CHAPTER-2

LITERATURE REVIEW

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The role of copper in plants depends greatly on its concentration. Copper in trace amounts is an essential micronutrient for algae and higher plants for its role as a cofactor for metabolic processes like photosynthesis, respiration, carbohydrate distribution, nitrogen fixation, protein metabolism, ethylene perception, oxidative stress reduction, cell expansion and cell-wall lignification. At higher concentrations, copper can induce several negative effects including generation of reactive oxygen species, exchange of essential metal ions from the active sites and visible symptoms such as chlorosis, necrosis and growth inhibition (Marschner, 1995; Prasad, 2004). A well coordinated procedure of uptake, buffering, translocation and storage processes is necessary to uphold essential concentrations of the metal in various tissues and compartments within the narrow physiological limits (Clemens *et al.*, 2002).

The aim of this review is to summarize the toxic effects of Cu^{2+} and focus on the recent developments on the various underlying metabolic changes that bring about such toxic effects. We also focus on tea, which is the most popular drink in the world after water. Tea (*Camella sinensis* (L.) O. Kuntze) is a perennial evergreen plantation crop with productivity round the year. The harvest includes tender shoots that are plucked normally at one to three weeks interval. This induces further vegetative growth and ensures continuous supply of green flushes (Burgess and Carr, 1997; Karmakar and Banerjee, 2005). Fungal pathogens such as *Exobasidium vexans* are capable of infecting the pluckable tender leaves thereby warranting a regular spraying of copper fungicides in heavy doses especially during the six month long monsoon period (May-October) when fungal infections assume massive proportions. This causes a buildup of Cu^{2+} in the soil over the years and the concentration of Cu^{2+} can easily overcome the threshold limit for toxicity.

2.1 Heavy metal stress

Plants are contaminated by heavy metals due to the consequences of heavy metal pollution in soil, water and air. Heavy metals make a significant contribution to environmental pollution as a result of human activities such as mining, smelting, electroplating, energy and fuel production, power transmission, intensive agriculture, sludge dumping and military operations. They present a risk for primary and secondary consumers and ultimately humans (Zeller and Feller, 1999). Heavy metals have a molecular mass >5.0 g cm^{-3} which was distinctly higher than the average particle density of soils (2.65 g cm^{-3}). Several heavy metals such as iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), cobalt (Co), or molybdenum (Mo) are essential for the growth of organisms (Bothe, 2011). According to Nedelkoska and Doran (2000) heavy metals like As, Cd, Co, Cu, Ni, Zn, and Cr are phytotoxic either at all concentrations or above certain threshold levels. The inhibitory effect of heavy metal compounds on growth and the performance of photosynthetic apparatus of plants were examined by several authors from time to time. There are two aspects on the interaction of plants with heavy metals: (i) heavy metals produce negative effects on plants and (ii) plants have their own resistance mechanisms against toxic effects and for detoxifying heavy metal pollution (Cheng, 2003).

Heavy metal toxicity is one of the major abiotic stresses leading to hazardous health effects in animals and plants. Because of their high reactivity, they can directly influence growth, senescence and energy synthesis processes. Their mechanism is connected with the generation of reactive oxygen species and jasmonate and ethylene signaling pathways and shows that toxicity symptoms observed in plants may result from direct heavy metal influence as well as the activity of some signaling molecules induced by the stress action (Maksymiec, 2007). Beside these, a heavy metal in excess stimulates the formation of free radicals that directly damages the cellular macromolecules such as lipid, protein and nucleic acids (Dietz *et al.*, 1999). Chlorophyll synthesis mechanism was also found to be interfered by heavy metals either through direct inhibition of an enzymatic step or by inducing deficiency of an essential nutrient (Van Assche and Clijsters, 1990).

2.2 Copper in plants

Cu participates in many physiological processes because of its ability to exist in multiple oxidation states *in vivo*. Under physiological conditions Cu exists as Cu^{2+} and Cu^{+}. The cation Cu^{2+} is often bound by nitrogen in histidine side chains, whereas Cu^{+} prefers interaction with the sulphur in cysteine or methionine. Copper is transported into the plant cell by COPT family of transporters on the plasma membrane which has been described as a group of highly hydrophobic proteins; all its members contain 3 trans-membrane domains and specific Cu^{2+} binding site rich in methionine and histidine residues at the amino terminus (Kampfenkel *et al.*, 1995; Sancenon *et al.*, 2003; Andres-Colas *et al.*, 2006). Copper homeostasis is maintained inside the cell by copper chaperones which sequester copper to a non-reactive form and also interact with other transport proteins for delivering copper to its necessary destinations (Himelblau and Amasino, 2000; Company and Gonzalez-Bosch, 2003; Chu *et al.*, 2005). Two P-type ATPases, PAA1 and PAA2, are required for efficient copper delivery across the plastid envelope and the thylakoid membrane, respectively, in *Arabidopsis* (Shikanai *et al.*, 2003; Abdel-Ghany *et al.*, 2005). Inside the root, Cu^{2+} is said to be strongly accumulated in the cortex and the concentration decreases sharply from the outer to the inner cell layers (Arduini *et al.*, 1996; Ducic and Polle, 2005). Copper is poorly translocated by xylem and thus uptake by shoots is very low (Liao *et al.*, 2000).

One of the major sites of copper accumulation in plants is the chloroplast. This metal is directly involved as a component of plastocyanin (PC) in the photosynthetic electron transport chain. PC is one of the most abundant proteins of thylakoid lumen (Kieselbach *et al.*, 1998) and is essential for electron transfer between the cytochrome b_6f complex and photosystem II (Weigel *et al.*, 2003). The metal has a distinct regulatory role in electron transport between the photosystems as the constituent of PC (Maksymiec, 1997). In the chloroplast stroma, Cu/Zn superoxide dismutase (SOD) requires Cu^{2+}, along with Zn, as cofactors to catalyze the dismutation of superoxide radicals (O_2^{-}) threby forming H_2O_2 and O_2^{-}. In *Arabidopsis thaliana*, out of seven identified SOD genes, the most active CSD1 and CSD2 genes both encode a Cu/Zn-SOD with CSD1 activity in the cytosol and

CSD2 activity in the stroma (Kliebenstein *et al.*, 1998). Polyphenol oxidase is another Cu^{2+} protein found in the thylakoids of some plants, such as spinach (Kieselbach *et al.*, 1998), but not in other species such as *A. thaliana* (Schubert *et al.*, 2002). The enzyme has been proposed to be involved in the photoreduction of O_2^- by PS I (Vaughn *et al.*, 1988). Cu^{2+} mediates the activity of several other enzymes such as ascorbate oxidase which catalyses the reduction of O_2^- to water. The enzyme contains 8 Cu^{2+} ions which participate in the transfer of electrons in presence of ascorbate, the reducing substrate (Maksymiec, 1997). Other important Cu containing proteins within plant cells include the mitochondrial cytochrome-C oxidase enzyme, the ethylene receptors in the endomembrane system and various apoplastic oxidases (Pilon *et al.*, 2006). Copper is also necessary for amine oxidase function where it catalyses oxidative deamination of polyamines with the simultaneous formation of aldehyde, ammonia and H_2O_2 (Maksymiec, 1997).

2.3 Copper as a toxic element

Inspite of the indispensability of copper in plant metabolism, excess copper has strong toxic effects. Copper can be limiting to plant productivity in crops when below 5 µg g^{-1} dry weight (DW), whereas toxicity is reported above 30 µg g^{-1} DW (Marschner, 1995). The most common feature of copper toxicity is the decrease in mass of roots. Copper toxicity can be damaging to plant roots, with symptoms ranging from disruption of the root cuticle and reduced root hair proliferation, to severe deformation of root structure (Sheldon and Menzies, 2005; Lequeux *et al.*, 2010). Cu^{2+} is toxic to plant cell which lead to plant retardation and leaf chlorosis (Rhoads *et al.*, 1989; Yadav, 2010). High Cu^{2+} concentrations predisposes photosystem II to photoinhibition (Patsikka *et al.*, 2002) and causes reduction in chlorophyll content arising from partial destruction of grana and modification of the protein-lipid composition of thylakoid membranes (Lidon and Henriques, 1991; Maksymiec, 1997). Copper toxicity can also result in significant alteration in the concentration of minerals such as Fe, Mg, Ca, Zn, K and Na in both root and shoot (Lidon and Henriques, 1993; Lequeux *et al.*, 2010).

