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SUSTAINABLE IMPACT OF Zn^{2+} INDUCED MILD STRESS ON EDIBLE *SPIRULINA* SPECIES: RESPONSES OF PHOTOSYNTHESIS, OXIDATIVE BIOMARKER AND ANTIOXIDANT DEFENSE SYSTEM

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Abstract— **Since Zinc (Zn^{2+}) is an important microelement due to its ability to defend organisms against free radicals, we investigated the effects of Zn^{2+} (as $ZnCl_2$) on growth, photosynthetic and oxidative response of exponential phase cultures of two cyanobacterial species, *Spirulina maxima* (*S. maxima*) and *Spirulina platensis* (*S. platensis*) as a method of producing Zn^{2+} enriched medium for their cultivation. The cyanobacterial strains were cultured in Zarrouk's medium containing 46 µg Zn^{2+} L^{-1} as control, and further treated with additional Zn^{2+}, considered as low, middle and high doses which correspond to 2.5, 3.5 and 4.5 mg L^{-1} respectively. Measurement was based on differential response of organism with and without Zn^{2+} treatment after 72 and 144 h of incubation. Results showed that Zn^{2+} at low dose stimulated the growth and other physiological parameters, and such stress adapted biomass of *Spirulina* may be used as a matrix for functional food containing zinc for human wellbeing.**

Keywords— ***Spirulina maxima, Spirulina platensis,* mild oxidative stress; Zinc (Zn^{2+}); photosynthetic and antioxidant activity.**

I. INTRODUCTION

Zn^{2+}, an essential trace element, is known to serve as the active center of several hundreds of enzymes and thousands of protein domains; hence it has great importance in human nutrition and health[1, 2]. It maintains the structural integrity of various proteins such as superoxide dismutase and zinc finger transcription factors and modulates protein–protein interactions[3]. At the cellular level it plays an important role in cell proliferation and survival, contributes to genomic stability and antioxidant defense, hence it has crucial role in aging and age-dependent degenerative diseases in humans[4]. The daily dietary Zn^{2+} intake ranges from 5.6 to 13 mg day^{-1} in infants and children and from 8.8 to 14.4 mg day^{-1} in adults[5]. Zn^{2+} deficiency in human causes hypogeusia, hyposmia, growth retardation, dermatitis, and delayed wound healing[6]. Conversely, more accumulation of Zn^{2+} in biotic systems such as plants, cyanobacteria, animals and other organisms creates oxidative stress and as a contaminant it enters into food chain and affects human health at high concentration[7]. Zn^{2+} is the main industrial pollutant deriving from the production of rubber, batteries, petrol, steel and paints. Although Zn^{2+} is an essential requirement for good health as it acts as an essential trace element for physiological processes, in human, plants and other living being, but at high concentrations it becomes toxic and declines the growth of organism[8]. According to WHO (World health organization) the maximum acceptable limit of Zn^{2+} is 5.0 mg L^{-1} in drinking water[9]. A far more common risk to human health is Zn^{2+} deficiency than intoxication; caused by malnutrition and foods with low bioavailability, aging, certain diseases, or deregulated homeostasis[10].

Cyanobacteria also known as blue green algae are diverse group of photosynthetic prokaryotes and have a high capability to tolerate various kinds of stresses in their natural habitats. They are often exposed to external conditions, such as temperature, heat, ultraviolet radiation, desiccation/drought, salinity, and metals. Earlier it was noticed that in Zn^{2+} enriched waters with high pH, cyanobacteria are abundant and can be the dominant organisms[11]. Among the various species of *Spirulina*, *S. platensis* and *S. maxima* are the most important[12], as they are

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largely used as human dietary supplement, whole food, and feed supplement in aquaculture, aquarium and poultry industries due to high amount of balanced proteins, wide range of nutraceuticals[13], carotenes, and other pigments. Phycocyanin obtained from *Spirulina* is such pigment has an antioxidant activity[14], hence receiving attention of researchers as well as different industries. It was reported that environmental stress is the major limiting factor for cultivation of *Spirulina*[15] as it affects the growth performance as well as cellular constituents[16].

The heavy metals in trace amounts are essential for various metabolic processes in organisms; though in large amounts can induce physiological stress leading to generation of free radicals[17]. Zn^{2+} at higher concentration inhibits photosynthesis by the binding to the oxidation sites as well as the reduction sites of PSII due to the sulphydryl group binding property in proteins[18, 19]. It can substitute the central Mg^{2+} in the chlorophyll molecule, hence alters the spectral property and also lowers the fluorescence quantum yield[20, 21]. This in turn induces the production of reactive oxygen species (ROS). Cyanobacteria have ability to perceive ROS and to rapidly initiate antioxidant defenses for their survival. Every cyanobacteria have complex array of enzymatic antioxidant defense system which comprises mainly the enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione S-transferase (GST) [22, 23]. So far there is need of renewed efforts that bring together studies addressing the interaction between these cyanobacteria and the environment at all levels of organization and precise/mild stressing might prove an efficient way to boost up the productivity[24] as a mechanism of fast, but reversible, modulation of growth and maintains the balance between growth and survival[25]. Therefore, for better exploitation of cyanobacteria as functional food application in near future; their degree of tolerance and tolerance strategies against precise/mild stresses must be known.

