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Review Article

TOLERANCE AGAINST HEAVY METAL TOXICITY IN CYANOBACTERIA: ROLE OF ANTIOXIDANT DEFENSE SYSTEM

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ABSTRACT

The toxicity of heavy metals is reviewed with reference to Cyanobacteria. Cyanobacteria also known as blue green algae are primitive photosynthetic prokaryotic microorganism which has high economic importance due to various bioactive compounds. Cyanobacteria are exposed to heavy metal stress since these are widely distributed. Heavy metal enters into the cell through various interactions between metal ions and functional groups present at the cell surface and cause toxicity. Heavy metals also cause oxidative stress by generation of reactive oxygen species, including superoxide, hydrogen peroxide and hydroxyl radical which are highly reactive and toxic and cause damage to nucleic acid, protein, and lipid. Cyanobacteria have evolved strategies to overcome the effect of reactive oxygen species that include antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase and Glutathione reductase.

Keywords: Cyanobacteria, Reactive oxygen species, Oxidative stress, Antioxidant enzymes.

INTRODUCTION

The environment is deteriorating day by day through various human activities leading to extensive chemical use in a country like India as a consequence of rapid industrialization in order to the development and to sustain over growing large problem of population. A broad range of chemicals including both organic and inorganic contaminants such as heavy metals, combustible and putrescible substances, hazardous wastes, explosives and petroleum products, phenol and textile dyes. The main component of inorganic contaminants are heavy metals [1-3] since these are used on a large scale in various industrial operations such as electroplating, mining and smelting of ores for metal extraction, etc. And in agricultural practice by using fertilizers, herbicides and pesticides. An increase in level of heavy metals is posing a serious trouble to our ecosystem. Since these are transmitted to human along their food chain and cause various disorders. Although some heavy metals are required for human beings in trace amounts, but these become toxic at higher concentration. Some heavy metals are well established for the generation of reactive oxygen species (ROS) and reactive nitrogen species (NO) as well as replacement of enzyme cofactors and transcription factors, inhibitions of antioxidative enzymes, cellular redox imbalance, ionic transport imbalance, DNA damage and protein oxidation [4-7]. The ROS are reduced form of molecular oxygen including superoxide radicals ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet-}$) and hydrogen peroxide (H_2O_2), which are more toxic and reactive than O_2. These active molecules are involved in the free radical chain reaction of membrane lipids and proteins. Thus causing oxidative decomposition of them [8,4]. Therefore, a defense mechanism is required to reduce the toxic effect of ROS. This mechanism includes various antioxidant enzymes such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione reductase (GR) and Ascorbate peroxidase (APX), etc.

Cyanobacteria are one of the most primitive prokaryotes inhabiting almost all biological niches including extreme environments [9]. Due to this wide-ranging distribution, algae are exposed to all kinds of stresses, whether tropospheric or aquatic. Therefore, algae have been widely used in understanding environmental problems in a variety of ways. The information related to the response due to algal antioxidant systems against environmental stresses seems to be meagre and widely scattered.

Adverse effects of heavy metals

The term "heavy metal" refers to those metallic elements which have relatively high density (5 gm/cm^3) and atomic mass between 54.63 and 200.59, and posses toxicity even at low concentration [10, 11]. There are 90 naturally occurring elements that are heavy metals, but not all of them are biologically significant. Under physiological conditions 17 heavy metals, based on their solubility may be available for living cells and of importance for organisms and ecosystems [12]. The eight most common pollutant heavy metals listed by the Environment Protection Agency (EPA) are: As, Cd, Cr, Cu, Hg, Ni, Pb, and Zn [13]. Heavy metals can be broadly divided into two groups. The first group consists of metals that are essential as nutritional requirements at trace amounts for many organisms, but are toxic when present in greater amounts. This group includes As, Fe, Cr, Co, Cu, Ni, Se, Va and Zn. The second group includes Pb, Hg, Cd, Ur, Ag and Be, all of them are highly poisonous even at very low concentration [14].

Heavy metals are natural constituents of the earth's crust and are persistent in nature since they cannot be degraded. As a result of persistent, they enter into the body system to a small extent along the food chain from drinking water and agricultural soil, which are contaminated through the discharge of industrial wastes into the soil and rivers, and they cause harmful effects to human. Although some heavy metals are required by living organism as they are used to stabilize protein structures, easy electron transfer reactions and catalyze enzymatic reactions [15]. Heavy metals such as Cu, Zn, Fe, Ni, Co, etc. are required for catalytic activity of enzymes [16] which are used to catalyze several biological reactions. Some heavy metals (like Zn, Fe, Co, Ca, and Mg) are known to have a biological function. For example, Fe, Cu, Zn and Mn are required for superoxide dismutase enzyme that provides a protection against oxidative stress. Zinc is one of the most important elements, found in the body. It is used as a cofactor by many enzymes such as dehydrogenating enzyme and carbonic anhydrase [17] in the body that is involved in different processes occurs in the human body. Zinc deficiency causes anaemia and retardation of growth and development [18]. Its deficiency also results in poor immunity since it is known to have a role in the immune system. Fe is one of the most copious metals found on the earth, including human beings. It is required for a number of processes that continually take place on a molecular level, such as oxygen transport, RBC production, DNA synthesis, electron transport, metabolism for energy generation. Calcium is also an important element in human metabolism, which is required for production of strong bones and teeth in human beings [17]. Even Cr (VI) is taken up in amount of 150 μg/day that are required for collagen, but it leads to irritation of the stomach, leading to ulcers and kidney and liver damage at higher concentration [19]. In humans, higher concentration of lead and mercury is associated with autoimmune disease such as rheumatoid arthritis as well as interfere with the proper functioning of the kidney and circulatory system that leads to injury of central nervous system [20].

Heavy metals such as Fe, Cu, Zn, Ni, Co, Cr, and Mn are vital to human only at lower concentration, but they become more toxic when they are taken up more than a bio-recommended limits. Heavy metals like Cd, Cr, Pb, Cu, Hg, etc. Are discharged into rivers and soil from different industries. These metals accumulate in the tissues of plants through polluted agricultural soil [21], as well as heavy metals are also accumulated in tissues of aquatic animals such as fish that survive in polluted water. Ultimately, heavy metals are accumulated into human beings through such plants, animals and drinking water and cause severe biochemical disorders. There are known common symptoms such as gastrointestinal (GI) disorders, diarrhea, stomatitis, tremor, hemoglobinuria causing a rust–red color to stool, ataxia, paralysis, vomiting and convulsion, depression, and pneumonia when volatile vapors and fumes are inhaled that are allied with heavy metals like Cd, As, Hg, Pb, Zn, Cr, Cu, etc. [18]. These metals are also linked with mental retardation, cancers, kidney damage, neuropsychiatric disturbances, memory loss, and even death [22]. The nature of the effects could be toxic (acute, chronic or sub-chronic), neurotoxic, carcinogenic, mutagenic or teratogenic.

