevine increased with increasing availability of Cu in soil. Similar results were obtained by Peng *et al.*, (2005) and Ansari *et al.*, (2009) for Cu and

Hg uptake in *Elsholtzia splendens* and *B. juncea* plants. It has been reported that transfer of Cu from root to shoot is low.

Plants grown from seed soaking in castasterone (CS) and raised in soil containing different concentration of Cu showed increase in Cu accumulation in roots, shoots and leaves. This might have been due to the increase in translocation of Cu from roots to aerial parts. The total metal uptake per plant has also been found to increase with the application of CS as seed pre-soaking. It might be due to the removal of toxicity by activation of defence system of plant and leading to increase in its growth and biomass. The increasing biomass will help in the distribution of Cu and decrease toxicity. The increase in Cu uptake was observed with the increasing stage of plant.

5.2.2 Growth parameters:

It was observed in the present study, that exposure of plants to increasing levels of Cu (0.25, 0.50 and 0.75 mM) had reduced the growth which is reflected by decrease in the length (root and shoot), biomass (fresh and dry) and dry matter content. The enhanced uptake of Cu with increasing concentrations produced more damaging effects on the plant metabolism leading to the reduced plant growth. Plant cell wall acts as main site for metal detoxification (Allan and Jarrell, 1989). Cu stress leads to the production of H₂O₂ which can make cross-links between cell wall polymers making cell wall non-elastic and stiff (Fry, 1988; Schopfer, 1996). It prevents elongation of cells by reducing the plant growth. The decrease in growth may be due to the involvement and disturbance in cell processes by some metals like Cu, Zn, Ni etc. when are in excess amounts. It has been reported by Battah (2009) that metabolic activity of plants reduces thus leading to the reduced growth under stressful conditions. The results are in accordance with the earlier studies of Sharma and Bhardwaj (2007), Fariduddin *et al.*, (2013) and Feigl *et al.*, (2015) who validated that under Cu stress, decrease in growth parameters takes place in *B. juncea*, *Cucumis sativus* and *B. napus* respectively. Due to Cu toxicity mitotic activity of plants decreases, which further affects cell elongation (Alaoui-Sosse *et al.*, 2004; Maksymiec and Krupa 2007). Cu toxicity is also linked with the membrane permeability and leads to reduced nutrient content mainly Fe, P, K, Ca, N

and Mg (Chen *et al.*, 2005; Yusuf *et al.*, 2011; Rozentsveta *et al.*, 2012). The decrease in plant height (shoot height and root height) was recorded under Cu stress in wheat plants (Kumar *et al.*, 2012; Baek *et al.*, 2012, Ashagre *et al.*, 2013). The decreased nutrient content inhibits cell division and cell elongation (Gajewska *et al.*, 2006). The excess Cu decreases root biomass in most plants (Lidon and Henriques 1992; Makesymiec 1997). The decrease in biomass under excess Cu exposure might be endorsed due to reduced photosynthetic rates as a consequence of alteration in the structure of chlorophyll (Hill *et al.*, 2000). Similar result of reduced biomass under Cu stress was observed by Khurana *et al.*, (2006).

Seed pre-soaking with CS improved root and shoot length, fresh and dry weight and dry biomass content in *Brassica* plants grown under Cu stress. BRs have been found to increase cell elongation in very minute quantities. Application of only 0.005 to 0.02 ppm of BRs to wheat plants make them taller than the untreated plants (Wimolphan, 2004). Cell elongation is linked with enhanced acid secretion. Therefore BRs action confirms the stimulation of cell elongation. Under Cu stress, BRs promote cell elongation along with progression of cell cycle and stimulates genes encoding for expansins and xyloglucanses leading to promoted growth (Gonzalez-Garcia *et al.*, 2011; Zhang *et al.*, 2009). These results are in accordance with Fariduddin *et al.*, (2009), who also observed that application of EBL improved root/shoot length and fresh/ dry biomass of the *B. juncea* plants under Cu stress. It might be due to the role of brassinosteroids in cell elongation or due to modification of plasma membrane permeability under stressful conditions. In present study, improvement in the plant growth with application of CS might be due to the improvement in the mineral contents (N, K, Ca and Mg) and auxin content. Similar results were obtained by Choudhary *et al.*, (2010) who observed reduction in copper toxicity by exogenous application of 24-EBL in radish seedlings and noted increase in root and shoot length. In the present study, improvement in the fresh and dry weight is observed in both treatments of Cu and CS as compared to only Cu treated plants. Similar enhancement in the fresh and dry weight was observed by Anuradha and Rao (2007) in radish seedlings under Cd toxicity by application of EBL and HBL. It might be due to the stimulatory effect of BRs on growth.