Copper is relatively abundant in the earth's crust and better soluble, therefore more mobile than other heavy metals in the surface environment

(Flemming and Trevors, 1989). Copper concentration in non-polluted soils range from 10 to 80 ppm Cu^{2+} but soils located near mining areas or metal-processing industries may be contaminated by very large amounts of Cu^{2+} (Hagemeyer, 2004). The bioavailability is determined by the form taken by the metal (ionic, complex or precipitated) which depends on environmental factors and therefore, varies widely, giving rise to possible conditions of toxicity (Flemming and Trevors, 1989; Greger, 2004). The level of bioavailable copper is increased by human activities which either increases the abundance or causes changes in soil chemistry thus affecting the solubility (Rhoads *et al.*, 1989; Flemming and Trevors, 1989). In the soil, copper remains immobilized onto the organic materials such as fulvic and humic acids and to clay and mineral surfaces. The bioavailibility in soil is strongly dependent on factors such as pH, cation exchange capacity (CEC), clay content, water hardness and organic matter content (Flemming and Trevors, 1989; Greger, 2004; Rooney *et al.*, 2006). Low pH increases the metal availability since the hydrogen ion has a higher affinity for negative charges on the colloids, thus competing with the metal ions of these sites, therefore releasing metals (Greger, 2004). Rhoads *et al.* (1989) found that growth of tomato plants was reduced at soil pH below 6.5 with soil-copper levels above 150 mg. Thus soil properties have a significant impact in the expression of toxicity of copper in plants.

According to Brun *et al.* (2001) agricultural soil in many parts of the world are contaminated by heavy metals. The use of Bordeaux mixture for almost one century against vine downy mildew has caused severe copper contamination of soil in many wine-producing regions (Van-Zwieten *et al.*, 2004). Copper contamination also caused serious problems in cereals such as rice (Lidon *et al.*, 1993), wheat (Lanaras *et al.*, 1993) and barley (Vassilev *et al.*, 2003). Graham *et al.* (1986) found that excess fungicidal copper reduced seedling growth in citrus and also inhibited colonization of the roots by mycorrhizal fungus. In citrus orchards, stunted trees were produced with less mycorrhizal colonization under higher Cu concentrations and low pH (<5) conditions of the soil. In India, the major tea cultivation area comprises the eastern sub-Himalayan region where the soil is mainly acidic (pH 4.2-5.8) in nature (Singh and Singh, 2006). While this is good for tea cultivation (Sarkar, 1994), but it increases the possibility of Cu^{2+} ions accumulated in

the tea garden soils to become more available for absorption by plants which may lead to toxicity.

PSII is the most sensitive site for Cu toxicity, thereby affecting photosynthetic electron transport system (Droppa and Horvath, 1990; Baron *et al.*, 1995). The most apparent affect of Cu toxicity on PSII is the inhibition of oxygen evolution accompanied by quenching of variable fluorescence (Mohanty *et al.*, 1989). Both the acceptor and the donar sides of PSII were affected under copper toxicity. Vassilev *et al.* (2003) reported the effects of excess Cu on both growth and photosynthetic performance of twenty days old barley (*Hordeum vulgare*) plants with treatments like 0, 10, 15 and 20 mg kg^{-1} sand for ten days. Their results indicated that stomata conductance and photosynthetic electron transport linked to PS-II + OEC exhibited the highest sensitivity to excess Cu, followed by plant dry weight accumulation, leaf area formation, net photosynthetic rate and photosynthetic electron transport linked to PS-I and PS-II-OEC.

Gonzalez-Mendoza *et al.* (2013) observed that photosynthesis and chlorophyll fluorescence measured after 30 h of copper stress in *Avicennia germinans* using two $CuSO_4$ concentrations (0.062 and 0.33 M) in Hoagland's solution resulted in a general reduction of the stomatal conductance (28 and 18% respectively) and 100% of inhibition of net photosynthesis. Wang *et al.* (2013) investigated the effect of copper stress (10 µM, 100 µM and 1 mM) in two cultivars of Chinese cabbage and found that 1mM copper significantly inhibited the photosynthesis and plant growth of both cultivars. Copper stress caused damage of photosystem resulting in reduction of PSII efficiency while non-photochemical-quenching parameter NPQ increased. Besides, at low concentration with 10µM, copper promoted the photosynthesis and plant growth. In another study, Kalaikandhan *et al.* (2014) observed that various pigments like chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content of *Sesuvium portulacastrum* increased at low levels (copper, 100-200 mg kg^{-1}) and decreased further with an increase in the soil copper levels (300-600 mg kg^{-1}).

Wang *et al.* (2004) observed that the application of 8µM Cu caused 50% reduction in biomass production in treated roots as compared to control. Sheldon and Menzies (2005) reported that the copper toxicity damaged the

plant roots of Rhodes grass (*Cloris gayana*), with symptoms ranging from disruption of the root cuticle and reduced root hair proliferation to severe deformation of root structure. An external concentration of 4 µM caused a reduction in root growth, with damage evidence resulting from an external concentration of 0.2 µM. Sfaxi-Bousbih *et al.* (2010) reported the relationships among excess copper treatment in seeds of bean (*Phaseolus vulgaris*) with germination rate, dry weight, sugar contents and carbohydrate activities in cotyledons. Heavy metal stress provoked a diminution in germination rate and biomass mobilization, as compared with control. A drastic disorder in soluble sugar export, especially glucose and fructose liberation, was also imposed after exposure to excess copper.

Singh *et al.* (2007) performed a study on the seedling growth in wheat under the influence of different concentrations of copper. The germination percentage, plumule and radicle length, and number of lateral roots decreased with increase in copper concentration (5, 25, 50, 100 mg g^{-1}). According to Khurana and Chatterjee (2007) excess copper (100 µM) in carrot induced interveinal chlorosis on upper leaflets. Excess copper caused morphological changes and poor development of root. Reduction in carrot yield and deterioration of its quality, nutrition value by reducing the concentration of sugars, starch, protein, protein nitrogen, carotene and increase of the non-protein nitrogen and phenols in carrot root were reported by them. Manivasagaperumal *et al.* (2011) made an attempt to study the influence of copper on growth of greengrams (*Vigna radiate*) plants. Copper sulphate was applied to the soil in different concentrations (0, 50,100, 150, 200, 250 mg kg^{-1}) in which the greengrams plants were grown. The plant samples were analyzed 45 days after sowing. The results indicated that moderate to high copper concentrations (100-250 mg kg^{-1}) decreased the growth, dry matter production and nutrient content of green gram in a glass house earthen pot experiment. Kumar *et al.* (2012) observed a decline in growth, chlorophyll contents, protein and carbohydrate content, and DNA and RNA content. Further, the proline, total phenol and H_2O_2 content increased at high concentration of Cu. Filova *et al.* (2013) examined the sensitivity of the 6 sunflower cultivars to copper ions and found that level of lipid peroxidation was increased by 11% and 30% in two different cultivars. Rout *et al.* (2013) investigated the effect of copper (0, 25, 50, 100

and 200 µM) in *Withania somnifera* seedlings for 7 and 14 days in MS media. The growth parameters (root length, shoot length, leaf length and total number of leaves per plant) showed a declining trend in the treated plants in a concentration dependant manner. In plant samples grown with 25 and 50 µM of Cu, a rapid increase in antioxidant activities were reported but at higher concentration (100 and 200 µM), the activities declined. Isoforms of CAT, SOD and GPX were examined and concentration specific new isoforms were reported. The authors suggested that isoforms of the antioxidant enzymes synthesized due to Cu stress was useful as biomarkers for other species grown under metal stress. In another study on the same plant grown in a field pot experiment, Singh *et al.* (2014) observed that increasing Cu^{+2} concentrations (0, 10, 20, 50, 100 and 200 mM) led to decreased stem length, root elongation and leaf area, decreased chlorophyll and carotenoids content and increased lipid peroxidation in different plant parts. They further reported that phenol content of leaves, stems and roots peaked at 50 mM Cu^{+2} treatment and thereafter declined.