It has been reported that the amount of Zn^{2+} ingested per day may be insufficient relative to the daily requirement in some groups of individuals resulting in Zn^{2+} deficiency. As is the case for all nutrients, the challenge is to achieve intakes of bioavailable Zn^{2+} and tissue levels within a physiological range.While taking another aspect i.e. Zn^{2+} (at high doses) tolerance in cyanobacteria, it has been extensively studied aimed at revealing their phytoremedial properties and metal sorption activities with regard to utilization in environmental safety. However, there are very few reports pointing to the precise/mild stress with low dose treatment and their effects on cyanobacterial strains from the prospects of possible functional food applications[26].

Therefore, keeping the above fact in mind the aim of this study was set a forth to understand phenomena precise /mild stress induced by Zn^{2+} on the two species of *Spirulina* i.e. *Spirulina maxima* and *Spirulina platensis* with their respective antioxidant defense system response. Different physiological parameters were analyzed such as growth, pigments, protein content, photosynthesis, respiration, fluorescence parameters (Fv/Fm, NPQ and qP) carbohydrate content, free radical like superoxide radical (SOR) and hydrogen peroxide (H_2O_2) as oxidative stress markers, lipid peroxidation (malondialdehyde; MDA) as indices of oxidative damage, antioxidant enzymes superoxide dismutase (SOD), peroxidase (POD), some non enzymatic antioxidants such as cystein, proline and ascorbate, total antioxidant activity (DPPH) and total phenolic content (TPC), which more importantly deals with oxidative stress and antioxidant system despite of ecological and economic importance of *Spirulina* sp. (*S. maxima* and *S. platensis*) which can be utilized as antioxidant of natural origin in different food and feed supplements to enhance their functionality and nutritional quality. We were especially interested in determining whether the precise/mild stress conditions may have either positive or negative impacts on growth, photosynthesis, chemical and enzymatic antioxidants of the test organisms which may further have broad application in the development of food and feed supplements via utilizing extracts and/or whole cell of *Spirulina* grown under these conditions.

II. Materials and Methods

Organism and culture condition

Two strains of cyanobacterium *Spirulina viz S. maxima* and *S. platensis* were collected from paddy field of Allahabad, isolated and auxenic cultures of this strains were grown in Zarrouk medium[27] at 27±2 ^{0}C under the photosynthetically active radiation (PAR) of 75 µmol photon $m^{-2}s^{-1}$ with a 16/10 h of light/dark period[28]. Manual shaking of cultures was done 3-4 times regularly. The early exponential phase cultures were used for each experiment.

Micronutrient Zinc (Zn^{2+}) treatment

Exponentially grown *S. maxima* and *S. platensis* culture were exposed to varying concentrations i.e. 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 8.0 and 10.0 mg L^{-1} of Zn^{2+} to understand the survival behavior of cyanobacterium against Zn^{2+} (screening experiments). The cyanobacterial strains were cultured in Zarrouk medium containing 46 µg Zn^{2+} L^{-1} as control, and further treated with additional Zn^{2+} (as $ZnCl_2$), considered as low, middle and high doses which correspond to 2.5, 3.5 and 4.5 mg L^{-1} respectively. Treated and untreated cells were kept in 250 ml conical flask under 75 µmol photon $m^{-2}s^{-1}$ of PAR in culture room at 27 ± 2 ^{0}C under 16/8 h of light/dark period.

Analysis of different physiological and biochemical parameters

Different physiological and biochemical parameters were analyzed after 72h (exponential growth phase) when medium was rich in nutrient providing maximum specific growth rate

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to the organisms and 144 h (early stationary growth phase)when medium becomes limiting resulting in lower specific growth rate of organism and decreased photosynthetic potential (probably due to self-shading effect) [29]. The results were compared with untreated culture (control) of *S. maxima* and *S. platensis*.

Measurement of growth

Measurement of survival behavior of both species of *Spirulina* was estimated by recording absorbance of culture at 750 nm using UV-VIS spectrophotometer (model 1700, Shimadzu, Japan). For the measurement of biomass accumulation each sample was centrifuged and dried at 80 ^{0}C for 24h. Finally, the dry weight of sample was determined by single pan electronic balance (Contech- CA 223, India). For protein estimation a definite amount of cell suspension was taken, centrifuged and 1N NaOH was added to pellets and the sample was kept on boiling water bath for 10 min. After cooling, the reagents were added to the sample and the amount of protein was determined according to the method of Lowry et al. (1951) [30].