5.2.3 Changes in photosynthesis

It is evident from the study that the levels of pigments (chlorophyll and carotenoids), and gas exchange parameters like photosynthetic rate, stomatal conductance, transpiration rate and intercellular CO₂ concentration reduce under various Cu treatments (0.25, 0.50 and 0.75 mM) in *Brassica* plants. Maximum decrease in the pigment contents was observed in the 60 d old plants. The decline in photosynthetic processes of plants due to the Cu toxicity is shown in Fig. 5.2 Copper is known for disturbing the photosynthetic apparatus by altering the structure of chloroplast and composition of thylakoid membrane (Quartacci *et al.*, 2000). It has been hypothesized that Cu treatment interferes the process of photosynthesis by changing the pigment and protein composition of photosynthetic membranes. The changed fatty acid composition of thylakoid membranes, lipid peroxidation and decrease in lipid content was also observed under excess copper (Luna *et al.*, 1994; Maksymiec *et al.*, 1994). These alterations lead to the modifications in PSII membrane fluidity (Quartacci *et al.*, 2000). The decrease in chlorophyll content under Cu stress in leaves might be due to changes in structure of chloroplast and thylakoid membrane (Yruela, 2005). Feigl *et al.*, (2015) has also reported decrease in pigment concentration in *B. juncea* and *B. napus* under Cu stress. At toxic levels, copper replaces Mg²⁺ by inhibiting synthesis of aminolevulinic acid (precursor of chlorophylls) and protochlorophyllide reductase (enzyme to catalyze the reductive formation of chlorophyllide). Kanoun–Boule *et al.*, (2009) has reported decrease in chlorophyll content under Cu stress in duckweeds. They also revealed that chl a and carotenoids content were more sensitive to Cu toxicity than chl b. Similarly, Kumar *et al.*, (2012) has observed decrease in chlorophyll a, chlorophyll b and carotenoids content in wheat plants treated with Cu and Zn. However, it has been observed in present study that anthocyanin content was increased with copper treatment. Our findings confirm report of Baek *et al.*, (2012) who observed increase in anthocyanin content in *Arabidopsis thaliana* plants under Cu treatment. It indicates that anthocyanin acts as antioxidant under Cu stress. It has been observed that anthocyanin content increase under some metal stresses and enhanced the protection against metal stresses (Hale *et al.*, 2001; Neill *et al.*, 2003).