Effect of heavy metals on aquatic organisms

Although heavy metals are being discharged into rivers and sea from various industries in a limited quantity, yet these metals are taken up by aquatic organisms since they have high solubility in the aquatic environment. Aquatic organisms such as fish which are present at the top of the aquatic food chain take both essential and toxic metals through their gills and skins from their surrounding environment and along the food chain [23, 24]. Heavy metal uptake rate by fish depends on a number of factors such as the concentration of metals, exposure period of metals to fish, surrounding pH, salinity and temperature [25]. Although Heavy metals are accumulated within a specific organ of organisms, but their distribution among organs is not uniform [26]. Metal distribution within a fish varies between fish species; depend upon age, developmental stage and other physiological factors. Fish accumulates metals in Gonads, liver, kidney, and gills resulting in pathological changes than the muscle tissues [27]. These metals transmit to human beings through fish consumption since fish provides a good source of proteins, essential fatty acids, minerals and vitamins that are used to treat heart diseases and reduce blood cholesterol level [28]. Fish accumulates substantial concentrations of mercury in their tissues and thus can represent a major dietary source of this element for humans. Fish is the single largest sources of mercury and arsenic for man. Mercury is a known human toxicant and the primary sources of mercury contamination in man are through eating fish. Biotransformations of mercury and methyl mercury formation constitute a dangerous problem for human health [29]. Effects of cadmium on aquatic organisms are analogous to those in humans, and include skeletal deformities and impaired functioning of kidneys in fish. Skeletal deformities in fish can result in an impaired ability of the fish to find food and avoid predators; hence, this sub lethal effect becomes a lethal effect [30]. Hexavalent chromium is mobile in the environment and is acutely toxic, mutagenic, teratogenic and carcinogenic to aquatic organisms [31]. Copper (Cu) and Zinc (Zn) that are often present in industrial wastewaters are hazardous to the aquatic ecosystem. Other than the fishes, Cyanobacteria which is one of the major microflora of aquatic life, is also affected adversely.

Cyanobacteria, also known as blue green algae are prokaryotic microorganisms that perform photosynthesis like higher plants by using chlorophyll a and different accessory pigments to capture sunlight for energy. Cyanobacteria are globally distributed and they are frequently found in lakes, ponds, hot springs, wetlands, streams, and rivers and they play a major role in the nitrogen, carbon, and oxygen dynamics of many aquatic environments [32]. It represents a large group within the prokaryotic kingdom Cyanobacteria are bluish green due to the presence of pigment phycocyanin and chlorophyll *a.* The morphology of cyanobacteria is commonly gelatinous, slimy and infrequently filamentous clusters with a range of colors; blue- green, yellow, brown to black and rarely red depending upon the concentration of chlorophyll-a and various accessory phyco-biliprotein pigments, phycocyanin (blue) and phycoerythrin (red) [9].

At present cyanobacteria comprise 2000 species organized in 150 genera [32]. The most common genera being *Microcystis, Synechococcus, Anacystis, Gloeocapsa, Agmenellum (syn. Merismopedia)* as unicellular and colonial types and *Oscillatoria, Lyngbya, Spirulina, Anabaena, Aphanizomenon, Nostoc, Cylindrospermopsis, Planktothrix, Calothrix, Rivularia* and *Gleoetrichia* as filamentous types. Some species of cyanobacteria are frequently found in marine intertidal and littoral ecosystem. They are *Lyngbya majuscula, Microcoleus ethnoplasts* (a common mat former), *Spirulina sp.* (a highly nutritious cyanobacterium) and several species of *Oscillatoria* [9]. Cyanobacteria species of *Anabaena* (or *Nostoc*), *Azollae* have the ability to fix atmospheric nitrogen. Some of these species such as *Azolla* are used as biofertilizers, mainly in rice production [33,34]. *Spirulina sp*. is a nutritious cyanobacterium and It is used as human food as an important source of protein [35]. It has gained considerable popularity in the human health food industry and it is used as a protein supplement.

As, the use of synthetic antioxidants is decreased due to their suspected activity as carcinogenesis [36] as well as general consumer rejection of synthetic food addition [37]. There is a great demand throughout the world in finding new natural sources of antioxidants to prevent oxidative damage to living cells and to reduce the deterioration of food by oxidation [38]. Traditionally, some antioxidants such as tea, wine, fruits, vegetables and spices are used from the ancient days. Cyanobacteria also represent a good source of antioxidants and contain a wide variety of antioxidant pigments than the plants and most algal source [39]. Carotenoids, which have potent antioxidant activity are the most widely distributed and structurally diverse classes of natural pigments predominantly produced by Cyanobacteria and that are doing important functions in photosynthesis and nutrition. Cyanobacteria is also known to have a rich source of novel bioactive metabolite, comprising many cytotoxic, antifungal and antiviral compounds [40].

Effect of heavy metals on cyanobacteria

Cyanobacteria are found almost at all biological niches including extreme condition. Therefore, they are exposed to various environmental stresses, whether tropospheric or aquatic. Cyanobacteria are considered as an excellent model to understand the environmental problems and the protective process against those problems. Heavy metal stress is one of the environmental stresses that is being extensively increased due to rapid industrialization. During evolution, these organisms have developed diverse strategies to maintain an equilibrated relation with heavy metal ions present and available in the surrounding medium [41]. Cells face two tasks, the first is to select those heavy metals essential for growth and exclude those that are not, and the second to keep essential ions at optimal intracellular concentrations [42]. Complexation, ion exchange, adsorption, inorganic microprecipitation, oxidation and/or reduction have been proposed to elucidate the uptake process [43]. The metal uptake process is considered as a two step process [44]. Firstly, they are adsorbed to the cell surface through interaction between different heavy metal ions and the functional group such as carboxyl, phosphate, hydroxyl, amino, sulphur, sulphide, thiol, etc. That is offered by the component of the cell wall like polysaccharides, proteins, lipids [45]. After that they get an entry into the cell through penetration to the cell wall [46] and are localized into organelles. It was noted that *Spirulina platensis* can take up a significant amount of Cr(VI) and Pb(II) from the culture medium and proposed that the *S. platensis* could be used as a biosorbent in the treatment of industrial wastewater for the removal of Cr(VI), Pb(II), and other heavy metals [47, 48]. *Spirulina fusiformis* were also found to be effective for removal of Chromium (93-99%) [49]. Further, *S. platensis* may be a suitable candidate for column design or large-scale batch biosorption systems [47]. Toxicity of heavy metals to Cyanobacteria has been thoroughly studied in the past few decades. Reduction in growth and chlorosis are common symptoms to algae of metal toxicity. Growth inhibition of micro algae is related to the concentration of heavy metal ions bound to the algal cell surface, in some cases, to intracellular heavy metal ion concentration [45] and to the chemical nature of the heavy metal ions [50]. Alterations in morphology and ultra structure have also been frequently reported [51], which may be also a possible reason of growth reduction. As observed earlier, *Spirulina platensis-S5* showed the reduction in growth with increasing metal concentration of initial exposure to lead, copper, and zinc as was observed by yellowing and fragmentation of filaments, and reduction in the number of spirals [52]. The effect of metals on different algal species including *Chlorella sp*., *Pediastrum duplex, Nitzschia palea*, and *Anacystis nidulans* also showed reduction in growth rate [53] and it was found that *Chlorella* sp. was the most tolerant of either metal ion having the highest EC_{50} value than the different algal species tested. Another Cyanobacterium *Chlorella vulgaris* also decreased the growth and chlorophyll content with increasing concentration of Cr (VI) along the exposure time [54]. Similarly, in the presence of heavy metals, growth and Chlorophyll content of *Spirulina sp.* [55] and *Oscillatoria sp.* [56] was found to be decreased. Mostly, growth reduction in microorganisms was observed in a dose dependent manner. Nitrogen fixing Cyanobacteria, *Anabaena doliolum* [57] and *Anabaena Variabilis* [58] showed the inhibition of growth rate in the presence of heavy metals. The growth of *Anabaena doliolum* was promoted at lower concentration of nickel and followed by a decrease at higher concentration [59]. Other than heavy metals, growth of *Anabaena sp.* was also inhibited in the presence of UV radiation [60]. The growth reflects metabolism of the cell, which depends on various metabolic processes such as photosynthesis, respiration and nutrient uptake. The growth inhibition could also be related to the decrease in the rate of cell division caused by metals is primarily attributed to their binding to sulfhydryl groups which are important for regulating the plant cell division [61]. As Indole Acetic Acid (IAA) stimulates the plant growth and multiplication, the degradation of indole acetic acid (IAA) could be a possible cause of growth inhibition in algal tissues. Heavy metals stimulates the activation of peroxidase enzyme that degrade the IAA and chlorophyllase activity, disorderness of membrane system and inactivation of electron transport functions in the photosystem could also be involved in the growth inhibition. The toxicity of heavy metals to algae depends on types of algae, chemical nature and concentrations of the metal, and the environmental conditions [45]. Other than, the inhibition of growth, higher concentration of heavy metals could lead to the generation of toxic oxygen species known as reactive oxygen species (ROS) which include superoxide, hydrogen peroxide and hydroxyl radical. These molecules are very reactive and affect the cellular function by reacting with nucleic acid, lipid, and proteins.