Photosynthetic pigment extraction and estimation

Five ml of each test culture was centrifuged at 10,000x*g* for 10 min in CPR 30 (Remi, India), and the pellets obtained were suspended in 100% methanol, mixed well and kept at 4^{0}C for overnight for pigment extraction. After 24 h each sample was centrifuged and supernatant was used for estimation of Chl *a* and carotenoids[31, 32]. The absorbance recorded by spectrophotometer (UV-VIS-1700, Shimadzu, Japan) at 665 and 450 nm was used to calculate Chl *a* and carotenoids respectively and the amount of Chl *a* and carotenoids is expressed as µg ml^{-1}culture.

Phycocyanin was extracted by adding 1-2 drops of pure toluene in pellets of treated and untreated cultures of the strains and mixed well. The samples were kept for overnight and pellets were added with 50 mM, pH 7.0 phosphate buffers, and after centrifugation the absorption of supernatant was read at 620 nm[33]. The amount of phycocyanin was expressed as µg ml^{-1}culture.

Measurements of whole cell photosynthetic O_2-evolution and respiration

Photosynthetic activity in terms of whole cell O_2-evolution and respiration of treated and untreated cells of both the test organismswere estimated by measuring O_2-evolution and O_2-consumption, respectively for 5 min using Digital Oxygen System (Rank Brothers, UK) in a temperature controlled air-tight reaction vessel at 25 ^{0}C. Photosynthetic O_2 evolution was measured under saturating light intensity of 380 µmol m^{-2} s^{-1} PAR, provided by projector lamp, and respiration was measured as O_2 consumption in darkness. The photosynthetic O_2 evolution activity is expressed as µmol O_2 evolved mg^{-1} Chl *a* h^{-1} whereas respiration is expressed as µmol O_2 consumed mg^{-1}Chl *a* h^{-1}.

Chlorophyll (Chl a) fluorescence determination

For the assessment of photosynthetic performance, chlorophyll *a* fluorescence measurements were taken in dark adapted cultures of control and treated test organism using hand held culture fluorometer (AquaPen-CAP-C 100, Photos System Instruments, Czech Republic). Fluorescence parameters: maximum photochemical efficiency of photosystem II (Fv/Fm), photochemical quenching (qP) and non-photochemical quenching (NPQ) were assessed. Measurements were taken from each set in triplicates after 72 and 144 h of experiments.

Estimation of superoxide radicals and hydrogen peroxide

Superoxide radicals (SOR; $O_2^{\bullet-}$) in treated and untreated cells were measured by following the method of Elstner and Heupal (1976) [34] based on formation of $NO_2^-$from hydroxylamine in the presence of $O_2^{\bullet-}$. Cells were homogenized in 65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 10,000x*g* for 10 min at 4 ^{0}C. The reaction mixture consisted of 65 mM potassium phosphate buffer (pH 7.8), 10 mM hydroxylamine hydrochloride and supernatant and it was incubated for 20 min at 25 ^{0}C. After this, 17 mM sulfanilamide and 7 mM naphthylethylene diamine dihydrochloride were mixed to the incubated reaction mixture. After 15 min of reaction, diethyl ether was mixed to the same reaction mixture gently and centrifuged at 2,000x*g* for 5 min. The absorbance of the colored aqueous phase was recorded at 530 nm. A standard curve was prepared with $NaNO_2^-$ and used to calculate the production of $O_2^{\bullet-}$.

Hydrogen peroxide (H_2O_2) in treated and untreated cells was measured by following the method of Velikova et al. (2000) [35]. For this cells were homogenized in 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000x*g* for 10 min at 4 ^{0}C. The reaction mixture (2 ml) contained tissue extract (0.5 ml), 10 mM potassium phosphate buffer (pH 7.0) and 1 M KI solution. Absorbance of reaction mixture was read at 390 nm. Hydrogen peroxide concentration was calculated by using a standard curve prepared with H_2O_2.

Measurement of lipid peroxidation

Damage to lipids was determined as lipid peroxidation by measuring production of malondialdehyde (MDA) [36]. Treated and untreated cells were crushed in 5% (w/v) TCA centrifuged at 10,000 g for 10 min at 4 ^{0}C. Reaction mixture contained thiobarbituric acid-TCA solution and cell extract and heated at 90^{0}C for 20 min and then quickly cooled in ice bath followed by centrifugation. The absorbance of reaction mixture was recorded at 532 and 600 nm. The concentration of MDA was calculated by using an extinction coefficient 155 mM^{-1} cm^{-1}.