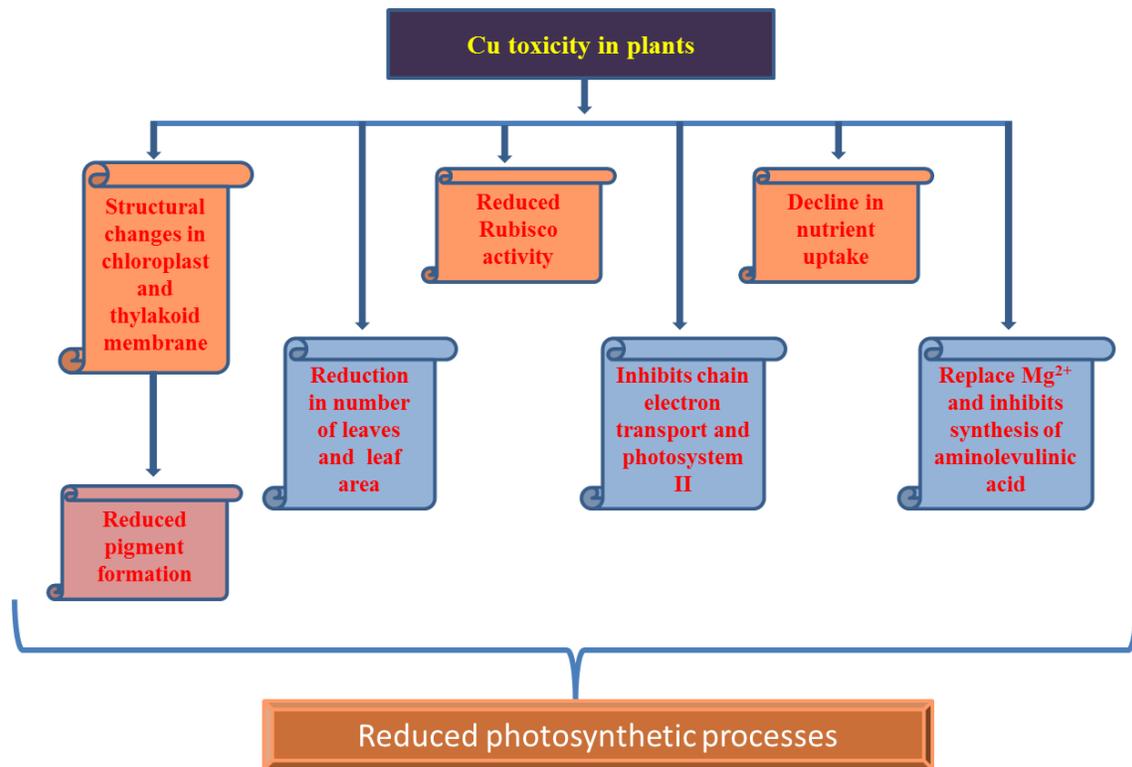


Fig. 5.2 Effect of copper toxicity on photosynthesis

Under Cu stress, the photosynthetic efficiency was observed to decrease. The plants raised in under Cu stress showed lower photosynthetic rate along with significant decrease in stomatal conductance, intercellular CO₂ concentration and transpiration rate. Copper has been reported to damage the structure of chlorophyll molecules by displacing Mg²⁺ ion (Van Assche and Clijsters, 1986). Copper has inhibitory role on chain electron transport and photosystem II catalyzed electron transport resulting into reduced photosynthesis (Swarna *et al.*, 2012). The Cu toxicity effects PSII by inhibiting oxygen evolution along with quenching of variable fluorescence (Mohanty *et al.*, 1989). The acceptor as well donor sides of PSII both suggested as main targets of attack during Cu toxicity. Direct interactions between Cu and Ca at oxidizing sides are also reported (Sabat, 1996; Maksymiec and Baszynski, 1999). It has been suggested by Azmat and Riaz, (2012) that Cu interferes with the photosynthetic machinery by modifying the pigment and thylakoid protein composition which results in decrease in internal CO₂ concentration. Moreover, Cu also decreased stomatal opening i.e. stomatal conductance. The collective effect of all these changed processes is accompanied by reduction in chlorophyll content which finally reduced the rate of photosynthesis. Similar results were obtained by Faridudin *et al.*, (2013) in *Cucumis sativus* plants under Cu stress.

In the present investigation, it has been observed that pre-soaking of seeds in CS caused improvement in the pigment content and gas exchange parameters. The improvement in photosynthesis mediated by BRs might be due to enhanced activity and synthesis of enzymes of chlorophyll synthesis and those associated to photosynthesis (Xia *et al.*, 2009). BRs enhance Rubisco activity and protect PS II under various environmental stresses and have positive effect on various gas exchange parameters (Wu *et al.*, 2012). BRs also improve water uptake and relative water content causing enhanced photosynthetic rate (Ali *et al.*, 2005). It has been suggested that BRs transcript for various photosynthetic genes, activates Calvin-Benson cycle thus leading to improvement in the photosynthetic capacity (Jiang *et al.*, 2012). Earlier studies also validated that co-application of BRs shield photosynthesis and related characteristics (Ali *et al.*, 2008; Fariduddin *et al.*, 2013).

5.2.4 Reactive Oxygen Species

Under heavy metal stress the production of reactive oxygen species (ROS) like H_2O_2 , and superoxide anions (O_2^-) increase (Vardhini and Anjum 2015). Accumulation of these free radicals is associated with changes in cellular redox status resisting the plants against these stresses (Maksymiec, 2007). In present study, the generation of O_2^- and content of H_2O_2 has been found to increase with the increasing copper concentrations (0.25, 0.50 and 0.75mM). Confocal microscopic studies also revealed same observations. The present work gets support from the studies on heavy metal toxicity where overproduction of free radicals (Anuradha and Rao, 2007; Srivastva and Dubey, 2011; Fariduddin *et al.*, 2013; Ramakrishna and Rao, 2015; Mir *et al.*, 2015). The overproduction of free radicals cause oxidative burst, damage biomolecules like membrane lipids, proteins, enzymes and nucleic acids and affect the detoxification processes. Plasma membranes are regarded as prime target of metal toxicity. Linoleic acid and linolenic acids are major fatty acids of plant membranes (Moller *et al.*, 2007). The free radicals attack these polyunsaturated fatty acids and produce lipid hydroperoxides. The damage to the membrane lipids is termed as biomarker of lipid peroxidation caused by free radicals and leads to enhanced leakage during stress (Chaoui *et al.*, 1997; Cuny *et al.*, 2004). MDA content (rate of lipid peroxidation) is regarded as stress marker. In present study the MDA content has been observed to increase with the increasing concentration of metal under Cu stress. Similar results were

observed under Cu metal stress in different plant species by different research groups (Szollosi *et al.*, 2012; Fidalgo *et al.*, 2013; Xu *et al.*, 2011; Poonam *et al.*, 2014).

It has been found in the present study that application of CS reduced the free radicals level in Cu treated *Brassica* seedlings. Ramakrishna and Rao, (2012) observed that increased ROS levels were found to decrease in radish seedlings under Zn stress by application of brassinosteroids. The reducing levels of free radicals are associated with the up-regulation of antioxidative defence system including both enzymatic and non-enzymatic antioxidants with the help of BRs. Other protective molecules like proline, sugars, free amino acids etc. also increase with the BR supplementation. The MDA levels were also found to decrease in binary combinations of Cu and CS treated *Brassica* plants. The lowering of MDA content with BRs is associated with decreased levels of free radicals and thus BRs helps in maintaining membrane integrity. Similar results were also found by various research groups in various plants under metal stress (Wang *et al.*, 2009; Ramakrishna and Rao, 2012; Fariduddin *et al.*, 2015; Poonam *et al.*, 2014; Filova *et al.*, 2013).

5.2.5 Antioxidant system

Abiotic stresses force the plants to change their physiology and biochemistry to adjust under excessive reactive oxygen species (ROS). This adjusting system is made up of antioxidants including enzymes such as SOD, POD, CAT, APOX, DHAR, GR, GST, GPOX and PPO and non-enzymatic antioxidants including ascorbic acid and glutathione (Munne-Bosch 2005; Kanoun-Boule' *et al.*, 2009; Maleva *et al.*, 2009; Shu *et al.*, 2012; Kumar *et al.*, 2012). The present study indicates that Cu treatment has increased the levels of antioxidative enzymes as well non-enzymatic antioxidants. SOD acts as first line of defence to scavenge ROS and catalyzes superoxide anion to H₂O₂ and O₂ (Alscher *et al.*, 2002). In the present study, significant enhancement in the SOD activity was recorded in the Cu stressed *Brassica* plants, suggesting its importance in the removal of superoxide anion induced by Cu toxicity. Superoxide dismutase reduces superoxide and thus decreases the threat of formation of hydroxyl radical from superoxide *via* metal catalysed Haber-Weiss reaction. Three isoenzymes of SOD (Mn-SOD, Cu/Zn-SOD and Fe-SOD) are reported in various plants under stress. The application of CS further enhanced the SOD activity showing enhanced superoxide