2.4 Copper in tea gardens

An example of an industry in India which depends primarily on copper fungicides is the tea industry. India is second only to China in tea production and the largest consumer of tea in the world. Currently, India produces 23% of total world production. It is the second largest industry in terms of employment and generally controls the economies of the regions where the tea gardens are clustered. This is common in the north-eastern states of India (Fig. 2.1 and 2.2) especially in Assam and sub-Himalayan West Bengal (Selvakumar and Jeyaselvam, 2012). Tea plants are cultivated extensively as large plantations where it is often allowed to grow under variant soil and climatic condition thereby making them prone to attacks by fungal pathogens. Perennial habit of the tea plant and warm humid climate of the tea growing areas are highly conducive for disease development (Hajra, 2001). Major diseases include blister blight, brown blight, grey blight and black rot in leaves, and branch canker, thorny blight and pink disease in stems. Most of the tea diseases are of fungal origin and only a few are caused by bacteria, algae and viruses. Chen and Chen (1990) described nearly 400 pathogens that attack tea plants in varying intensities.

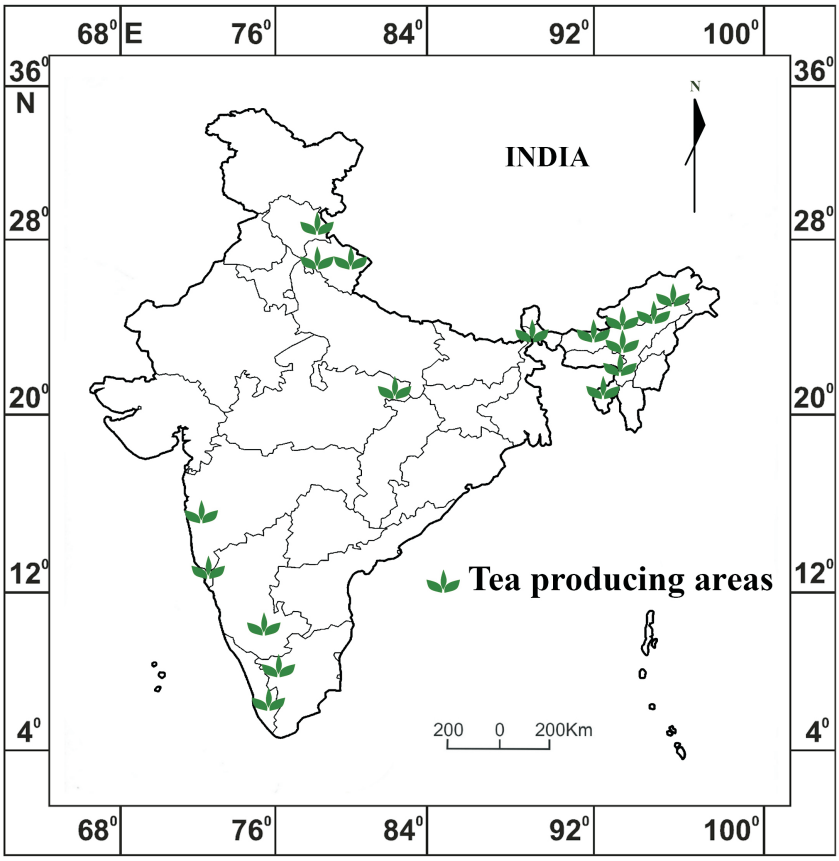

Fig. 2.1: Tea growing regions of India.

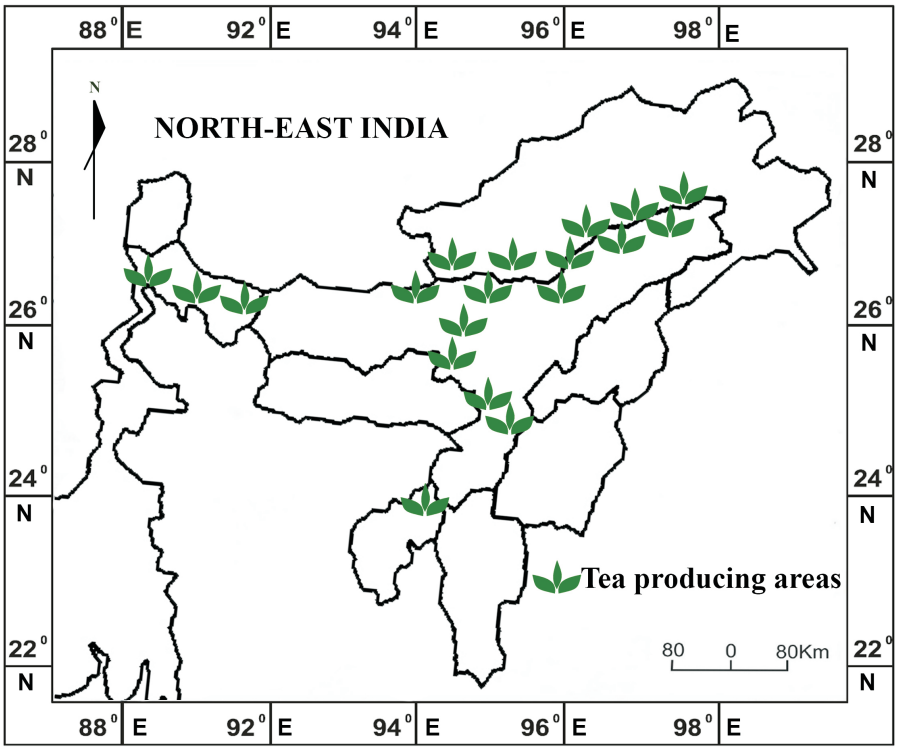

Fig. 2.2: Tea growing regions of North East India.

Sometimes the disease symptoms get manifested as debilitation, defoliation and leads to death of the bushes. The economic loss of tea due to diseases is generally higher compared to losses due to animal pests (Lehmann-Danzinger, 2000). Hence, the microclimatic conditions and monoculture habitats invites a wide range of phytopathogens which leads to severe losses which was estimated to be around 25-33% during 2001 to 2010 (Ponmurugan *et al.*, 2010).

To control the diseases, copper based fungicides (Table 2.1) are used excessively in tea gardens of North East India including Assam and sub-Himalayan West Bengal (Barua, 1988). The fungicides that are used most commonly include basic copper sulphate, Bordeaux mixture (a combination of hydrated lime and copper sulphate), Bicoxy (a new formulation of copper oxychloride 50% WP) and various customized formulations of copper sulphate and copper oxychloride (Worthing, 1983; Singh, 2005). A survey covering several tea gardens of the Darjeeling and adjoining Jalpaiguri district of sub-Himalayan West Bengal conducted at the onset of the current study has revealed that copper-fungicides are extensively used in the tea gardens of the Dooars and Terai region and also in the hilly regions of West Bengal. Copper based fungicides are used in large scale because they have multisite activity with a low risk of pathogens developing resistance (Van-Zwieten *et al.*, 2004) and are relatively less phytotoxic than Ni based fungicides. In fact, copper based fungicides are highly recommended in literature and are often regarded as the most efficacious and economic fungicide for controlling the foliar diseases of tea (Singh, 2005).