Estimation of total antioxidant activity (2, 2-diphenyl-1-picrylhydrazyl assay)

The measurement of hydrogen donating capability of extract was assessed using DPPH (2, 2’ diphenyl-1- picrylhydrazyl)

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radical as substrate, following the method described by Athavale et al. (2012) with some modification[37]. DPPH radical gives strong absorbance at 517 nm (deep violet color) due to its unpaired electron. When this radical pairs off in presence of a free radical scavenger, the absorption vanishes and the resulting discoloration is stoichiometric with respect to the number of electrons taken up. In this assay, 0.2 ml of extract solution was added to 3ml of 0.1 mM methanolic DPPH solution and absorbance was read at 517 nm after 30 minutes incubation in dark at room temperature. The decrease in absorbance at ambient temperature was correlated with the scavenging action of the test compound and compared with ascorbic acid (used as standard). The radical scavenging activity was calculated using equation (1-AS/AC) × 100; AC = Absorbance of Control, AS = Absorbance of Sample solution.

Estimation of enzymatic antioxidants

For measurement of activity of each antioxidant enzyme, treated and untreated samples were harvested by centrifugation at 4,000x*g* for 10 min, and cells obtained were used for estimating enzymatic antioxidants.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed by crushing cells in EDTA-phosphate buffer (pH 7.8) and centrifuged at 15,000x*g* for 20 min at 4 ^{0}C and supernatant was used as enzyme. Reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7.8), 1.3 µM riboflavin, 0.1 mM EDTA, 13 mM methionine, 63 µM nitrobluetetrazolium (NBT), 0.05 M sodium carbonate (pH 10.2) and enzyme extract (0.1 ml). The reaction mixtures were illuminated for 20 min under white light intensity of 100 µmol photons m^{-2} s^{-1}. The photo reduction of NBT (formation of purple formazone) was recorded spectrophotometrically at 560 nm and compared with blank samples having no enzyme extract[38]. One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition in reduction of NBT.

Peroxidase (POD, EC 1.11.1.7) activity was determined by using the enzyme extract prepared in 50 mM potassium phosphate buffer (pH 7). The reaction mixture (3 ml) contained 0.9 ml of 100 mM potassium phosphate buffer, 100 mM pyragallol, 25 mM H_2O_2 and 0.1 ml enzyme extract. Increase in the absorbance due to oxidation of pyragallol was measured at 430 nm for 1 min[39].

Estimation of total phenolic content

Total phenolic contents (TPCs) were estimated following the method of Waterhouse (2001) [40]. Cells, obtained from 20 mL culture suspension of each sample, were extracted in 1 mL ethanol, centrifuged at 10,000×*g* for 10 min. Then, 0.1 mL of ethanolic extract was mixed with 1.5 mL distilled water and 0.1 mL of folin phenol reagent (2 N), and after gentle shaking, 0.3 mL of sodium carbonate (20 %) was added. Samples were incubated for 30 min in a water bath maintained at 40 ^{0}C. The absorbance was read at 765 nm and compared with the standard curve prepared by gallic acid. The amount of TPCs was expressed as µg gallic acid equivalent mg^{-1} dry weight.

Estimation of non-enzymatic antioxidants

Proline content was estimated according to the method of Bates et al. (1973) [41]. Cells obtained from 20 mL culture suspension of each sample were crushed in 3 % (w/v) sulfosalicylic acid, centrifuged at 10,000×*g* and 1 mL of this extract was mixed with 3 % (v/v) glacial acetic acid and acid ninhydrin solution. Samples were heated for 1 h in a water bath maintained at 95 ^{0}C, cooled, and extracted with 4 mL toluene by vortexing for 5 min with a test tube mixer. The toluene layer was taken for recording the absorbance at 520 nm using toluene as blank. The proline content in each sample was calculated with the help of standard curve.

Total ascorbate (reduced ascorbate + dehydroascorbate; AsA + DHA) was determined by the method of Gossett et al. (1994) [42]. This assay is based on reduction of Fe^{3+} into Fe^{2+} with ascorbic acid in acid solution followed by the formation of red chelate between Fe^{2+} and 2,2-bipyridyl. Treated and untreated cells (20 mL) were homogenized in 5 % (w/v) m-phosphoric acid using a mortar and pestle in cool condition. The homogenate was centrifuged at 22,000×*g* for 15 min. Total ascorbate was determined in a reaction mixture consisting of 0.2 mL of supernatant, 0.5 mL of 150 mM potassium phosphate buffer (pH 7.4) containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mL of 10 mM dithiothreitol (DTT) to reduce dehydroascorbate (DHA) into reduced ascorbate (AsA). After 10 min of incubation at room temperature, 0.1 mL of 0.5 % (w/v) N-ethylmaleimide was mixed in samples and stirred for 5 min to remove excess DTT. Color developed in the reaction mixture by the addition of 0.4 mL of 10 % (w/v) trichloroacetic acid (TCA), 0.4 mL of 44 % (v/v) O-phosphoric acid, 0.4 mL of 4 % (w/v) 2, 2-bipyridyl in 70 % (v/v) ethanol, and 0.2 mL of 3 % (w/v) $FeCl_3$. The reaction mixture was incubated at 40 ^{0}C for 1 h and quantified spectrophotometrically at 525 nm. Total ascorbate content was calculated by using standard curve prepared with L-ascorbic acid.