anion scavenging under both normal and Cu stressed *Brassica* plants. Similar results were obtained by Ramakrishna and Rao, (2012) in radish seedlings under Zn stress with the application of EBL. The decomposition of superoxide anion radical leads to the formation of H_2O_2 which diffuse through plasma membrane and is toxic as it can act as antioxidant and reductant both (Foyer *et al.*, 1997). Catalase (CAT) and peroxidases catalyze H_2O_2 to water and O_2 . Under Cu stress in this study, increase in the CAT, guaiacol peroxidase (POD), ascorbate peroxidase (APOX) and glutathione peroxidase (GPOX) concentration increase. These enzymes act as second line of defence against enhanced production of free radicals (Gasper *et al.*, 1991). Whereas application of CS alone or to Cu stressed *Brassica* plants increased the activities of all enzymes illustrating the protective role of BRs through efficient ROS scavenging. These results are supported by Ramakrishna and Rao (2012) who reported increased SOD, POD, CAT, APOX and GPOX activities under Zn metal stress in radish seedlings. Similarly Ali *et al.*, (2008) reported BRs up-regulated antioxidant defence system in *B. juncea* plants under Ni and salinity stress. For the protection of plant cells from damaging effects of free radicals, the APOX induction occurs through AsA-GSH cycle, where AsA is used as hydrogen donor by APOX. The GR catalyzes the GSSG to reduced GSH form by NADPH dependent reduction and helps to maintain high ratio of GSH/GSSG. Reduced ascorbate is regenerated by enzymes MDHAR and DHAR using NAD(P)H to recycle ascorbate. Increased activities of these enzymes were observed in this study under application of Cu and CS both. Activity of glutathione-s-transferase also was observed to increase under the presence of Cu in growth media. Similar increase in GST activity was recorded by Kapoor *et al.*, (2014a) in 30 d old *B. juncea* plants under Cd stress. Gajewska *et al.*, (2006) also reported enhanced activities of POD and GST under increased levels of Ni stress. The application of CS also enhanced the activity of GST in *Brassica* plants grown under Cu stress. These results are in accordance with the Sharma *et al.*, (2014) who experienced similar increase in radish plants under Cu stress with the application of 28-HBL. Similarly, PPO activity was also recorded to increase under Cu stress in *Brassica* plants. PPO has been observed to behave as antioxidative defence enzyme under various metal stresses. Enhanced enzymatic activity along with PPO in *Jatropha curcas* under Cd, Cr and Hg has been recorded by Devi Chinmayee *et al.*, (2014). Cu stress increased activity of PPO in *Chlorella vulgaris* (Kebeish *et al.*, 2014).

The activity of PPO was further enhanced with the application of CS. These results are supported by Sharma *et al.*, (2014) who observed enhancement in the PPO activity under metal stress and 28-HBL application in radish plants. It was observed that this BR reduces Cu toxicity by enhancing the activities of various enzymes (Fig. 5.3).