Generation of ROS upon heavy metal stress

The ROS are partially reduced forms of atmospheric oxygen. Although atmospheric oxygen is a totally harmless molecule, and it is used as a terminal oxidant so it has the potential to be partially reduced and forms ROS. Molecular oxygen must accept four electrons at once for the production of two molecules of water. Since it is free radical and has two impaired electron with the same spin quantum number. So, molecular oxygen can accept only one electron at a time due to spin restriction [62].

Therefore, molecular oxygen is converted into ROS either by transfer of energy or electron. The first one leads to the generation of singlet oxygen, whereas the second results in the sequential reduction to superoxide, hydrogen peroxide, and hydroxyl radical (fig. 1) [63].

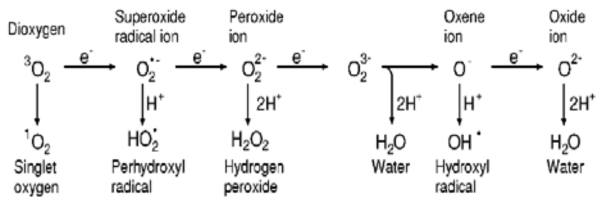

Fig. 1: Generation of ROS by sequential reduction of molecular O_2 adapted from [64].

ROS are continuously produced as byproducts of various metabolic pathways that are localized in different cellular compartments such as chloroplast, mitochondria and peroxisomes [65]. In algae and higher plants, photosynthesis occurs in chloroplasts, which contain a highly organized thylakoid membrane system consisting all components of the light-capturing photosynthetic apparatus and provides all structural properties for optimal light harvesting. O_2 generated in the chloroplasts during photosynthesis can accept electrons passing through the photosystems, thus forming superoxide radicals ($O_2^{\cdot-}$). Normally in the PSI, the electron flow is directed to $NADP^+$ from excited photosystem and reducing it into NADPH. Then it enters into calvin cycle and reduces the terminal electron acceptor CO_2. But in case of overloading of ETC (under various stresses), a part of electron flow is diverted to O_2 from ferredoxin and reduce O_2 into superoxide by Mehler reaction [66]. PSII also provides the sites (QA, QB) of electron transfer to O_2. Finally, CuZn-SOD present in the chlorolplast, convert superoxide into H_2O_2. Therefore, triplet chl, ETC in PSI and PSII makes the chloroplast a major site of ROS production. In the darkness, the mitochondria appear to be the main ROS producers. It has been estimated that 1-5% of the O_2 consumption of isolated mitochondria results in ROS production [67]. In mitochondria, complex I, II and ubiquinone of electron transport chain (ETC) are the major sites for the generation of $O_2^{\cdot-}$. Further, it is reduced into superoxide by SOD. In the presence of suitable transition metals, especially Fe, superoxide and hydrogen peroxide is converted to $OH^{\cdot}$ at neutral pH and ambient temperature by $O_2^{\cdot-}$ driven Fenton reaction (fig. 2) [68].

$$H_2O_2 + O_2{\bullet}^- \xrightarrow{Fe^{2+},Fe^{3+}} OH^- + O_2 + OH{\bullet}$$

Fig. 2: Generation of hydroxyl radicals by Fenton reaction

Under physiological steady state conditions, the ROS molecules are regularly scavenged by various antioxidative defense components that are often confined to particular compartments [69]. So, there is an equilibrium between production and scavenging of ROS molecules which is always maintained. This equilibrium may be perturbed by a number of adverse environmental factors such as drought [70], heavy metals [71], high salt concentration [72], extremes of temperature [73], and UV irradiation [74], etc. Heavy metal toxicity enhances the ROS production up to 30-fold [75]. A rapid increase in intracellular production of ROS molecules under various stresses could lead to the generation of oxidative stress. Although these ROS molecules play an important role in signaling that alter gene expression by targeting and modifying the activity of transcription factors. But at higher concentration, ROS molecules present toxicity to cells [64]. ROS affect many cellular functions by damaging nucleic acids, oxidizing proteins, and causing lipid peroxidation (LPO) [68]. It is important to note that whether ROS will act as damaging, protective or signaling factors depends on the delicate equilibrium between ROS production and scavenging at the proper site and the time [76].

ROS induce lipid peroxidation

It is well known that chloroplast, mitochondria and peroxisomes are the major site of ROS production. ROS are highly toxic that adversely affect many cellular functions by damaging nucleic acids, oxidizing proteins, and causing lipid peroxidation (LPO). As lipids are the major constituents of cell membranes and it is the primary target of ROS. So, the peroxidation of lipids is considered as the most cellular damaging process that occurs in every living organism. In algae photosynthesis takes place in chloroplast, are highly organized and formed by a complex system of membranes rich in polyunsaturated fatty acids, which are potential targets for peroxidation [77]. It is well known that lipid peroxidation yields a wide range of cytotoxic products from polyunsaturated precursors that include small hydrocarbon fragments such as ketones and aldehydes, like malon-dialdehyde (MDA), 4- hydroxynonrnal (HNE) [78]. Some of these compounds react with thiobarbituric acid (TBA) to form colored products called thiobarbituric acid reactive substances (TBARS) [79]. It has been noted that Cyanobacteria exposed to heavy metal stress enhanced the level of lipid peroxidation as a result of generation of ROS. Treatment with Pb, Cu and Zn significantly enhanced the level of LPO measured as MDA in *Spirulina platensis* [52]. But in the presence of Cu, MDA content was found to be enhanced as Cu is a redox active transition metal that catalyze the generation of $OH^{.}$, most toxic and reactive to lipid, from O_2^{-} by Fenton reaction. Deniz *et al* (2011) also noted a significant increase in MDA of *Spirulina sp*. under salinity stress and Cu (II). Similarly, an increase in MDA level was noted in *Chlorella vulgaris* with increasing concentration of Cr (VI) [54]. Nitrogen fixing Canabacteria *Anabaena doliolum* [57] and *Anabaena sp.* PCC7120 [60] enhanced MDA content under heavy metal stress. Therefore, Under various stresses, Lipid peroxidation products such as malionaldehyde (MDA) is used as an indicator of free radical production and LPO linked to tissue damage.