Table 2.1: Inorganic copper compounds used as fungicides

Name	Chemical formula	Uses
Cupric sulfate	$CuSO_4.5H_2O$	Seed treatment and preparation of Bordeaux mixture
Copper dihydrazine Sulfate	$CuSO_4(N_2H_5)_2SO_4$	Control of powdery mildew
Copper oxychloride	$3Cu(OH)_2.CuCl_2$	Control of powdery mildew
Copper zinc chromates	$15CuO\text{-}10ZnO.\ 6CrO_3.\ 25H_2O$	Control of many fungal diseases.
Cuprous oxide	Cu_2O	Control of powdery mildew
Basic copper sulfate	$CuSO_4.Cu(OH)_2.H_2O$	Seed treatment and preparation of Bordeaux mixture
Cupric carbonate	$Cu(OH)_2.CuCO_3$	Many fungal diseases
Copper hydroxide	CuH_2O_2	Many fungal diseases

2.5 Mechanisms of Cu^{2+} toxicity

Copper is a redox active metal with an electrochemical potential of -260V. The redox nature of Cu^{2+} ions makes it very useful as a cofactor in electron transfer reactions (Ducic and Polle, 2005). However, the reversible oxidation–reduction property of Cu^{2+} could also result in oxidative stress if Cu^{2+} would be present as a free ion. Heavy metals in general have been recognised as a major toxicant in plant cells due to their capability of generating reactive oxygen species (ROS) such as hydroxyl radical ($OH^{\bullet}$) superoxide (O_2^{-}) and hydrogen peroxide (H_2O_2), which can damage the biomolecules such as membrane lipids, proteins and nucleic acids. During the reduction of oxygen to water, ROS may be produced by a chain of reactions which initially need energy input but subsequently occur spontaneously. O_2^{-} is a short-lived and moderately reactive ROS which reduces quinines and transition metal complexes of Fe^{3+} and Cu^{2+} thereby affecting the metal containing transporters and enzymes. O_2^{-} can additionally combine with protons in aqueous medium and form hydroperoxyl radicals ($HO_2^{\bullet}$) which can induce lipid auto-oxidation in membranes (Shaw *et al.*, 2004). H_2O_2 is relatively long-lived and moderately reactive which oxidises the thiol groups of some enzymes (e.g. enzymes of the Calvin cycle and Cu/Zn-SOD) and inactivates them (Vranova *et al.*, 2002). However, the most reactive of all the ROS is the hydroxyl radical ($OH^{\bullet}$) which can potentially react with all types of biomolecules and in excess can cause cell death because cells do not have any enzymatic antioxidant system to quench it. The radical is formed from H_2O_2 by the Haber Weiss and Fenton reactions and Cu^{2+} being a redox active metal catalyzes the formation of this most harmful active radical (Arora *et al.*, 2002; Vranova *et al.*, 2002) as summarized below:

$$H_2O_2 + O_2^{-} \xrightarrow[\text{Haber Weiss reaction}]{Cu+,\ Cu2+} OH^{-} + OH^{\bullet} + O_2^{-}$$

One of the richest sources of ROS in plants is the chloroplast. These can be formed due to the highly energetic electron transfer reactions triggered by chlorophyll excitation along with an excess supply of oxygen. Singlet oxygen ($^1O_2$) can be formed during de-excitation of chlorophyll which causes major oxidative damage to biomolecules. High light intensity can cause over reduction of PSI and generation of excessive NADPH which cannot be utilized

by the CO_2 fixation process thereby reducing the $NADP^+$ pools. O_2^- which is abundant in the chloroplast, can take up electrons from PSI in such a situation, which leads to production of ROS through the Mehler reaction (Sharma *et al.*, 2012). Under conditions of low CO_2 fixation such as cold temperature or low CO_2 availability, excess reduction of PSI and increase in ROS levels can occur even at moderate light intensities. As H_2O_2 or O_2^- are only moderately reactive, therefore, the main responsible factor for the intense biological damage is the metal ion which catalyzes the formation of the highly toxic hydroxyl free radical ($OH^\bullet$) from H_2O_2 (Maksymiec, 1997). Thus ROS may be generated in the plant due to several abiotic as well as biotic causes but true damage is caused by the additional metal toxicity.

The hydroxyl radical ($OH^\bullet$) can either add onto the biological molecules or eliminate hydrogen from them by forming water. The hydroxylated biomolecules can in turn hydroxylated other molecules thereby initiating a chain of reaction or change to stable oxidized products. The activated hydroxylated molecules can also dismute themselves by forming intermolecular cross links (Shaw *et al.*, 2004). Oxidized Cu^{2+} ions can be actively involved in electron transfer during formation of stable oxidized products. In reactions where the $OH^\bullet$ radical eliminates H from biomolecules, it leaves an unpaired electron in the organic molecule thereby forming a reactive organic radical which can then react with oxygen to form peroxy radical ($ROO^\bullet$). The peroxy radical is again a reactive species and can eliminate hydrogen from other biomolecules and change them into organic radical products thereby creating a chain of reactions. The peroxidation reaction is evident in lipid peroxidation reactions that take place in cell membranes to form lipid peroxides (ROOH) (Shaw *et al.*, 2004; Arora *et al.*, 2002). However, in presence of reduced Cu^{2+} ions which can participate in Fenton reaction (shown below), the highly reactive alkoxy radical (RO^-) is formed from the ROOH which is as damaging as the hydroxyl radical thus opening up another cascade of immensely damaging oxidative reactions.

$$ROOH + Cu^+ \longrightarrow OH^- + Cu^{2+} + RO^-$$

Two common sites of ROS attack on the phospholipid molecules are the unsaturated (double) bond between two carbon atoms and the ester linkage between glycerol and the fatty acid. The polyunsaturated fatty acids (PUFAs)

present in membrane phospholipids are particularly sensitive to attack by ROS. A single •OH can result in peroxidation of many polyunsaturated fatty acids because the reactions involved in this process are part of a cyclic chain reaction. A single initiation event thus has the potential to generate multiple peroxide molecules by a chain reaction (Gill and Tuteja, 2010). The overall effects of lipid peroxidation are to decrease membrane fluidity; make it easier for phospholipids to exchange between the two halves of the bilayer; increase the leakiness of the membrane to substances that do not normally cross it other than through specific channels and damage membrane proteins, inactivating receptors, enzymes, and ion channels (Sharma *et al.*, 2012). A study on the toxicity mechanisms suggest that the generation of reactive oxygen species is a natural phenomenon but is increased to alarming proportions due to presence of stress factors. Presence of Cu^{2+} ions above the threshold limit is immensely stressful to plants due to its redox nature as it can catalyze and enhance the formation of all types of ROS by participating actively in several types of oxidative reactions.

2.6 Plant response to Copper toxicity

Plants have developed a wide range of protective mechanisms for mitigating copper toxicity. Primary defence mechanisms prevent metal to enter into the cell via exclusion, or binding of metal to cell wall and other ligands, organic acids, amino acids, glutathione (GSH) or phytochelatins (PCs) to render them harmless (Antosiewicz and Wierzbicka, 1999; Gao *et al.*, 2008). Antioxidative mechanisms that control the level of ROS and shield the system before the sensitive parts of the cellular machinery gets damaged are mediated by molecules which have been broadly divided into two types, the high molecular weight enzymatic catalysts and the low molecular weight antioxidants (Pinto *et al.*, 2003). The enzymes involved in scavenging ROS include superoxide dismutases (SOD), catalase (CAT), peroxidases (POD) and glutathione peroxidase and those involved in detoxifying lipid peroxidation products include glutathione-S-transferases (GST), phospholipid-hydroperoxide glutathione peroxidase and ascorbate peroxidase (APX). Besides these, a whole array of enzymes is needed for the regeneration of active forms of the antioxidants such as monodehydroascorbate reductase (MDAR), dehydroacscorbate reductase (DHAR) and glutathione reductase

(GR) (Blokhina *et al.*, 2003; Pinto *et al.*, 2003). At least four of them participate in a highly developed detoxification system named the ascorbate glutathione cycle (Halliwell-Asada cycle) (Bartosz, 1997). The low molecular weight compounds that act as cellular antioxidants are ascorbate, glutathione, phenolics, flavonoids, carotenoids and tocopherols. Non-enzymatic scavengers are essential in the protection of cellular components from most ROS, but they cannot cope with reducing radicals such as superoxide or metastable hydroperoxides (Chaudiere and Ferrari-Iliou, 1999).