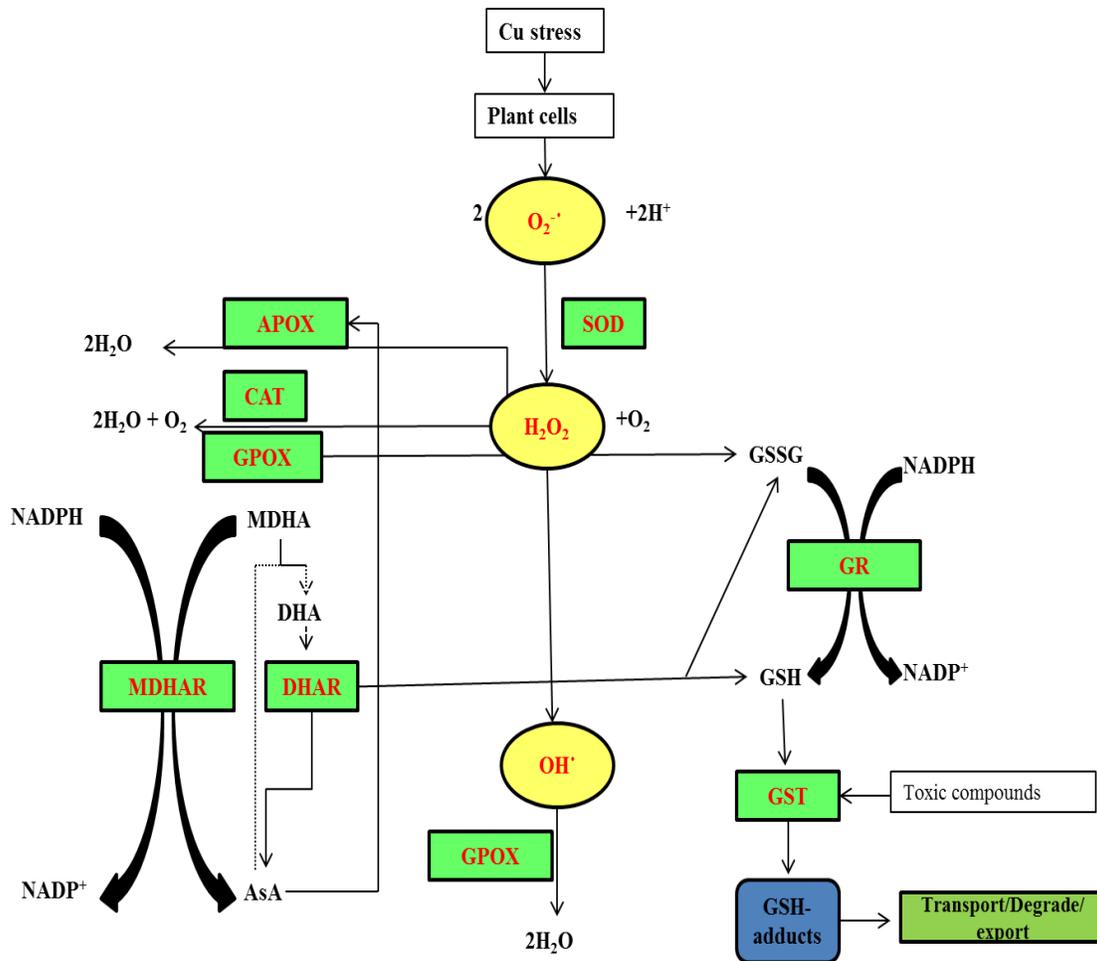


Fig. 5.3 Cu induced enzymatic antioxidative defence system

Non-enzymatic low molecular weight molecules like ascorbate, glutathione and tocopherol accompany the enzymatic antioxidant systems to counteract the stress induced ROS accumulation. These molecules have the capacity to chelate metal ions, leading to reduction in catalytic activity of metal to generate ROS and also scavenge these free radicals. In plant cells, this trio (ascorbate, glutathione and tocopherol) constitute an integral part of abiotic stress response (Szarka *et al.*, 2012). In the present investigation, increase in these molecules with both Cu and CS applications has been recorded. Glutathione play an important role in antioxidative system, in ROS scavenging and keeping cellular redox state balanced. Glutathione is available in two

reduced and oxidized states. Glutathione takes part in AsA-GSH cycle, which successively oxidizes and reduces ascorbic acid and glutathione with cyclic transfer of reducing equivalents. Our results are in accordance with Choudhary *et al.*, (2012) who also observed the increase in ascorbic acid and glutathione content under Cu stress as well as EBL treatment in *Raphanus sativus* seedlings. Similar increase in the ascorbic acid and glutathione content in rice seedlings was reported by Thounaojam *et al.*, (2012). The increase in ascorbic acid content in seeds of pea and lupine by application of BRs has been reported by Biesaga-Koscielniak *et al.*, (2014).

5.2.6 Carbohydrate metabolism

In the present investigation total sugars content was increased under Cu treatment. The results are in accordance with the Aly and Mohammad, (2012) who observed enhancement in the total sugar content in maize cultivars under Cu stress. Sugars help in maintaining the osmotic balance in plants and increase storage capacity of carbohydrates to sufficient level by providing nutrition for basic metabolic activities under stressed conditions (Dubey and Singh, 1999). Sugar accumulation also helps in the protection of biomolecules and membranes by regulating internal osmolarity. It has been reported by Guzel and Terzi, (2013) that copper toxicity increases the total sugars in maize leaves. Similarly, sucrose which is one of the soluble sugar also increased in cucumber leaves under Cu stress (Alaoui-Sosse *et al.*, 2004). In the present study, plants grown from seeds pre-soaked in CS and raised in Cu containing growth media, revealed increase in the total sugar contents when compared to only Cu treated plants. Improvement in the photosynthesis with the application of BRs can be a reason behind this increased content of total sugars (Vardhini *et al.*, 2012).

In the present study, a number of sugars were expressed in copper treated plants. Sugars are one of the soluble solutes which get accumulated under various biotic and abiotic stresses and help in the osmoregulation of plant cells (Aly and Mohamed, 2012). Increase in soluble sugars can be related directly to tolerance of plants towards stress conditions (Su and Wu, 2004). Various secondary metabolites like mannitol, sorbitol etc. accumulates during adverse growth conditions and help in increasing tolerance of plants (Ramakrishna and Ravishankar, 2011). Polyols (sorbitol, mannitol) accumulation has two functions: they support redox control and help in osmotic balance. They also

regenerate NAD^+ and phosphate. It has been reported that high amount of sorbitol was accumulated in stroma of chloroplasts, which suggest its role in osmotic adaptation and shielding of this compartment (Nadowodnik and Lohaus, 2008). Increase in the number of sugars expressed in treated with Cu and CS was observed in Cu treated *Brassica* plants. Janeczko *et al.*, (2009) and Bajguz (2011) reported increase in the soluble sugars content under various metal stresses.