Antioxidant defense system

Exposure of higher level of heavy metals to Cyanabacteria may lead to the generation of ROS at higher concentration. Hydrogen peroxide may be reduced to hydroxyl radicals in the presence of transition metal ions by superoxide. $OH^{.}$ radical is more reactive than superoxide and hydrogen peroxide and there is no known scavenger of hydroxyl radical. To reduce the risk of $OH^{.}$, the reactions that lead to the production of $OH^{.}$ and the concentration of O_2^{-} and H_2O_2 are to be kept under tight control [64].

To neutralize the toxic effect of ROS, cyanobacteria have evolved a mechanism known as antioxidant defense systems in its organelles like chloroplast, mitochondria. Antioxidant defense mechanisms are of two types, namely enzymatic and non-enzymatic reactions. The enzymatic antioxidants include superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase.

The non enzymatic antioxidants include nutrient antioxidants like carotenoids, α-tocopherol, ascorbic acid, glutathione, flavonoids, uric acid and plasma proteins such as transferrin, albumin, metalothionein etc. [80].

Enzymatic antioxidants

Superoxide dismutase (SOD, EC 1.15.1.1)

Superoxide dismutase (SOD) is metalloprotein that catalyzes the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen (fig. 3) [81] and provides the first line of defense against ROS, produced in different organelles such as chloroplast, mitochondria and peroximes. It is the most effective intracellular enzymatic antioxidant in all aerobic organisms and in all subcellular compartments prone to ROS mediated oxidative stress. It removes superoxide radical ($O_2^{.-}$) hence decrease the risk of the most effective OH. by the metal catalyzed Haber- Weiss type reaction [68].

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

Fig. 3: Reduction of superoxide radical by Superoxide dismutase

In plant system, three types of SOD are found in different organelles which are distinguished by their metal cofactors. The first one is copper/zinc (Cu/Zn-SOD), which are localized in chloroplast. It is sensitive to CN^-. The other two are the manganese (Mn-SOD) and the iron (Fe-SOD), which are CN^- insensitive. In plants, MN-SOD is found in mitochondria. Fe-SOD is observed in some families of plant and associated with chloroplast [62]. The SOD enzymatic activity, is found to be increased under various environmental stresses and employ a defense mechanism. Table 1 compiles the effect of heavy metal stress on the SOD activity of Cyanobacteria. Choudhary *et al* (2007) studied the effect of heavy metals (Pb, Cu and Zn) in *Spirulina platensis* and observed increased SOD activity with increasing concentration of metals that pointed to the occurrence of scavenging mechanism of ROS ($O_2^{.-}$) and enhance the resistance capability of *Spirulina platensis*. Similar increased activity of SOD was noted in nitrogen fixing Cyanobacterium, *Anabaena variabilis* in the presence of metals [58]. A bell shaped dose response curve of SOD activity was noted in *Chlorella vulgaris* under Chromium, which is suggested to use as a biomarker for Cr pollution in water [54].

Catalase (CAT, EC 1.11.1.6)

Catalase, a tetrameric heme containing enzyme plays an important role to decompose H_2O_2 into H_2O and O_2 (fig. 4), generated in peroxisomes by oxidases involved in β-oxidation of fatty acids, photorespiration, and purine catabolism. When, cells are energy stressed and rapidly generating H_2O_2 through catabolic processes. Then, catalase provides an energy efficient mechanism to degrade H_2O_2. Hence, Catalase removes H_2O_2 without consuming cellular reducing equivalents such as NADPH [62].→

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$$

Fig. 4: Reduction of H_2O_2 by catalase

Table 1 compiles the effect of heavy metal stress on the CAT activity of Cyanobacteria. It was noted that chromium treated *Chlorella vulgaris* increased the catalase activity linearly with increasing concentration of chromium (0 to 100 µg/ml) [54]. Sultan *et al* (2007) noted that *Anabaena doliolum* enhanced the activity of CAT under heavy metal, but it was decreased at higher doses of heavy metals.

Table 1: Response of antioxidant enzymes induced in Cyanobacteria upon heavy metal exposure.

S. No.	Heavy Metals	Cyanobacteria	Antioxidant Enzymes	Response: Increased (I) or Decreased (D)	References
1	Pb, Cu, and Zn	*Spirulina platensis*	SOD	I	[52]
2.	Cr(VI)	*Chlorella vulgaris*	SOD, APX and CAT	I (SOD, up to 1µg/ml and APX up to 10µg/ml), I (linearly up to 100µg/ml)	[54]
3	Cd and Cu	*Anaebaena doliolum*	SOD and APX, GR and CAT	I, I (but decreased at higher concentration)	[57]
4	Mg, Mn, Cd, Hg, Zn, Co, and Pb	*Anabaena variabilis*	SOD, CAT	I, D	[58]
5	Cd, and UV-B	*Anabaena sp.*	SOD, CAT, APX and GR	I	[60]
6.	As	*Hapalosiphon fontinalis*	SOD, CAT, APX and GR	I	[82]
7	H_2O_2	*Spirulina platensis*	SOD, CAT, APX and GR	I	[83]

Ascorbate Peroxidase (APX, EC 1.11.1.11) and Glutathione reductase (GR, EC 1.6.4.2)

Ascorbate peroxidase and Glutathione reductase are mainly enzymatic antioxidants of Ascorbate- Glutathione cycle, also referred to as Halliwell- Asada pathway. Ascorbate- glutathione cycle represents the successive oxidation and reduction of ascorbate, glutathione, and NADPH by the enzyme APX, GR and dehydroascorbate reductase (fig. 5). APX and GR play an important role to detoxify ROS generated in a subcellular compartment of higher plants, algae and other organisms.

APX is a heme-containing protein, which scavange the H_2O_2 into H_2O, generated in chloroplast by dismutation of $O_2^{.-}$. To decompose the

H_2O_2 in chloroplast is essential to avoid the inhibition of the calvin cycle enzymes [84]. In another compartment such as proxies, degradation of H_2O_2 is catalyzed by cattails. But CAT is absent in the chloroplast, so degradation of H_2O_2 is catalyzed by APX in chloroplasts. APX has a higher affinity for H_2O_2 (mM range) than CAT and POD (mM range) [68].

APX detoxify the H_2O_2 into H_2O molecule by using ascorbate as electron donor and oxidize it into monodehdroascorbate (MDHA). MDHA can spontaneously dismutate into dehydroascorbate (DHA). Ascorbate is regenerated by dehydroascorbate reductase using NAD(P)H as reducing equivalents. Regeneration of ascorbate is mediated by oxidation of GSH (reduced glutathione) into GSSG (oxidized glutathione). Finally, GSH is regenerated from GSSG by GR using NADPH as reducing equivalent. Glutathione reductase (GR) is a flavoprotein oxidoreductase, found in both eukaryotes and prokaryotes [85]. It is localized predominantly in chloroplasts, but a small amount of this enzyme has also been found in mitochondria and cytosol [86, 87]. It is an important enzyme of ascorbate-glutathione cycle to maintain the GSH pool which is very important for many metabolic regulatory and antioxidative processes in plants [88, 89] Like Ascorbate peroxidase, Glutathione peroxidase also decomposes H_2O_2 to H_2O by using GSH directly as a reducing agent.