2.6.1. Binding of copper and its sequestration

Plant adapt to heavy metal stress by acquiring several strategies, the most prominent being the synthesis of phytochelatins and metallothioneins which contribute to metal detoxification by chelation of the metal ions. Phytochelatins are simple thiol rich metal binding peptides containing glutamate, cystein and glycine in ratios of 2:2:1 to 11:11:1 (Grill *et al.*, 1985; Prasad, 2004). They are $(\gamma\text{-glutamylcysteinyl})_n$- glycines with n = 2 to 11, (Grill *et al.*, 1985; Steffens, 1990). These peptides are synthesized non-translationally from glutathione in the presence of heavy metals by the enzyme phytochelatin synthase (Grill *et al.*, 1989). Apart from being a precursor to phytochelatins, glutathione is also an important antioxidant molecule, which plays a predominant role in protection against free radicals (Alscher, 1989). Copper induced increase in phytochelatin synthesis results in oxidative stress through the depletion of the antioxidant glutathione. Phytochelatin production induced by copper was reported to be accompanied by an increase of total glutathione in maize (Tukendorf and Rauser, 1990). De Vos *et al.* (1992) showed that copper tolerance in the plant species *Silene cucubalus* does not depend on the production of phytochelatins but is related to the ability of this plant to prevent glutathione depletion resulting from copper-induced phytochelatin production.

Metallothioneins are low molecular weight proteins with high cystein content, which bind metal ions to form metal thiolates and metal thiolate clusters. Class III metallothioneins are found in plants and is reported to be induced by the presence of a variety of metals including Cd, Cu, Zn, Pb, Hg and Ag (Hamer, 1986; Prasad, 2004). However, phytochelatins rather than metallothioneins are mainly responsible for detoxification of toxic heavy

metals (Yadav, 2010). Moreover, metal binding ability is higher in phytochelatins than in metallothioneins on a per-cysteine basis (Mehra and Mulchandani, 1995). In addition, phytochelatins possess the ability to scavenge ROS and thereby aid in mitigating oxidative stress (Tsuji *et al.*, 2002).

Phytochelatins are thus the major if not the only thiol-rich compounds induced in metal-exposed plants (Grill *et al.*, 1985; Steffens, 1990), although it has been reported that copper induces metallothionein-like compounds as well (Tomsett *et al.*, 1989). Phytochelatins probably play a central role in the homeostatic control of metal ions in plants (Steffens, 1990). They may also be involved in the physiological mechanism of metal tolerance of selected cell lines and intact plants (Steffens *et al.*, 1986; Tomsett *et al.*, 1989).

2.6.2 Antioxidant response through enzymatic antioxidants

Plants possess well developed defense system against ROS which restricts its formation and maneuver its removal. The antioxidant system comprises several enzymes such as superoxide dismutase, catalase and peroxidases which play a crucial role to protect plants from oxidative damage. It is widely accepted that the amount and activities of these antioxidant enzymes determine the degree of tolerance in plant (Teisseire and Guy, 2000). The importance of antioxidant enzymes is in their ability to scavenge ROS and thereby prevent oxidative damage. The balance between ROS generation and eradication determines the survival of the system (Khatun *et al.*, 2008; Golshan *et al.*, 2011). There has been a large body of literature describing the role of these antioxidant enzymes in managing stressful conditions in plants. Table 2.2 enlists several enzymes that have been reported in literature as having antioxidant potential or play prominent role in alleviating environmental stress. The list is selective rather than comprehensive and is limited to the findings published in the last 20 years.

2.6.2.1 Superoxide dismutase (SOD)

Inside the plant cell, superoxide dismutases (EC 1.15.1.1) provide the first line of defence against ROS. The enzyme is located in different cell compartments including mitochondria, chloroplast, glyoxisomes, peroxisomes, microsomes, apoplast and cytosol (Alscher *et al.*, 2002) and

catalyzes the disproportionation of O_2^- to H_2O_2 and molecular oxygen (Scandalios, 1993). SOD enzymes are classified into three known types based on the metal cofactors: the Cu/Zn-SOD, the Mn-SOD and Fe-SOD (Bowler *et al.*, 1994). The distribution of SOD isoenzymes is also distinctive. The Cu/Zn-SOD is found in the cytosolic fraction and also in chloroplasts in higher plants. The Fe-SOD isozymes, often not detected in plants (Ferreira *et al.*, 2002) are usually associated with the chloroplast compartment when present (Alscher *et al.*, 2002). In *Arabidopsis thaliana* genome, three Fe-SOD genes (FSD1, FSD2 and FSD3), three Cu/Zn-SOD genes (CSD1, CSD2 and CSD3), and one Mn-SOD gene (MSD1) have been reported (Kliebenstein *et al.*, 1999). The Mn-SOD is found in the mitochondria of eukaryotic cells and in peroxisomes (Del Rio *et al.*, 2003). Although each type of SOD predominates in specific cell compartments, their occurrences are not restricted, and all types can be detected in most of the cellular locations (Arora *et al.*, 2002). An increased level of SOD has been correlated to enhanced oxidative stress protection in plants (Sen Gupta *et al.*, 1993). The SODs remove O_2^- by catalyzing its dismutation, one O_2^- being reduced to H_2O_2 and another oxidized to O_2 (Table 2.2). It removes O_2^- and hence decreases the risk of OH^- formation via the metal catalyzed Haber Weiss - type reaction. This reaction has a 10,000 fold faster rate than spontaneous dismutation. The up regulation of SODs has been observed in plants subjected to both abiotic (Boguszewska *et al.*, 2010) and biotic stresses (Torres, 2010). Over expression of SODs in transgenic plants resulted in higher stress tolerance (Badawi *et al.*, 2004). Thus, SOD_S have a critical role in the survival of plants under environmental stresses.

Increase in SOD activity has been reported against copper induced stress in several plants such as *Arabidopsis thaliana* (Drazkiewicz *et al.*, 2004); *Prunus cerasifera* (Lombardi and Sebastiani, 2005); *Elsholtzia haichowensis* (Zhang *et al.*, 2008); *Jatropha curcas* (Gao *et al.*, 2008); *Holcus lanatus* (Hartley-Whitaker *et al.*, 2001); *Daucus carota* (Ke *et al.*, 2007); *Ceratophyllum demersum* (Rama Devi and Prasad, 1998); *Brassica juncea* (Wang *et al.*, 2004); *Hydrilla verticillata* (Srivastava *et al.*, 2006); *Zea mays* (Nie *et al.*, 2012), *Triticum aestivum* cv. Hasaawi (Azooz *et al.*, 2012); *Allium sativum* (Meng *et al.*, 2007) etc. However, Weckx and Clijsters (1996)

observed that SOD was not involved in the defence mechanism against copper induced oxidative stress in primary leaves of *Phaseolus vulgaris*.