5.2.7 Phenol contents:

Increase in the total phenol content under Cu stress in *Brassica* plants has been observed in this study. Increase in the total phenolic content was also observed in wheat plants under Cu and Zn stress (Kumar *et al.*, 2012). Similarly, Hamid *et al.*, (2010) have also reported the increase in total phenolic content under heavy metal stress. Plants produce a variety of secondary metabolites with known functions in protection against various biotic and abiotic stresses. Compounds with phenol group are one of these secondary metabolites with known importance in stress management. Polyphenols are also involved in free radical scavenging due to their chemical structure and show better expression than vitamin E and C. Hydroxyl and carboxyl group of phenols help them to bind with heavy metals like Fe and Cu (Jung *et al.*, 2003). Increase in total phenolic content under Cu and salinity stress was observed by Hejazi-Mehrzi *et al.*, (2012). Similar results were obtained by Bizzo *et al.*, (2014) who recorded increase in phenolic content under Cu toxicity in *Salvinia auricularia*. Accumulation of phenolic content under Cu stress might be due to induced synthesis of shikimate dehydrogenase (Diaz *et al.*, 2001). Increase in the phenolic content in leaves, stems and roots of *Withania somnifera* under Cu stress has been observed by Singh *et al.*, (2014). Total phenolic content was further increased with the application of CS which shows that this brassinosteroids help in the efficient removal of free radicals produced and ameliorating Cu toxicity.

In the present study, number of polyphenols identified increased under Cu stress in *Brassica* plants. Role of polyphenolic compounds in different biological functions like UV protection, pollen tube growth, antimicrobial activity, biotic and abiotic stress resistance make them important for plants. Simple phenols (trans-cinnamic acid, coumaric acid) act as precursor for the formation of complex phenolic compounds like

flavonoids, tannins, lignins and anthocyanin (Šebestík *et al.*, 2011). A group of phenolic acids such as caffeic acid, trolox, rosmarinic acid has been recognized as good antioxidants. Polyphenols behaves as antioxidant and scavenge free radicals produced under heavy metal toxicity. They also make chelates with metals thus lead to reduced metal toxicity (Lavid *et al.*, 2001). Chelation of Cu by polyphenolic precipitates in *Armeria maritime* has been studied by Neumann *et al.*, (1995). The number of identified polyphenols varied with the treatment of Cu and CS in combination as compared to Cu treatment. Application of 24-EBL increased the total phenolic content in berries of *Vitis vinifera* plants (Xu *et al.*, 2014). They found a direct correlation between increase in phenolic content and antioxidant potential of plants. Decrease in the total phenolic content and antioxidant capacity was observed with the application of brassinazole, which inhibits formation of BRs. Spraying of brassinosteroids analogues enhanced the phenolic content and total antioxidant activity of pepper plants (Serna *et al.*, 2012). Similarly, Ahammed *et al.*, (2013) observed increase in total phenolic content with the application of EBL under phenanthrene stress in tomato plants. This can be assumed that increase in total phenolic content as well as changes in various polyphenols levels lead to the enhanced antioxidant capacity against metal stress.

5.2.8 Osmoprotectants levels

In the present work, accumulation of osmoprotectants like flavonoids and proline increased under Cu stress in *Brassica* plants in comparison to control untreated plants. Various osmoprotectants produce and accumulate under abiotic stress and play role in the tolerance mechanisms. Flavonoids have metal chelating properties suggesting their role in oxidative stress conditions where transition metals ions like iron and copper are involved. Flavonoids have higher reducing capacity for Cu ions than Fe ions (Mira *et al.*, 2002). Flavonoids containing multiple hydroxyl substitutions displayed higher antiperoxy radical activities (Cao *et al.*, 1997). Flavonoids locate and neutralize free radicals before they harm the cells, thus act as ROS scavengers under hostile environmental conditions. Increase in total flavonoids content was observed by Mammat *et al.*, (2015) in *Orthosiphon stamineus* under Cu stress. Similar increase in flavonoids content under Cd stress in *Brassica juncea* plant is reported by Kapoor *et al.*, (2014). Enhancement in total flavonoid content in *Orthosiphon stamineus* under Pb stress and decrease under Cu stress has been recorded (Manan *et al.*, 2015). The

increase in total flavonoids content suggests its role in ROS scavenging. Cu and CS treatment further enhanced the flavonoids content in the *Brassica* plants. These results are in accordance to the results obtained by Ahammed *et al.*, (2013a). They found significant enhancement in the flavonoids content with the application of 24-EBL to tomato under phenanthrene. Similarly, Xi *et al.*, (2013b) observed increase in the secondary metabolites including flavonoids content in grape berry thus, leading to better antioxidant capacity. Kapoor *et al.*, (2014b) also reported 24-EBL mediated enhancement in the flavonoids content in radish seedlings under Cd and Hg stress.