Table 1 compiles the effect of heavy metal stress on the APX and GR activity of Cyanobacteria. Hanna *et al* (2007) studied the direct effect of H_2O_2 in *Spirulina platensis* and found that the APX and GR activity was linearly increased with increasing concentration of H_2O_2. Similarly, increased activity of APX and GR of N_2 fixing cyanobacterium *Anabaena doliolum* was observed, but GR activity was decreased at higher doses of metals. And it was also noted that GR was highly correlated with APX [57]. A bell shaped dose response curve for APX was found in chromate treated *Chlorella vulgaris* which could be used as a biomarker [54].

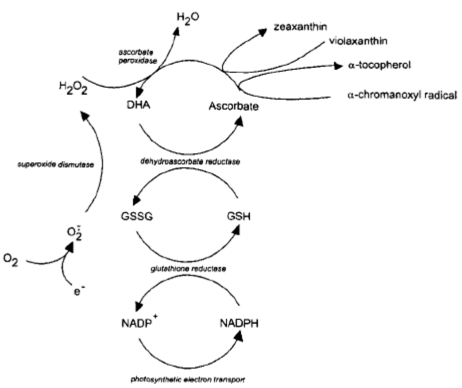

Fig. 5: Ascorbate- glutathione cycle and associated reactions adapted from [86].

Non enzymatic antioxidants

Ascorbic acid (Vitamin C)

Ascorbic acid is the most important water soluble vitamin which acts as an antioxidant to remove H_2O_2 by involving in ascorbate-glutathione cycle. It is found in all plant tissues, generally higher in photosynthetic cell. The increased cellular content of ascorbic acid in *Spirulina platensis* in response to increasing H_2O_2 concentration, indicating the contribution of ascorbic acid to remove H_2O_2 [83]. Ascorbic acid occurs higher in mature leaves with fully developed chloroplast and higher chlorophyll. It is reported that about 30 to 40% of total ascorbate is found in the chloroplast. Under normal physiological conditions, ascorbate remains in reduced form in leaves and chloroplast. Ascorbate has two oxidized forms, monodehydroascorbate and dehydroascorbate. These oxidized forms are formed in two sequential oxidation reactions, producing first monodehydroascorbate. Production of monodehydroascorbate is catalyzed by APX in chloroplasts. Monodehydroascorbate is also produced from univalent oxidation of ascorbate e. g. in reduction of superoxide and hydroxyl radicals. Monodehydroascorbate is spontaneously reduced to ascorbate and dehydroascorbate. Regeneration of ascorbate is important because oxidized form, dehydroscorbate is unstable due to short lifetime and is lost unless it is reduced to ascorbate. In plants, mitochondrion plays central role in the metabolism of ASH. Plant mitochondria are not only synthesize ASH by L-galactono-γ-lactone dehydrogenase but also take part in the regeneration of ASH from its oxidized forms [90]. Ascorbate also play important role in regeneration of α- tocopherol from α-chromanoxyl radical generated during lipid peroxidation, carotenoid and of zeaxanthin from its diepoxide, violaxanthin. Other than, the role of ascorbate in the ascorbate- glutathione cycle, it also plays an important role to preserve the activities of enzymes that include prosthetic transition metal ions [91].

Reduced Glutathione (GSH)

Tripeptide glutathione (γglu-cys-gly), the major low molecular weight thiol is found in both prokaryotes and eukaryotes, and represents a major pool of non- protein reduced sulphur [62]. It is major water soluble antioxidants in plants which provide intracellular defense against ROS induced oxidative damage by reducing them. It was observed as increased level of GSH in *Spirulina platensis* in the presence of H_2O_2 concentration [83], and in CD and UV-B treated *Anabaena sp.* [60]. It occurs abundantly in reduced form (GSH) in plant tissues and is localized in all cell compartments like cytosol, endoplasmic reticulum, vacuole, mitochondria, chloroplasts, peroxisomes as well as in apoplast [92, 93] and plays an important role in numerous physiological processes, including sulfate transport regulation, signal transduction, conjugation of metabolites, detoxification of xenobiotics [94]. Reduced glutathione helps the regeneration of ascorbate (water soluble antioxidant) by oxidizing itself into GSSG (oxidized glutathione), catalyzed by dehydroascorbate reductase via ascorbate- glutathione cycle [95]. It was observed as a decrease in GSH corresponding to increasing level of GSSG in arsenic treated aquatic cyanobacteria *Hapalosiphon fontinalis* [82]. For most of the cellular reaction, glutathione must be available in reduced form (GSH) to prevent the harmful effects of ROS induced oxidative stress [96]. It is a potent scavenger of $^1O_2$, H_2O_2 [97, 91] and most dangerous ROS like $OH^{\cdot}$ [98]. The reduction of GSSG is catalyzed by GR to glutathione (GSH). Therefore, the balance between the GSH and GSSG is necessary to maintain the cellular redox state.

Carotenoid

The plant has adapted several mechanisms to protect the photosynthetic apparatus from excess light by energy dissipation and deactivating $^1O_2$ at extremely high rates [68]. Some of which involve isoprenoid compounds consisting the carotenoids β-carotene and zeaxanthin and tocopherols serve an important photoprotective role in all photosynthetic organisms. Carotenoid, is an accessory lipid soluble pigments found in plants and microorganisms, that are organized in pigment protein complexes in thylakoid membranes where they have light harvesting and protective. There are over 600 carotenoids occurring in nature. Carotenoid performs three major functions in plants. First, the accessory light harvesting role in which, they absorb light in the wavelength region between 400 and 550 nm and transmit it to the Chlorophyll [99]. Second, an antioxidant function in which, they provide protection to the photosynthetic apparatus from photooxidative damage by quenching a triplet sensitizer (Chl^3), $^1O_2$ and other harmful free radicals which are naturally formed during photosynthesis [100]. Third, a structural role in which, they are necessary for the PSI assembly and the stability of light harvesting complex proteins as well as thylakoid membrane stabilization [101, 102]. Increased level of carotenoid in *Spirulina platensis* under H_2O_2 indicating its scavenging activity [83]. Similarly, it was also observed in heavy metals treated N_2 fixing Cyanobacterium *Anabaena doliolum* [57].

Proline

Proline plays significant roles in osmoregulation [103, 104], enzyme protection [104, 105], stabilization of the protein synthesis machinery [106], cytosolic acidity regulation [107]. In additional, proline is found to be a potent antioxidant and inhibitor of PCD. Therefore, proline is to be considered as non-enzymatic antioxidant that reduces the adverse effect of ROS. Sorbitol, mannitol, myo-inositol and Proline have been tested for OH· Scavenging capacity and it have been found that Pro appeared as an effective scavenger of OH· [108]. Free Pro have been proposed to act as an osmoprotectant, a protein stabilizer, a metal chelator, an inhibitor of LPO, and OH· and $^1O_2$ scavenger [109, 110]. An increasing concentration of accumulated proline was reported in *Spirulina Sp*. in the presence of NaCl and Cu (II) [55] and Pb, Cu and Zn [52]. And they have concluded that proline might have a role to reduce the level of NADH and acidity (2NADH + $2H^+$) for the production of proline from glutamic acid. Proline also provides a defense mechanism to tolerate the heavy metal stress by binding with metal ions due to its chelating ability. Accumulation of proline was also noted for different algal species including *Chlorella sp*., *Pediastrum duplex*, *Nitzschia palea,* and *Anacystis nidulans* in the presence of heavy metals [53].