Cysteine content was extracted and estimated of by the method of Gaitonde (1967) [43]. Cells (40 mL) were centrifuged and crushed in 5 % chilled perchloric acid, and homogenates were centrifuged at 8000×*g* for 10 min. A required amount of glacial acetic acid and acid ninhydrin was added to the extract, and reaction mixture was kept at 95 ^{0}C for 30 min. After cooling, the absorbance of reaction mixture was read at 560 nm, and the amount of cysteine was calculated by the standard curve.

Statistics

Results were statistically analyzed by analysis of variance (ANOVA). Duncan's multiple range test was applied for mean separation for significant differences among treatments

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at P<0.05 levels. The results presented are the means of three independent experiments.

III. Experiment and Result

The results of this study showed Zn^{2+} induced precise/mild stress effect on growth, photosynthetic pigment contents, photosynthesis, florescence parameters, protein content, total antioxidant activity, reactive oxygen species generation, lipid peroxidation and antioxidants in the two species of cyanobacterium *Spirulina maxima* and *Spirulina platensis*. Test cyanobacterium experiencing stress have major impact on its nutritional properties which directly affect health of neighboring organism including human as the stressor itself is passed on into the food chain.

Growth

Both spiral cyanobacterial cells (*S. maxima* and *S. platensis*) were treated with different concentrations; 2.5, 3.5 and 4.5 mg L^{-1} considered as low, middle and high dose of Zn^{2+} to evaluate the effect of these variable ranges of Zn^{2+} on biomass productivity, measured as optical density of cell suspension at 750 nm (as shown in supplemental data, Fig. S1 and S2). The Zn^{2+} concentration response curve showed that the growth was significantly (P<0.05) higher at the 2.5 mg L^{-1} while 3.5 and 4.5 mg L^{-1} of Zn^{2+} concentration caused significant (P<0.05) reduction in growth of tested species of cyanobacterium as compare to control (containing 46 µg Zn^{2+} L^{-1})

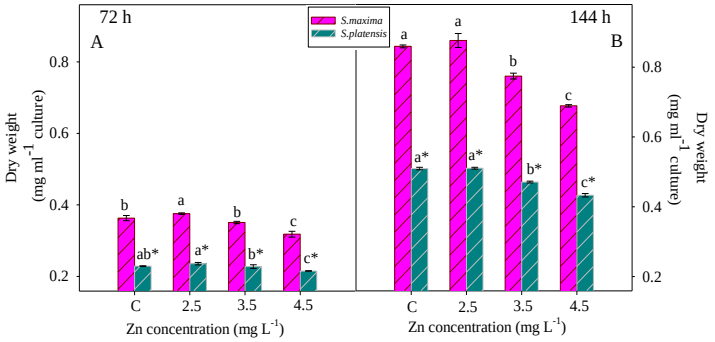

Fig. 1 Impact of precise/mild stress of Zn^{2+} on dry weight of *S. maxima* and *S. platensis* after 72 and 144 h of incubation. Data are mean ± standard error of three independent experiments. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

Further the effect of increasing Zn^{2+} concentrations on the growth as dry weight of *S. maxima* and *S. platensis* are also shown in Fig. 1A and 1B at two different time intervals i.e. 72 and 144 h. The results of cyanobacterial growth revealed that tested low dose (2.5 mg L^{-1}) of Zn^{2+}caused stimulatory effect on dry weight accumulation, resulting in growth stimulation by 3.4 and 2.9 % after 72 h and by 1.9 and 0.2 % after 144 h of Zn^{2+} treatment in *S. maxima* and *S. platensis*, respectively. However, in the current study species showed growth inhibitions by 3.3 and 0.5 % at middle dose (3.5 mg Zn^{2+} L^{-1}) and by 5.9 and 12.4 % at high dose (4.5 mg L^{-1}) of Zn^{2+} in *S. maxima* and *S. platensis*, respectively after 72 h of incubation. After 144 h of incubation growth was inhibited by 9.9 and 7.6 % at middle dose and by 15 and 19.8 % at high dose of Zn^{2+} in *S. maxima* and *S. platensis*, respectively as compared to control (100 %).

Photosynthetic pigment

The effect of increasing Zn^{2+} concentrations on the chlorophyll *a* content is shown in Fig. 2A and 2B. There was asignificant (P<0.05) increase in chlorophyll *a* content at low dose of Zn^{2+} which was found to be 3 and 7.6 % in *S. maxima* and 1.6 and 0.1 % in *S. platensis* after 72 and 144 h of incubation respectively. Further the chlorophyll *a* content decreased as the concentration of Zn^{2+} increased to 4.5 mg L^{-1} in dose dependent manner as compared to control. The results pertaining to carotenoids (Fig. 2C and 2D) and phycocyanin (Fig. 2E and 2F) showed similar trend. At low dose of Zn^{2+} more carotenoids and phycocyanin were produced; at middle dose the content of carotenoids slightly decreased and at high dose these photosynthetic pigments content markedly reduced as compare to control.