Enhancement in the proline levels is recorded in *Brassica* plants under Cu stress in the present investigation. Proline is an amino acid which accumulates under various abiotic stresses. It behaves as an osmoprotectants and help in maintaining osmotic balance of cells under various abiotic stresses. Other than role of osmoprotectants, proline also acts as antioxidant to protect the cell from free radicals damage and inhibits PCD (Moussa and El-Gamal, 2010; Gill and Tuteja, 2010). Thus, proline is regarded as non-enzymatic antioxidant required for the mitigation of adversative effects of ROS (Chen and Dickman, 2005). It is well reported that proline accumulation increase dramatically under salt, drought and metal stress due to up-regulated synthesis or down-regulated degradation. Proline has been suggested to behave as osmoprotectant, metal chelator, protein stabilizer, and inhibitor of lipid peroxidation, singlet oxygen, and hydroxyl radicals (Shah and Dubey, 1998; Rauser, 1999; Ashraf and Foolad, 2007; Trovato *et al.*, 2008). It has been reported by Gajewska and Sklodowska (2005) that accumulation of proline along with stimulated GST activity caused response of pea plants to Ni stress. Siripornadulsil *et al.*, (2002) has observed that free proline levels are associated with glutathione redox state and MDA content under heavy metal toxicity to algae, thus suggesting its role as an antioxidant under Cd stress. Bizzo *et al.*, (2014) have found that proline content significantly enhanced under Cu stress in *Salvinia auriculata*. Fidalgo *et al.*, 2013 reported enhanced accumulation of proline in the roots of *Solanum nigrum* under higher levels of Cu. The larger accretion of proline in plants exposed to higher levels of Cu suggest its role in detoxification from Cu toxicity and its role in Cu tolerance of various plants has been well reported (Singh *et al.*, 2010; Andrade *et al.*, 2010; Thounaojan *et al.*, 2012). The proline content further increased in the plants grown from seeds pre-soaked in CS and raised in growth media containing

Cu. These results are supported by Ali *et al.*, (2008) who found increase in proline content in mung bean under Al stress with the foliar spray of BRs (EBL and HBL). Hayat *et al.*, (2007) also reported significant increase in the proline content under Cd stress and BR application. BR and Cd induced proline accumulation is also reported by Hasan *et al.*, (2011) in tomato and Anuradha and Rao, (2007) in radish. It seems possible that BRs shows protective role for plants under stressed conditions by accumulating higher levels of proline.

5.2.9 Amino acids

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 role in the heavy metal tolerance. Amino acids chelate heavy metals intracellularly. Enhancement in the free amino acid content was observed in the present study. In agreement to this, El-Tayeb *et al.*, (2006) reported accumulation of free amino acids in stems and leaves of sunflower under higher Cu levels. Similarly, Mazen (2004) reported Cu induced accumulation of free amino acids. Zhi-Ting *et al.*, (2006) reported increased free amino acids content in *Brassica perkinsensis* under Cu stress. Chen *et al.*, (2014) suggested that changes in the levels of aromatic amino acids under Hg stress contribute to the tolerance strategy of rice seedlings. 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. Cysteine content is low in the unstressed cells, but it has been reported that under heavy metal stress increase in the cysteine content is observed along with the increase in glutathione. 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). Content of cysteine enhanced under Cu stress in present investigation. These results are supported by Aly and Mohamed, (2012), who observed gradual increase in the cysteine content under various levels of Cu stress in maize plants. Cysteine is needed for synthesis of methionine and glutathione thus; it acts as important part of antioxidant system and metal sequestration. Genetic modification in capacity of metal induced synthesis of cysteine supported tolerance of *Arabidopsis* under acute Cd toxicity (Dominguez-Solis *et al.*, 2004). The combination of Cu and CS enhanced the

free amino acids and cysteine levels. BRs induced accumulation of free amino acids content and cysteine content shows their role in detoxification of Cu induced stress.

Amino acid profiling showed that proline, methionine, cysteine, glutamine, alanine and aspartic acid were the major amino acids whose levels enhanced under Cu stress. Bhatia *et al.*, (2005) also reported increase in the levels of aspartic acid, alanine and glutamine under Ni stress showing its role in complexation of Ni in xylem. Synthesis of stress related amino acids shows the molecular response of plants towards heavy metals (DalCorso *et al.*, 2008). Methionine helps in the translocation of Cu in plants. The interaction of Cu and CS further enhanced the levels of various amino acids. These amino acids participate in metal chelation, thus help in the removal of Cu toxicity.

5.2.10 Thiols content

Enhanced levels of total thiols, non-protein thiols and protein thiols were observed in this study. It was reported that metal stress increase the sulfur reduction pathway by affecting the sulfur uptake and transport but also change the activity of pathway enzymes (Rausch and Wachter, 2005; Herbette *et al.*, 2006). An increase in the protein thiol content in rice seedlings under different Cu (0, 50, 100, 200 μM) concentrations was reported by Zhang *et al.*, (2009). The non-protein thiols are composed of various acid soluble sulfhydryl-components like cysteine, glutathione, phytochelatin (PCs) and g-glutamylcysteine (De Vos *et al.*, 1993). Since the phytochelatin are well known for binding with the toxic metals like Cd and Cu, the induction of PCs synthesis is considered to provide plant tolerance towards metal toxicity (Cobbett, 2000). Increase in the non-protein thiols were observed in the present study under Cu stress in *Brassica* plants. These results were in accordance to the Wang *et al.*, (2004) who observed enhancement in the non-protein thiol content under Cu stress in *B. juncea* plants. Aly and Mohamed, (2012) reported a significant increase in the total thiols and protein thiols while gradual decrease in non-protein thiols was observed in maize plants. Tari *et al.*, (2002) also noticed change in the level of thiols content in roots of wheat plants exposed to Cu. Strong increase in the non-protein thiols were recorded in the roots of maize plant under Cd and Zn metal stress. Application of CS as seed pre-soaking further enhanced the thiols content in plants in the present

investigation. This increase in thiols content with CS application shows BR induced tolerance in plants under Cu stress.