CONCLUSION

Environmental pollution of heavy metals such as Pb, Cu, Fe, Zn, Hg, Co, Ni, and Cr, etc. is being widely spread due to anthropogenic activities. Heavy metals are transmitted to human along their food chain. It has brought a serious threat to the ecosystem. At lower concentration, heavy metals are necessary for proper metabolic function of an organism. But they become toxic at higher concentrations and target the lipid which is a key biomolecules of the cell membrane and results in serious disorder to human. To treat the heavy metal pollution, Cyanobacterial cells are extremely used as they can accumulate a significant concentration of heavy metals. But, exposure of higher concentration of heavy metals to cyanobacteria induces the generation of reactive oxygen species which are very toxic and possess oxidative threat to the cell. Cyanobacteria tolerate the heavy metal stress and reduce the toxic effect of ROS by action of several antioxidant enzymes and an accumulation of proline. The antioxidant enzymes include SOD, CAT, APX and GR. Among them SOD provides the first line of defense by reducing the concentration of superoxide. Therefore, cyanobacterial cells can be genetically modified for different antioxidant enzymes and make them resistant against heavy metals as well as exploited for enhancement of production of antioxidant compound that can be used for pharmaceutical purposes.

CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Gad NS, Saad AS. Effect of environmental pollution by phenol on some physiological parameters of oreochromis niloticus. Global Vet 2008;2:312-9.
2. Ghosh M, Singh SP. Review on phytoremediation of heavy metals and utilization of its by products. Appl Ecol Res 2005;3:1-18.
3. Jadhav JP, Kalyani DC, Telke AA, Phugare SS, Govindwar SP. Evaluation of the efficacy of a bacterial consortium for the removal of color, reduction of heavy metals, and toxicity from textile dye effluent. Bioresour Technol 2010;101:165-73.
4. Ann C, Karen S, Jos R, Kelly O, Els K, Tony R, *et al*. The cellular redox state as a modulator in cadmium and copper responses in Arabidopsis thaliana seedlings. J Plant Physiol 2011;168:309–16.
5. Aravind P, Prasad MNV, Malec P, Waloszek A, Strzalka K. Zinc protects Ceratophyllum demersum L. (free floating hydrophyte) against reactive oxygen species induced by cadmium. J Trace Elem Med Biol 2009;23:50–60.
6. Gangwar S, Singh VP, Srivastava PK, Maurya JN. Modification of chromium (VI) phytotoxicity by exogenous gibberellic acid application in Pisum sativum (L.) seedlings. Acta Physiol Plant 2011;33:1385–97.
7. Huang H, Gupta DK, Tian S, Yang X, Li T. Lead tolerance and physiological adaptation mechanism in roots of accumulating and non-accumulating ecotypes of Sedum alfredii. Environ Sci Pollut Res 2012;19:1640–51.
8. Kanoun-Boule M, Vicente JAF, Nabais C, Prasad MNV, Freitas H. Ecophysiological tolerance of duckweeds exposed to copper. Aquat Toxicol 2009;91:1–9.
9. Kulasooriya SA. Cyanobacteria: pioneers of planet earth. Ceylon J Sci (Biol Sci) 2011;40:71-88.
10. Dias MA. Removal of heavy metals by an Aspergillus terreus strain immobilized in polyurethane matrix. Lett Appl Microbiol 2002;34:46-50.
11. Lenntech Water Treatment and Air Purification: Water Treatment Published by Lenntech, Rotterdamseweg, Netherlands; 2004.
12. Weast RC. CRC handbook of chemistry and physics, 6^{4th} edn. CRC Press, Boca Raton; 1984.
13. Athar M, Vohora SB. Heavy metals and environment, New Delhi, New Age International Publisher; 2001. p. 3-40.
14. Inthorn D. Removal of heavy metal by using microalgae. Edited by hiroyuki kojima and yuan kun lee, photosynthetic microorganisms in environmental biotechnology. Springer Verlag Hong Kong Ltd 2001;310:111-69.
15. Ash C, Stone R. A question of dose. Sci 2003;300(5621):849-1036.
16. Allan R. Mining and metals in the environment. J Geochem Explor 1997;58(2):95–100.
17. Holum JR. Elements of General and Biological Chemistry, 6th Edition, John Wiley and Sons NY; 1983. p. 324, 326, 353, 469.
18. McCluggage D. Heavy Metal Poisoning, NCS Magazine, Published by The Bird Hospital, CO, USA; 1991.
19. Dayan AD, Paine AJ. Mechanisms of chromium toxicity, carcinogenicity and allergenicity. Hum Expos Toxicol 2001;20:439–51.
20. Patrick L. Lead toxicity, a review of the literature. Part 1: Exposure, evaluation, and treatment. Altern Med Rev 2006;11:2–22.
21. Trueby P. Impact of Heavy Metals on Forest Trees From Mining Areas. In: International Conference on Mining and The Environment III, Sudbury, Ontario, Canada; 2003.
22. Mudgal V, Madaan N, Mudgal A, Singh RB, Mishra S. Effect of toxicmetals on human health. Open Nutr 2010;3:94–9.
23. Saha N, Zaman MR. Evaluation of possible health risks of heavy metals by consumption of foodstuffs available in the central market of Rajshahi City, Bangladesh. Environ Monit Assess 2012;185:3867-78.
24. Hadson PV. The effect of metabolism on uptake, disposition and toxicity in fish. Aquat Toxicol 1988;11:3–18.
25. Akoto O, Bismark Eshun F, Darko G, Adei E. Concentrations and health risk assessments of heavy metals in fish from the fosu lagoon. Int J Environ Res 2014;8(2):403-10.
26. Terra BF, Araújo FG, Calza CF, Lopes RT, Teixeira TP. Heavy metal in tissues of three fish species from different trophic levels in a tropical brazilian river. Water Air Soil Pollut 2007;187:275-84.
27. Yilmaz AB. Levels of heavy metals (Fe, Cu, Ni, Cr, Pb, and Zn) in tissue of Mugil cephalus and Trachurus mediterraneus from Iskenderun Bay, Turkey. Environ Res 2003;92:277–81.
28. Al-Busaidi M, Yesudhanon P, Al-Mughairi S, Al-Rahbi WAK, Al-harthy KS, Al-Mazrooei NA, *et al*. Toxic metals in commercial marine fish in Oman with reference of national and international standards. Chemosphere 2011;85:67–73.
29. Emami Khansari F, Ghazi-Khansari M, Abdollahi M. Heavy metals content of canned tuna fish. Food Chem 2005;93:293-6.
30. Landis GW, Ming-Ho Yu. Introduction to Environmental Toxicology: Impacts of Chemicals Upon Ecological Systems. CRC Press, Lewis Publishers, Boca Raton, Fl; 2003.
31. Palmer CD, Puls RW. Natural attenuation of hexavalent chromium in ground water and Soils. USEPA Ground Water Issue, EPA/540/5-94/505, U. S. Govt. Print. Office, Washington, DC; 1994. p. 57-72.
32. Vincent WF. Cyanobacteria. Laval University, Quebec City, QC, Canada Elsevier Inc; 2009. p. 226-32.
33. Azolla Utilization: Proceedings of the Workshop on Azolla Use, Fuzhou, Fujian, China 1985. The International Rice Research Institute, Los Banos, Philippines; 1987. p. 296.
34. Venkataraman GS. Algal Biofertilizers and Rice Cultivation. Today and Tomorrows Printers and Publishers, Calcutta; 1972. p. 81.
35. Habib MAB, Parvin M, Huntington TC, Hasan MR. A review on culture, production and use of spirulina as food for humans and