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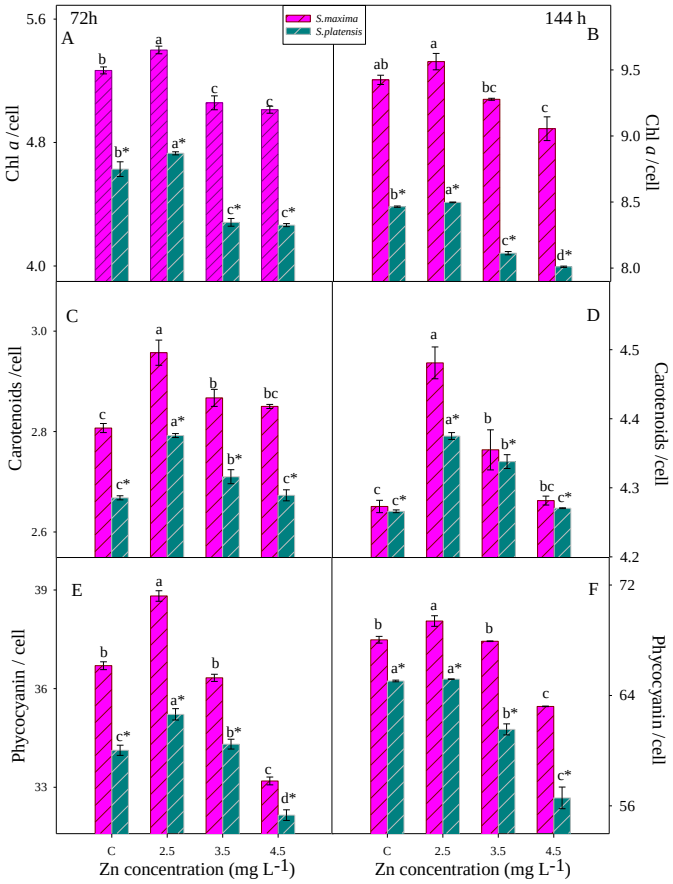

Fig. 2 Impact of precise/mild stress of Zn^{2+} on chlorophyll *a*, carotenoids and phycocyanin contents in *S. maxima* and *S. platensis*. Data are mean ± standard error of three independent experiments. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

Photosynthetic activity

The photosynthetic activity (Fig. 3A and 3B) showed a slight increase by 2.9 and 2.92% at low dose in *S. maxima* after 72 and 144 h, respectively which significantly (P<0.05) decreased at middle dose followed by high dose of Zn^{2+} by 11.4 and 15.4 % after 72 h and 14.1 and 19.2 % after 144 h of treatment. In case of *S. platensis* there was also increment in photosynthetic activity at low dose by 1.8 and 1.6 % after 72 and 144 h, respectively but at middle dose the activity reduced by 11.4 and 11.6 % with maximum reduction by 15.7and 21.4 % at high dose of Zn^{2+} after 72 and 144 h of treatment, respectively. In contrast to photosynthetic activity the respiration increased in dose dependent manner as concentration of Zn^{2+} increased.

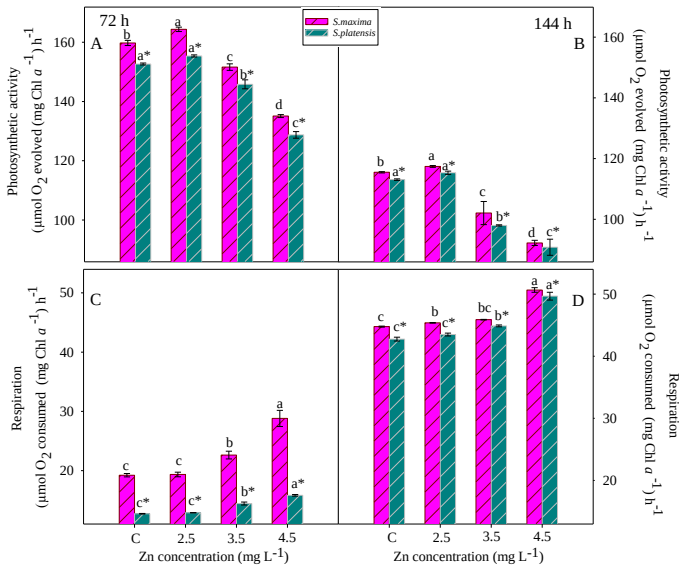

Fig. 3 Impact of precise/mild stress of Zn^{2+} on photosynthetic and respiratory activity in *S. maxima* and *S. platensis*. Data are mean ± standard error of three independent experiments. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

The measurement of photosynthetic efficiency (Fv/Fm) (Fig. 4A and 4B), and number of open reaction centres i.e. photochemical quenching (qP) (Fig. 4C and 4D) showed reduction with increased concentration of Zn^{2+} in both the species of *Spirulina* while a reverse trend was observed in case of amount of heat dissipation as energy i.e. non photochemical quenching (NPQ); as the concentration of Zn^{2+} increased from 2.5 followed by 3.5 to 4.5 mg L^{-1} of Zn^{2+} NPQ insignificantly (P<0.05) increased (Fig.4E and 4F).