2.6.2.2 Catalase (CAT)

Catalase (EC 1.11.1.6), the first antioxidant enzyme to be discovered and characterized represents one of the primary enzymatic mechanisms employed by aerobic organisms to decompose hydrogen peroxide, a toxic intermediate of oxygen metabolism (Mhamdi *et al.*, 2010). Catalase is a heme-containing enzyme that catalyzes the dismutation of two molecules of hydrogen peroxide to water and oxygen. Catalases are generally involved in scavenging H_2O_2 generated during the photo-respiration and beta-oxidation of fatty acids (Morita *et al.*, 1994). Catalase is particularly abundant in the glyoxysomes of germinating seedlings where it destroys H_2O_2 generated by flavin oxidation, and in the peroxisomes of green leaves, where it destroys H_2O_2 arising from the oxidation of photorespiratory glycolate (Gill and Tuteja, 2010). All forms of the enzyme are tetramers with each monomer of 50-70 kDa. Multiple forms of catalase have been described in many plants. Monocots and dicots contain three catalase genes. The CAT isozymes have been studied extensively in higher plants (Polidoros and Scandalios, 1999) e.g. two in *Hordeum vulgare* (Azevedo *et al.*, 1998), four in *Helianthus annuus* cotyledons (Azpilicueta *et al.*, 2007) and as many as 12 isozymes in *Brassica* (Frugoli *et al.*, 1996). Maize has three isoforms (CAT1, CAT2 and CAT3), found on separate chromosomes that are differentially expressed and independently regulated (Scandalias, 1990).

CAT has one of the highest turnover rates for all enzymes: one molecule of CAT can convert about 6 million molecules of H_2O_2 to H_2O and O_2 per minute. Environmental stresses which reduce the rate of protein turnover cause the depletion of catalase activity. This has significance in the plant's ability to tolerate the oxidative components of these environmental stresses (Boguszewska *et al.*, 2010; Mhamdi *et al.*, 2010). A literature survey reveals contradictory results regarding the response of catalase (CAT) against copper stress. Catalase activity did not increase in Cu^{2+} stressed roots of rice seedlings (Chen *et al.*, 2000) or in black gram (*Vigna mungo*) seedlings (Solanki *et al.*, 2011). On the other hand, CAT activity was reported to increase in *Atriplex halimus* leaves (Brahim and Mohamed, 2011), *Prunus*

cerasifera roots and shoots (Lombardi and Sebastiani, 2005), *Cucumis sativus* roots (Iseri *et al.*, 2011) and in maize roots and shoots (Pourakbar *et al.*, 2007) in response to excess Cu^{2+} concentrations.

2.6.2.3 Peroxidase (POD)

Peroxidases (EC 1.11.1.7) are heme containing proteins that utilize H_2O_2 in the oxidation of various organic and inorganic substrates (Asada, 1994). Peroxidases utilizing guaiacol as electron donor *in vitro* are guaiacol peroxidases and participate in developmental processes, lignification, ethylene biosynthesis, defense, wound healing, etc. (Asada, 1992). The other group of peroxidases scavenges H_2O_2 in cell and utilizes glutathione, Cyt c, pyridine nucleotide and ascorbate as electron donors *in vitro* (Asada, 1992). In most plants, about 90% of the peroxidase activity is referred to as guaiacol peroxidase (Foyer *et al.*, 1994). Guaiacol peroxidases are glycoproteins, located in cytosol, vacuole, cell wall and in extracellular space, while the other group is non-glycosylated and localized in chloroplasts and cytosol (Asada, 1992). POD therefore represents an important peroxidase group which oxidise a large number of organic compounds such as phenols, aromatic amines, hydroquinones etc. But the commonly used reducing substrates are guaiacol or pyrogallol. Peroxidases also take part in defense mechanism against heavy metal toxicity through lignification of cell walls (Diaz *et al.*, 2001) that confer rigidity and prevent growth.

Both CAT and POD are therefore involved in the removal of H_2O_2 that accumulates due to dismutation of O_2^- by SOD. The mobilization of POD in response to Cu^{2+} induced oxidative stress in plants is well accepted (Fang and Kao 2000; Diaz *et al.*, 2001; Cuypers *et al.*, 2002; Meng *et al.*, 2007; Solanki *et al.*, 2011).

2.6.2.4 Ascorbate peroxidase (APX)

Ascorbate peroxidase (EC 1.11.1.11) is related to a peroxidase family that includes the microbial peroxidase, yeast cytochrome C peroxidase and prokaryotic catalase-peroxidase (Takeda *et al.*, 2000; Wilkinson *et al.*, 2002). APX differs from guiacol peroxidase not only in the specific donor, but also in the amino acid sequence and physiological functions. Ascorbate peroxidase (Mr 28-58 kDa) contains heme iron. It was detected in all intracellular compartments such as cytosol, chloroplast, mitochondria and plant cell

thylakoids (Mittler and Zilinskas, 1992; Bunkelman and Trelerse, 1996; Ishikawa *et al.*, 1996, 2008; Jimenez *et al.*, 1998; Caverzan *et al.*, 2012). There are different isoforms of APX based on the localization: thylakoid APX (tAPX), glyoxysome membrane APX (gmAPX), chloroplast stromal soluble form (sAPX) and cytosolic form of APX (cAPX). APX is widely known to show an enhanced expression in plants growing under unfavourable environmental conditions (Boguszewska and Zagdanska, 2012).

APX uses ascorbic acid as a reductant in the first step of the ascorbate-glutathione cycle and is involved in scavenging of H_2O_2 into water. This is the most important peroxidase in H_2O_2 detoxification operating both in cytosol and chloroplasts (Mittova *et al.*, 2000; Smirnoff, 2000). APX has a higher affinity for H_2O_2 (mM range) than CAT and POD (mM range) and it may have a more crucial role in the management of ROS during stress (Gill and Tuteja, 2010). *Apx* gene expression is rapidly induced by various stress conditions (Inze and van Montage, 1995). All these enzymes along with ascorbate and glutathione have a pivotal role in defence against ROS induced oxidative damage (Arora *et al.*, 2002; Yruela, 2005; Sharma and Dietz, 2008; Shan *et al.*, 2012). De Vos *et al.* (1992) observed that glutathione depletion is the major cause of Cu^{2+} induced oxidative damage in Cu^{2+} sensitive *Silene cucubalus* plants. It has been shown that tolerance to a copper-enriched environment, and the accompanying oxidative stress in *Enteromorpha compressa* occurs through the accumulation of copper, activation of ascorbate peroxidase, synthesis of ascorbate (accumulated as dehydroascorbate) and consumption of glutathione and water-soluble phenolic compounds (Ratkevicius *et al.*, 2003).

2.6.2.5 Other enzymes of ascorbate-glutathione cycle

Apart from POD and CAT, the enzymes and metabolites of the ascorbate-glutathione cycle are also involved in the removal of H_2O_2. The majority of these enzymes such as, glutathione reductase and dehydroascorbate reductase have been found in chloroplasts, cytosol, mitochondria, and peroxisomes (Dat *et al.*, 2000). Glutathione and ascorbate accumulate in these cellular compartments and their redox state is maintained through glutathione reductase, monodehydroascorbate reductase and dehydroascorbate reductase.

Table 2.2: Enzymes/metabolites whose level has been studied after copper exposure

Enzyme/ metabolite	Plant	Enzymes activity	References
Peroxidase	*Zea mays*	Increased in both leaves and roots.	Mocquot *et al.*, 1996
	Lycopersicon esculentum	Increased in both root and stem but no significant change occurred in leaves	Mazhoudi *et al.*, 1997
	Oryza sativa	Induced activity in leaves.	Fang and Kao, 2000
	Capsicum annum	Induced activity in seedlings and two isoperoxidases were detected.	Diaz *et al.*, 2001
	Phaseolus vulgaris	Increased activity in both leaves and roots.	Cuypers *et al.*, 2002
	Allium sativum	Increased in both leaves and roots, but after 9th day activity dropped in only in root but was still higher than control.	Meng *et al.*, 2007
	Erica andevalensis	Activity was significantly increased in leaves and roots.	Oliva *et al.*, 2010
	Festuca arundinacea	Activity was increased first, reached maximum and then decreased in roots.	Zhao *et al.*, 2010
	Lolium perenne	Activity was lower than control in roots.	Zhao *et al.*, 2010
	Vigna mungo	Activity increased but reverses at highly toxic condition in seedlings.	Solanki *et al.*, 2011
	Beta vulgaris	Activity was increased in leaves.	Morales *et al.*, 2012
	Camellia sinensis	Activity was increased and two new isozymes were detected in leaves.	Saha *et al.*, 2012
	Astragalus neo-mobayenii	Activity increased in both root and shoot with increasing concentration of copper.	Karimi *et al.*, 2012
	Triticum aestivum	Activity increased upto a concentration then decreased in seedlings.	Olteanu *et al.*, 2013
	Nicotiana tabacum	The activity was significantly higher in leaves.	Martins *et al.*, 2014
	Withania somnifera	Activity increased at lower concentration but decreased at higher concentration in seedlings.	Rout *et al.*, 2013