feeds for domestic animals and fish. FAO Fisheries and Aquaculture Circular. No. 1034. Rome, FAO; 2008. p. 33.
36. Namiki M. Antioxidant/ antimutagenics in food. In: CRC. Crit Rev Food Sci Nutr 1990;29:273-300.
37. Mirada MS, Cintra RG, Barros SM, Mancini-Filho J. Antioxidant activity of microalga Spirulina maxima. Braz J Med Biol Res 1998;31:1075-9.
38. Pratt DE. Natural antioxidants from plant material, Phenolic compounds: Food and their effects on health. Am Chem Soc, Washington, (ACS Symposium Series 1992;507:54-71.
39. Robbins J. Anti-inflammatory and Antioxidant effects. Diet for A new America; 1987. p. 20.
40. Sudha SS, Karthic R, Francis M, Soumya TS, Ramanujam JR. Associated microorganisms from spirulina p-roducts. Isolation and preliminary characterization of associated microorganisms from Spirulina products and their silver mediated nanoparticle synthesis. J Algal Biomass Utln 2011;2:1–8.
41. Perales-Vela HV, Pen˜a-Castro J, Can˜izares-Villanueva RO. Heavy metal detoxification in eukaryotic microalgae. Chemosphere 2006;64:1-10.
42. Cobbett C, Goldsbrough P. Phytochelatin and metallothioneins: Roles in heavy metal detoxification and homeostasis. Ann Rev Plant Biol 2002;53:159–82.
43. Liu RX, Tang HX, Lao WX. Advances in bio-sorption mechanism and equilibrium modeling for heavy metals on biomaterials. Prog Chem 2002;14:87-92.
44. Goyal N, Jain SC, Banerjee UC. Comparative studies on the microbial adsorption of heavy metals. Adv Environ Res 2003;7:311-9.
45. Arunakumara KKIU, Xuecheng Z. Heavy metal bioaccumulation and toxicity with special reference to microalgae. J Ocean Univ Chin 2008;7:25-30.
46. Wang J, Chen C. Biosorption of heavy metals by *Saccharomyces cerevisiae*. Biotech Adv 2006;24:427-51.
47. Babu RL, Vijayalakshmi E, M Naveen Kumar, Patil RH, Devaraju KS, Sharma SC. Biosorption of Chromium(VI) and Lead(II): Role of Spirulina platensis in the Treatment of Industrial Effluent. Biorem J 2013;17(4):231-9.
48. Balaji S, Kalaivani T, Rajasekaran C, Shalini M, Siva R, Singh RK, *et al*. *Arthrospira (Spirulina)* species as bioadsorbents for lead, chromium and cadmium–a comparative study. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim; 2014.
49. Pandi M, Shashirekha V, Swamy M. Bioabsorption of chromium from retan chrome liquor by cyanobacteria. Microbiol Res 2009;164:420-8.
50. Tripathi BN, Gaur JP. Physiological behavior of *Scenedesmus sp*. during exposure to elevated levels of Cu and Zn and after withdrawal of metal stress. Protoplasma 2006;229:1-9.
51. Choudhury S, Panda SK. Toxic effects, oxidative stress and ultrastructural changes in moss Taxithelium nepalense (Schwaegr.) Broth under chromium and lead phytotoxicity. Water Air Soil Pollut 2005;167:73-90.
52. Choudhary M, Jetley UK, Khana MA, Zutshi N, Fatma T. Effect of heavy metal stress on proline, malondialdehyde, and superoxide dismutase activity in the cyanobacterium *Spirulina platensis-S5*. Ecotoxicol Environ Saf 2007;66(2):204-9.
53. Jiunn-Tzong W, Chang S, Chou T. Intracellular proline accumulation in some algae exposed to Copper and Cadmium. Bot Bull Acad Sin 1995;36:89-93.
54. Rai UN, Singh NK, Upadhyay AK, Verma S. Chromate tolerance and accumulation in *Chlorella vulgaris* L: role of antioxidant enzymes and biochemical changes in detoxification of metals. Bioresour Technol 2013;136:604–9.
55. Deniz F, Saygider SD, Karaman S. Response to copper and sodium chloride excess in spirulina sp. (cyanobacteria). Bull Environ Contam Toxicol 2011;87:11-5.
56. Jayashree S, Thangaraju N, Gnanadoss JJ. Toxic effects of chromium on the aquatic cyanobacterium Oscillatoria sp. and removal of chromium by biosorption. J Exp Sci 2012;3(5):28-34.
57. Sultan P, Williams Shah MI, Arif Jan P, Ahamad N. Biochemical basis of heavy metal induced stress tolerance in the N_2 fixing Cyanobacterium *Anabaena doliolum*. Afr J Cln Exper Microbiol 2007;8:8-22.
58. Khan UA, Iffat ZA. Alterations in antioxidative defense system of *anabaena variabilis* in the presence of heavy metals. APCBEE Procedia 2013;5:491-6.
59. Shukla MK, Tripathi RD, Sharma N, Dwivedi S, Mishra S, Singh R, *et al*. Responses of cyanobacterium *Anabaena doliolum* during nickel stress. J Environ Biol 2009;30(5):871-6.
60. Singh PK, Rai S, Pandey S, Agrawal C, Shrivastava AK. Kumar S, Rai LC. Cadmium and UV-B induced changes in proteome and some biochemical attributes of *Anabaena* sp. PCC7120. Phykos 2012;42(1):39-50.
61. Visviki I, Rachlin JW. The toxic action and interactions of copper and cadmium to the marine alga Dunaliella minuta in both acute the chronic exposure. Arch Environ Contam Toxicol 1991;2:271–5.
62. Mallick N, Mohn FH. Reactive oxygen species: response of algal cells. J Plant Physiol 2000;157:183-93.
63. Klotz LO. Oxidant-induced signaling: effects of peroxynitrite and singlet oxygen. Biol Chem 2002;383:443–56.
64. Apel K, Hirt H. Reactive oxygen species: metabolism oxidative stress and signal transduction. Annu Rev Plant Biol 2004;55:373–99.
65. Navrot N, Rouhier N, Gelhaye E, Jaquot JP. Reactive oxygen species generation and antioxidant systems in plant mitochondria. Physiol Plant 2007;129:185-95.
66. Wise RR, Naylor AW. Chilling-enhanced photooxidation. Evidence for the role of singlet oxygen and superoxide in the breakown of pigments and endogenous antioxidants. Plant Physiol 1987;83:278-82.
67. Moller IM. Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. Annu Rev Plant Physiol Mol Biol 2001;52:561-91.
68. Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 2010;48:909-30.
69. Alscher RG, Donahue JL, Cramer CL. Reactive oxygen species and antioxidants: relationships in green cells. Physiol Plant 1997;100(2):224-33.
70. Smirnoff N. The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol 1993;125:27-58.
71. Weckx JEJ, Clijsters H. Oxidative damage and defense mechanisms in primary leaves of Phaseolus vulgaris as a result of root assimilation of toxic amounts of copper. Physiol Plant 1996;96:506-12.
72. Meneguzzo S, Navari-Izzo F, Izzo R. Antioxidative responses of shoots and roots of wheat to increasing NaCl concentrations. J Plant Physiol 1999;155:274-80.
73. Doke N, Miura Y, Leandro MS, Kawakita K. Involvement of superoxide in signal transduction: responses to attack by pathogens, physical and chemical shocks and UV-irradiation. In: Foyer CH, Mullineaux P (eds) Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC Press Inc, Boca Raton, Fl; 1994. p. 177-97.
74. Murphy TM, Huerta AJ. Hydrogen peroxide formation in cultured rose cells in response to UV-C radiation. Physiol Plant 1990;78:247-53.
75. Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 2002;7:405-10.
76. Gratao PL, Polle A, Lea PJ, Azevedo RA. Making the life of heavy metal stressed plants a little easier. Funct Plant Biol 2005;32:481-94.
77. Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine, third ed. Oxford University Press, New York; 1999.
78. Garg N, Manchanda G. ROS generation in plants: boon or bane? Plant Biosys 2009;143:8-96.
79. Heath RL, Packer L. Photoperoxidation in isolated chloroplasts: kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 1968;125:180-98.
80. Luximon Ramma A, Bahorun T, Soobrattee M, Aruoma OI. Antioxidant activities of phenolic, proanthocyanidin and flavonoid components in extracts of Cassia fistula. J Agric Food Chem 2002;50:5042–7.
81. Giannopolitis CN, Ries SK. Superoxide dismutase: occurrence in higherplants. Plant Physiol 1977;59:309-14.
82. Zutshi S, Bano F, Ningthoujam M, Habib K, Fatma T. Metabolic adaptations to arsenic-induced oxidative stress in *hapalosiphon fontinalis-339*. Int J Inn Res Sci Eng Technol 2014;3(2):9386-94.
83. Hanaa H, Abd El-Baky, FKE Baz, Gamal S. El-Baroty: enhancement of antioxidant production in *spirulina plantensis* under oxidative stress. Am Eur J Sci Res 2007;2(2):170-9.