The respiration significantly (P<0.05) increased by 0.7, 17.7 and 49.9 % after 72 h of treatment which further decreased by 1.4, 11.3 and 39.0 % after 144 h of treatment at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* (Fig. 3C) while in case of *S. platensis* respiration showed significant (P<0.05) increase by 1.3, 13.6 and 24.5 % after 72h and 1.7, 7.3 and 18.5 % after 144 h of treatment (Fig. 3D) at low, middle and high dose of Zn^{2+}, respectively. Similar results were reported in a cyanobacterium *Plectonema boryanum*[44].

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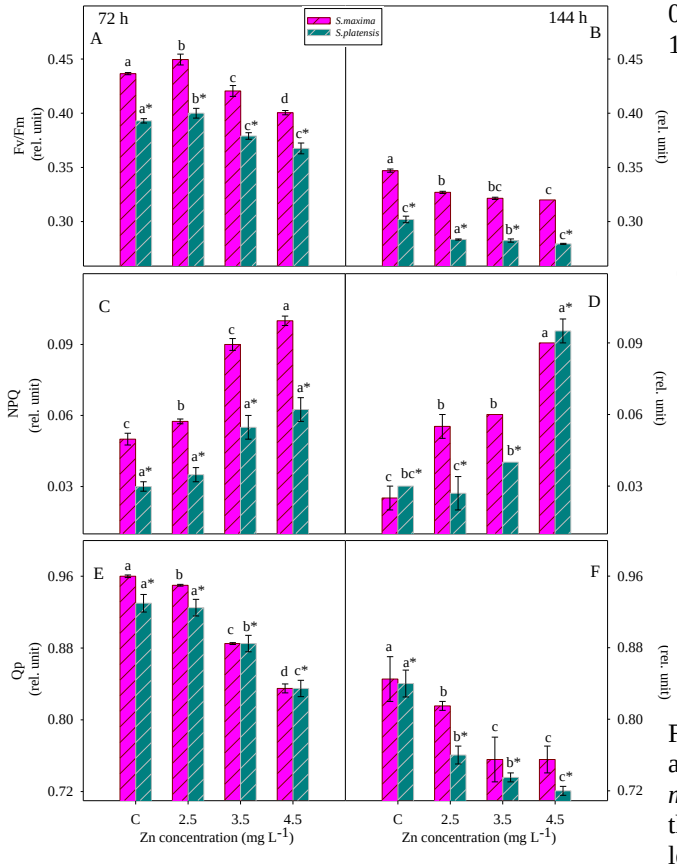

Fig. 4 Impact of precise/mild stress of Zn^{2+} on photosynthetic efficiency (Fv/Fm), photochemical quenching (QP) and non-photochemical quenching (NPQ) in *S. maxima* and *S. platensis*. Data are mean ± standard error of three independent experiments. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

Protein and Total antioxidative activity

The results of protein content are shown in Fig. 5A and 5B. The results revealed that the protein content and total antioxidative property of both the *Spirulina* species increased at all the three doses of Zn^{2+} as compare to control (100 %) but showed decreased trend from lower concentration (2.5 mg L^{-1}) to higher concentration (4.5 mg L^{-1}) of Zn^{2+}. The protein content significantly (P<0.05) increased by 19.8, 15.6 and 2.8 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* while in *S. platensis* protein increased by 16.5, 6.9 and 2.6 % at low, middle and high dose of Zn^{2+}, respectively after 72 h of incubation as compare to control (100 %). The results also showed that there is more production of protein content at 72 h as compare to144 h of treatment where the protein content significantly (P<0.05) increased by 9.0, 4.0 and 0.1 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* while in *S. platensis* it increased by 7.3, 2.19 and 0.48 % at low, middle and high dose of Zn^{2+}, respectively after 144 h of treatment as compare to control (100 %).