contd…

Table 2.2 (contd…): Enzymes whose level has been studied upon copper exposure

Enzyme/ metabolite	Plant	Enzymes activity	References
Peroxidase	*Aeluropus littoralis*	Activity decreased in leaves.	Rastgoo *et al.*, 2014
	Triticum aestivum	Activity increased when the concentration of copper is higher in seedlings.	Liu *et al.*, 2014
	Cicer arietinum	Increased activity with increasing concentration of copper in leaves.	Kumar *et al.*, 2014
Catalase	*Avena sativa*	Decrease in activity in leaves.	Luna *et al.*, 1994
	Lycopersicon esculentum	Activity was not modified in leaves and in stems, but it was decreased in roots.	Mazhoudi *et al.*, 1997
	Oryza sativa	No effect in seedlings.	Chen *et al.*, 2000
	Prunus cerasifera	Increased in roots and shoots, approximately five times greater than the control plantlets.	Lombardi and Sebastiani, 2005
	Zea mays	Higher activity in roots and shoots. Increased activity of catalase in leaf as compare to root.	Pourakbar *et al.*, 2007
	Vigna mungo	Activity declined in seedlings.	Solanki *et al.*, 2011
	Atriplex halimus	Increased activity in leaves. Three iso-enzymes were observed.	Brahim and Muhamed, 2011
	Cucumis sativus	Activity increased in roots	Iseri *et al.*, 2011
	Astragalus neo-mobayenii	Increasing activity from lower to higher concentration in roots and shoots.	Karimi *et al.*, 2012
	Solanum nigrum	Activity remained unaltered in root and shoot.	Fidalgo *et al.*, 2013
	Triticum aestivum	Activity decreased in seedlings	Olteanu *et al.*, 2013
	Nicotiana tabacum	Activity decreased in leaves.	Martins *et al.*, 2014

contd…

Table 2.2 (contd…): Enzymes whose level has been studied upon copper exposure

Enzyme/ metabolite	Plant	Enzymes activity	References
Catalase	*Withania somnifera*	Activity increased at lower concentration but decreased at the higher concentration in seedlings.	Rout *et al.*, 2013
	Aeluropus littoralis	Activity decreased in leaves	Rastgoo *et al.*, 2014
Superoxide dismutase	*Glycine max*	Increased activity in roots.	Chongpraditnun *et al.*, 1992
	Holcus lanatus	Activity was suppressed in roots.	Hartley-Whitaker *et al.*, 2001
	Brassica juncea	Low activity during first four days but increased late in roots.	Wang *et al.*, 2004
	Prunus cerasifera	Increased activity in roots and shoots.	Lombardi and Sebastiani, 2005
	Elsholtzia splendens	Activity increased in root, stem and leaves	Peng *et al.*, 2006
	Daucus carota	Higher activity in roots and leaves.	Ke *et al.*, 2007
	Allium sativum	Activity increased in leaves and roots.	Meng *et al.*, 2007
	Elsholtzia haichowensis	Increased activity in roots.	Zhang *et al.*, 2008
	Jatropha curcas	Slight increase in activity in roots, stems and leaves.	Gao *et al.*, 2008
	Zea mays	Stimulated activity in leaves.	Nie *et al.*, 2012
	Triticum aestivum	Activity was increased in seedlings.	Azooz *et al.*, 2012
	Astragalus neo-mobayenii	Activity was increased in roots and shoots with increasing copper concentration.	Karimi *et al.*, 2012
	Solanum nigrum	Activity remained unaltered in both root and shoot.	Fidalgo *et al.*, 2013
	Triticum aestivum	Activity increased at low concentration but decreased at higher concentration in seedlings.	Olteanu *et al.*, 2013

contd….

Table 2.2 (contd…): Enzymes whose level has been studied upon copper exposure

Enzyme/ metabolite	Plant	Enzymes activity	References
Superoxide dismutase	*Nicotiana tabacum*	Activity significantly increased in leaves.	Martins *et al.*, 2014
	Withania somnifera	Activity increased at lower concentration but decreased at higher concentration in seedlings.	Rout *et al.*, 2013
	Aeluropus littoralis	Activity increased in leaves.	Rastgoo *et al.*, 2014
	Cicer arietinum	Increased activity with increasing concentration of copper in leaves.	Kumar *et al.*, 2014
Ascorbate peroxidase	*Avena sativa*	Decreased activity in leaves.	Luna *et al.*, 1994
	Phaseolus vulgaris	Increased activity in leaves and roots.	Weckx and Clijsters, 1996
	Lycopersicon esculentum	Activity was unaltered in roots and in stems, while it was diminished in leaves.	Mazhoudi *et al.*, 1997
	Oryza sativa	Activity increased in seedling.	Chen *et al.*, 2000
	Brassica juncea	Activity in roots decreased initially but significantly increased after four days.	Wang *et al.*, 2004
	Morus rubra	Activity increased in leaves.	Tewari *et al.*, 2006
	Oryza sativa	Activity increased in roots and shoots.	Thounaojam*et al.*, 2012
	Camellia sinensis	Increase in activity in leaves.	Saha *et al.*, 2012
	Zea mays	Activity increased in leaves.	Nie *et al.*, 2012
	Triticum aestivum	Activity was reduced in seedlings.	Azooz *et al.*, 2012
	Nicotiana tabacum	Slight increase with increase in Cu concentrations.	Martins *et al.*, 2014

contd….

Table 2.2 (contd…): Enzymes whose level has been studied upon copper exposure

Enzyme/ metabolite	Plant	Enzymes activity	References
γ-glutamylcysteinyl synthetase	*Camellia sinensis*	Upregulated expression in leaves.	Yadav and Mohanpuria, 2009
	Triticum aestivum	Increased activity in leaves	Shan *et al.*, 2012
Glutathione reductase	*Silene cucubalus*	Depletion in activity was observed in root.	De Vos *et al.*, 1992
	Panax ginseng	Activity increased in roots.	Ali *et al.*, 2006
	Morus rubra	Activity increased in leaves.	Tewari *et al.*, 2006
	Zea mays	Activity was higher in both roots and leaves.	Pourakbar *et al.*, 2007
	Zea mays	Increased activity in roots.	Wang *et al.*, 2011
	Triticum aestivum	Activity was higher in leaves.	Shan *et al.*, 2012
Glutathione reductase	*Oryza sativa*	Activity increased in both root and shoot.	Thounaojam *et al.*, 2012
	Zea mays	Activity increased in leaves.	Nie *et al.*, 2012
Dehydroascorbater eductase	*Triticum aestivum*	Increased activity in leaves.	Shan *et al.*, 2012
	Panax ginseng	Induced activity in roots.	Ali *et al.*, 2006
Phenylalanine ammonia lyase	*Phyllanthus tenellus*	Induced activity in leaves.	Santiago *et al.*, 2000
	Camellia sinensis	Enhanced activity in leaves.	Basak *et al.*, 2001

contd….