5.2.11 Elemental analysis

In the present investigation, contents of C, P, O, Ca and K were decreased in *Brassica* plants grown in soil contaminated with Cu, whereas the contents of S, Na, Mg and N increased. Enhanced sulfur uptake in maize roots was observed under copper contamination (Nocito *et al.*, 2006). The up-regulation of sulfur transporters due to increased demand of S under Cu toxicity could also be the reason behind increased content of S (Shahbaz *et al.*, 2010). Various heavy metals (Pb, Cd, Ni) increase Na content in the shoots of *Vicia faba* (Karimi *et al.*, 2013). Cu ions in divalent form bind with the sulfhydryl group and cause damage to root membranes. Due to it, permeability of root cells change and lead to leakage of K ions and diffusion of Cu ions (Siedlecka, 1995). Presence of other cations in soil, decrease the uptake of Ca ions. It has been observed that under Cu stress, Mg content was increased in *Brassica* plants. This has been reported that Mg reduce the development of lateral root hairs in *Arabidopsis* plants. In the present study, combination of Cu with CS improved the contents of Mg, C, O, Ca and K. The levels of S decreased under CS treatment. Decrease in S content might be due to enhanced synthesis of glutathione and thiols. Normal uptake of elements is restored by application of BRs, because of BRs role in cell membrane integrity under adverse conditions. Foliar spray of EBL and HBL under salinity stress increased Ca content while reduced Na content in comparison to control plants (Vardhini *et al.*, 2012).

5.2.12 Change in NO levels

In the present investigation, increase in the nitric oxide (NO) levels has been observed. Similar accumulation of NO under Cu stress has been reported by Zhang *et al.*, (2008) in *Chlamydomonas reinhardtii*. NO is biologically active molecule and has appeared as an important plant signalling molecule under various biotic and abiotic stresses including heavy metals (Hsu and Kao, 2004; Wang and Yang, 2005). NO effects the accumulation of ROS and modulate components of antioxidative system (Laspina *et al.*, 2005). NO has been reported to behave as antioxidant in perspective to Cu, Cd and Al toxicity (Singh *et al.*, 2009). NO also controls Ca²⁺ homeostasis in plants

by changing the activity of plasma membrane and other intercellular Ca^{2+} permeable channels (Besson-Bard *et al.*, 2008). NO also enhances the activity of various enzymes like CAT, SOD, and APOX (Zhou *et al.*, 2005; Zhang *et al.*, 2007). Increased activity of antioxidative enzymes is directly related to enhanced tolerance of plants towards abiotic stresses. NO interact with ROS in many ways and can also block ROS-induced lipid peroxidation (Laspina *et al.*, 2005; Wang and Yang, 2005). NO also induce the production of various phytohormones including ABA. The NO content further increased in the combination of Cu with CS in *Brassica* plants. These results are in accordance to the Cui *et al.*, (2011) who observed enhanced NO content in cucumber after treatment with 24-EBL. This shows that NO plays role in the induction of plant stress tolerance by BR.

5.2.13 Stomatal Closure

Cu stress induced closing of stomata in the leaves of 7 days old *Brassica* seedlings. Inhibition in the movement of stomata in *Vicia faba* leaves with exposure to Hg and La has been reported by Yang *et al.*, (2004). It has been proposed that the reason behind this might be blocking of water channels by heavy metals or due to disturbed activities of ion channels. The enhanced production of NO has been observed in this study. The closing of stomata is also linked with the enhanced production of NO and ABA under stress (Neill *et al.*, 2008). In the present work, the leaves of seedlings grown from seeds soaked in CS and raised in Cu containing growth media showed open stomata. Xia *et al.*, (2014) described that stomatal movements are controlled by BRs and thus, any modulation in BRs biosynthesis cause reduced stomatal aperture.

5.3 Conclusion and Future prospects

In the present study, it was observed that plants grown in copper have reduced photosynthesis and growth due to enhanced oxidative stress induced by accumulation of reactive oxygen species. Application of castasterone as seed pre-soaking resulted in up-regulation of antioxidative defence system by enhancing the activities of various antioxidative enzymes (SOD, POD, CAT, APOX, GR, GST, GPOX, PPO) and non-enzymatic antioxidants (glutathione, ascorbic acid, total water soluble antioxidants and total lipid soluble antioxidants). The production of other stress molecules like proline, sugars, phenols, flavonoids, anthocyanin etc. also increased. Thus, application of

castasterone as seed pre-soaking method might help in the mitigation of adverse effects of Cu on photosynthesis and growth. Stress amelioration by castasterone has been confirmed by stomatal studies done with scanning electron microscopy and cell death studies carried out with confocal microscopy. This elaborative study helped in understanding the role of castasterone in plant tolerance towards copper stress. It provide a baseline data for molecular and genetics approaches to define the mechanism of castasterone action in plants. As BRs are natural and eco-friendly compounds and are effective at very low doses, these may be employed in plant stress protection strategies.