84. Kaiser W. The effect of hydrogen peroxide on $C0_2$ fixation of isolated intact chloroplasts. Biochim Biophys Acta 1976;440:476-82.
85. Romero-Puertas MC, Corpas FJ, Sandalio LM, Leterrier M, Rodriguez Serrano M, Rio LAD, *et al*. Glutathione reductase from pea leaves: response to abiotic stress and characterization of the peroxisomal isozyme. New Phytol 2006;170:43-52.
86. Creissen G, Edwards A, Mullineaux P. Glutathione reductase and ascorbate peroxidase. In: Foyer CH, Mullineaux P (eds) Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants. CRC Press Inc, Boca Raton, Fl; 1994. p. 344-64.
87. Edwards EA, Rawsthorne S, Mullineaux PM. Subcellular distribution of multiple forms of glutathione reductase in leaves of pea (Pisum sativum L.). Planta 1990;180:278-84.
88. Chalapathi Rao ASV, Reddy AR. Glutathione reductase: a putative redox regulatory system in plant cells. in: NA Khan, S Singh, S Umar (Eds.), Sulfur Assimilation and Abiotic Stresses in Plants. Springer, The Netherlands; 2008. p. 111-47.
89. Reddy AR, Raghavendra AS. Physiology and Molecular Biology of Stress Tolerance in Plants. Springer, The Netherlands; 2006. p. 157-86.
90. Szarka A, Horemans N, Kovacs Z, Grof P, Mayer M, Banhegyi G. Dehydroascorbate reduction in plant mitochondria is coupled to the respiratory electron transfer chain. Physiol Plant 2007;129:225-32.
91. Noctor G, Foyer CH. A re-evaluation of the ATP: NADPH budget during C3 photosynthesis. A contribution from nitrate assimilation and its associated respiratory activity? J Exp Bot 1998;49:1895-908.
92. Mittler R, Zilinskas BA. Molecular cloning and characterization of a gene encoding pea cytosolic ascorbate peroxidase. J Biol Chem 1992;267:21802-7.
93. Jimenez A, Hernandez JA, Pastori G, Rio LAD, Sevilla F. Role of the ascorbate-glutathione cycle of mitochondria and peroxisomes in the senescence of pea leaves. Plant Physiol 1998;118:1327-35.
94. Xiang C, Werner BL, Christensen EM, Oliver DJ. The biological functions of glutathione revisited in *Arabidopsis* transgenic plants with altered glutathione levels. Plant Physiol 2001;126:564-74.
95. Foyer CH, Halliwell B. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 1976;133:21–5.
96. Meyer AJ. The integration of glutathione homeostasis and redox signaling. J Plant Physiol 2008;165:1390-403.
97. Briviba K, Klotz LO, Sies H. Toxic and signaling effects of photochemically or chemically generated singlet oxygen in biological systems. J Biol Chem 1997;378:1259-65.
98. Larson RA. The antioxidants of higher plants. Phytochem 1988;27:969-78.
99. Sieferman-Harms D. The light harvesting function of carotenoids in photosynthetic membrane. Plant Physiol 1987;69:561-8.
100. Collins A. Carotenoids and genomic stability. Mutat Res 2001;475:1-28.
101. Gill SS, Khan NA, Anjum NA, Tuteja N. Amelioration of cadmium stress in crop plants by nutrients management: morphological, physiological and biochemical aspects. Plant Stress (Spl issue); 2010(in press).
102. Niyogi KK, Shih C, Chow WS, Pogson BJ, DellaPenna D, Bjorkman O. Photoprotection in a zeaxanthin-and lutein-deficient double mutant of Arabidopsis. Photosynth Res 2001;67:139-45.
103. Ahmad I, Hellebust A. The relationship between inorganic nitrogen metabolism and proline accumulation in osmoregulatory responses of two euryhaline microalgae. Plant Physiol 1988;88(2):348–54.
104. Laliberte G, Hellebust JA. Regulation of proline content of Chlorella autopica in response to change in salinity. Can J Bot 1989;67:1959–65.
105. Nikolopoulos D, Manetas Y. Compatible solutes and in vitro stability of Salsola soda enzyme: proline incompability. Phytochem 1991;30:411–3.
106. Kadpal RP, Rao NA. Alteration in the biosynthesis of proteins and nucleic acid in finger millet (Eleucine coracana) seedling during water stress and the effect of proline on protein biosynthesis. Plant Sci 1985;40:73–9.
107. Venekemp JH. Regulation of cytosolic acidity in plants under condition of drought. Plant Physiol 1989;76:112–7.
108. Smirnoff N, Cumbes QJ. Hydroxyl radical scavenging activity of compatible solutes. Phytochem 1989;28:1057-60.
109. Ashraf M, Foolad MR. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Env Exp Bot 2007;59:206-16.
110. Trovato M, Mattioli R, Costantino P. Multiple roles of proline in plant stress tolerance and development. Rendiconti Lincei 2008;19:325-46.