Table 2.2 (contd…): Enzymes whose level has been studied upon copper exposure

Enzyme/ metabolite	Plant	Enzymes activity	References
Phenylalanine ammonia lyase	*Matricaria recutita*	Increased activity in root and no effect in leaves.	Kovacik and Backor, 2007
	Jatropha curcas	Unchanged in stem but gradually increased in root and leaves.	Gao *et al.*, 2008
	Glycine max *Lupinus luteus*	Increased activity in *Lupinus luteus* roots but no significant effect in *Glycine max* seedlings.	Chmielowska *et al.*, 2008
Polyphenol oxidase	*Camellia sinensis*	Activity declined in leaves.	Basak *et al.*, 2001

2.6.3 Response through antioxidant molecules

2.6.3.1 Proline

Accumulation of amino acids like proline has been observed in response to several biotic and abiotic stresses in plants. Proline accumulation in plant tissues has been suggested to result from (a) a decrease in proline degradation, (b) an increase in proline biosynthesis, (c) a decrease in protein synthesis or proline utilization, and (d) hydrolysis of proteins (Charest and Phan, 1990). Content of free proline has been found to be related to Cu^{2+} tolerance in plants (Backor *et al.*, 2003; Chen *et al.*, 2004). Excess Cu^{2+} has been found to result in inadequate proline (Thomas *et al.*, 1998) and lead to the malfunctioning of copper exclusion machinery (Chen *et al.*, 2004). Copper complexes with amino acids such as proline, histidine or nicotinamine play important role in xylem sap transport (Liao *et al.*, 2000). Azooz *et al.* (2012) reported an increase in proline production with increase in copper in wheat (*Triticum aestivum* cv. Hasaawi) at early growing stage. They also proposed that, proline acts as a source of carbon and nitrogen for rapid recovery from the stress, and also functions as a stabilizer of plasma membrane and some macromolecules and free radical scavenger (Jain *et al.*, 2001). Moreover, proline was found to play a role in the detoxification of active oxygen in *Brassica juncea* and *Cajanus cajan* under heavy metal stress (Alia *et al.*, 1995).

2.6.3.2 Glutathion-ascorbate couple

Glutathione is a well-known antioxidant playing a prominent role in the defense against free radicals in plants (Sharma and Dietz, 2008). GSH and ascorbate accumulate to millimolar concentrations in chloroplasts and mitochondria owing to the ascorbate–GSH cycle, which also operates in peroxisomes. They have a pivotal role in defence against ROS induced oxidative damage. GSH functions as an HM-ligand (Canovas *et al.*, 2004) and an antioxidant. Upon HM exposure, GSH concentrations drop as a consequence of initiated phytochelatin biosynthesis. This causes oxidative stress and in turn short-term toxicity (Nocito *et al.*, 2006).

2.7 Stress in tea

A literature survey revealed that several studies have been conducted on different types of abiotic stresses in tea. Plants of different cultivars of tea have been grouped into the tolerance classes: susceptible and resistant, in response to drought stress (Chakraborty *et al.*, 2002; Damayanthi *et al.*, 2010), cold stress (Upadhyaya, 2012) and heavy metal stress (Zhang *et al.* 2013). Several parameters have been identified such as rates of photosynthesis and transpiration, relative water content, stomatal conductance and leaf total soluble sugar content (Damayanthi *et al.*, 2010), root and shoot extension (Burgess and Carr, 1997), levels of proline and antioxidative enzymes (Chakraborty *et al.*, 2002; Upadhyaya *et al.*, 2008), morphological characters (Waheed *et al.*, 2012) etc. in order to screen tea cultivars for drought tolerance. Additionally, studies on alterations in bioconstituents that determined quality of tea in the tea clones under soil moisture revealed a decrease in PAL activity in both tolerant and susceptible clones which correlated with a lower flavonol content and quality deterioration (Jeyaramraja *et al.*, 2003).

Tea plants exposed to excess heavy metals have shown several alterations in physiological and biochemical parameters. Increased level of lipid peroxidation and a reduction in photosynthetic rate, transpiration rate, chlorophyll and protein content and biomass production were found in plants exposed to excess Cd (Mohanpuria *et al.*, 2007; Shi *et al.*, 2009). Oxidative stress was evident as the transcript levels of glutathione biosynthetic genes showed up-regulation while glutathione-S-transferase

(GST), the enzyme which help in sequestration of high levels of metal ions to vacuole, did not show any change on Cd exposure (Mohanpuria *et al.*, 2007). In a similar study on cultivars differing in metal tolerance, expression of glutathione biosynthetic enzymes and phytochelatin synthase was found to be more elevated in the more tolerant Chinary cultivar than in Assamica under copper and aluminium excess (Yadav and Mohanpuria, 2009). Hajiboland and Bastani (2012) observed that CO_2 assimilation and dry matter production decreased while antioxidant enzyme activity and proline content increased significantly in tea plants under Boron deficiency and water stress. Mukhopadhyay *et al.* (2013) observed that both deficiency and excess in zinc caused a considerable decrease in shoot and root fresh and dry masses. Zinc stress decreased net photosynthetic rate, transpiration rate, stomatal conductance, and content of chlorophylls *a* and *b* and increased the content of superoxide anion, malondialdehyde, hydrogen peroxide, and phenols. Although the activities of ascorbate peroxidase, catalase, superoxide dismutase, and peroxidase as well as expression of respective genes were up-regulated, the authors concluded that the overall antioxidant system did not afford sufficient protection against oxidative damage (Mukhopadhyay *et al.*, 2013). Treatment of tea plants with excess heavy metals such as mercury (II) and nickel (II) decreased the chlorophyll content of the leaves, along with a significant reduction in Hill activity (Basak *et al.*, 2001). The activities of antioxidative enzymes viz. Superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) was increased by aluminium in the roots of cultured tea cells and also in intact plants (Ghanati *et al.*, 2005). Aluminum (Al) inhibited tea pollen tube growth but the effect was found to be alleviated by fluorine (Konishi and Miyamoto, 1983) which is accumulated by tea plants normally in high excess (Ruan and Wong, 2004). Tea plants tolerated fluorine at concentrations < 0.32 mM (Li *et al.*, 2011). Fresh and dry mass, chlorophyll content and net photosynthetic rate decreased while proline, malondialdehyde and hydrogen peroxide contents increased with increasing fluorine concentrations. Activity of antioxidant enzymes also showed significant alterations thereby suggesting that antioxidant defence system of leaves did not sufficiently scavenge excessive reactive oxygen species generated due to excess fluorine (Li *et al.*, 2011).

2.8 Cu^{2+} stress in tea

Although copper based fungicides are being used in tea gardens for several decades (Sarmah, 1960), we know little about the role of excess Cu^{2+} on tea plants and at what concentrations it may be considered as a pervasive threat (Saha *et al.*, 2012). Only a few studies have focused on Cu^{2+} toxicity in tea (Basak *et al.*, 2001; Yadav and Mohanpuria, 2009; Saha *et al.*, 2012) and these have revealed that number physiochemical parameters are altered on exposure to excess copper. For example, the chlorophyll and protein contents were found to decrease in Cu^{2+} treated plants (Basak *et al.*, 2001; Yadav and Mohanpuria, 2009; Saha *et al.*, 2012). Yadav and Mohanpuria (2009) observed that expression of the enzymes γ-glutamylcysteinyl synthetase, glutathione synthetase and phytochelatin synthase was elevated more in the tolerant tea cultivar than the susceptible one when exposed to excess Copper and Aluminium. Mandal *et al.* (2013) investigated the toxic effect of Cu^{2+} on seed germination, growth and morphological changes in tea seedlings.

Heavy metal stress is one of the major problems that limit agricultural productivity of plants. Plants show relative differences in their heavy metal tolerance capacity among the species and also among cultivars of the same species. Copper stress in general induces ROS and generates oxidative stress. It has been found that in addition to accumulated metal ions, high levels of ROS adversely affected the plants. Such ROS related damages have been observed in tea cultivars also. Although the negative impact of excess Cu^{2+} in tea plants have been documented, the level of Cu^{2+} accumulation caused due to long term application of Cu^{2+}-based fungicides in tea gardens and its bioavailability under tea garden conditions are yet to be studied. Additionally, more detailed studies on mechanisms of Cu^{2+} toxicity in the tea plant, especially at the gene level are necessary. Identification of genetic determiners of tolerance may make the resistant cultivars a potential source for genetic manipulation of other important elite cultivars.