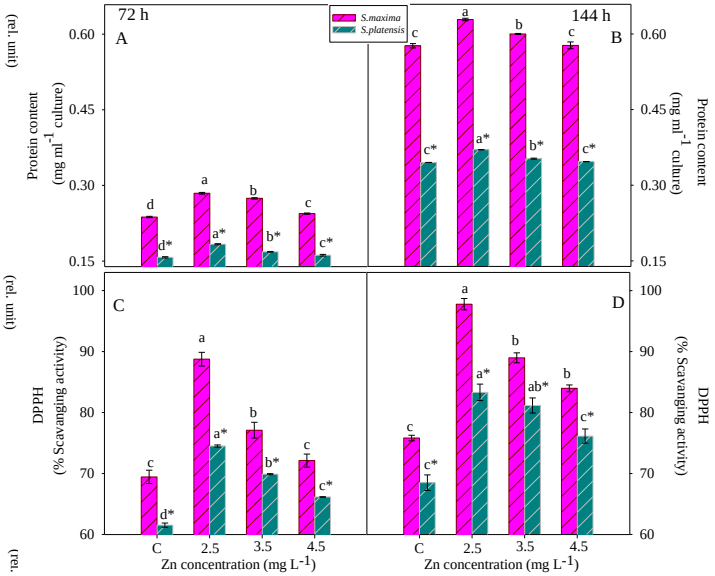

Fig. 5 Impact of precise/mild stress of Zn^{2+} on protein content and total antioxidant activity (% scavenging activity) in *S. maxima* and *S. platensis*. Data are mean ± standard error of three independent experiments. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

Exposing to mild/low level stress of Zn resulted in changes to the total antioxidant activity in the two species of *Spirulina*, as shown in Fig.5C and 5D. The total antioxidant activity significantly (P<0.05) increased by 27.8, 11.0 and 3.8 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* while in *S. platensis* the activity increased by 21.1, 13.6 and 7.5 % at low, middle and high dose of Zn^{2+}, respectively after 72 h of treatment as compare to control (100 %). The results also showed increased total antioxidant activity at 144 h as compare to72 h of treatment where it significantly (P<0.05) increased by 29.0, 17.4 and 10.8 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* while in *S. platensis* the activity increased by 21.6, 18.5 and 11.2 % at low, middle and high dose of Zn^{2+}, respectively after 144 h of treatment as compare to control (100%).

Reactive oxygen species and oxidative damage

Fig. 6A and 6B (H_2O_2 content) and 6C, 6D (SOR content) showed that Zn^{2+} at tested doses (2.5, 3.5 and 4.5 mg L^{-1}) increased H_2O_2 and SOR contents significantly (P<0.05) in both species of *Spirulina* compared to the control samples. The H_2O_2 content significantly (P<0.05) increased by 7.0, 20.0 and 30.8 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* while in *S. platensis* it increased by 5.0, 19.5 and

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27.9 % at low, middle and high dose of Zn^{2+}, respectively after 72 h of treatment as compare to control (100 %). The H_2O_2 content is more at 72 h as compare to 144 h and showed a significant (P<0.05) increase up to 6.9, 19.8 and 28.9 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* while in *S. platensis* it increased by 3.6, 13.4 and 23.2 % at low, middle and high dose of Zn^{2+}, respectively after 144 h of treatment as compare to control (100%).

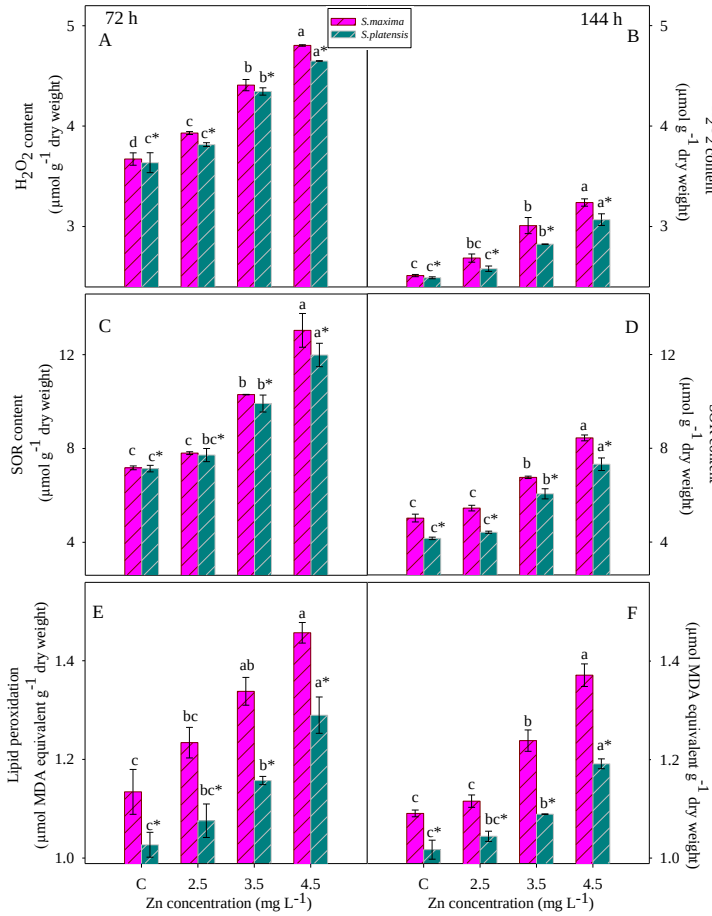

Fig. 6 Impact of precise/mild stress of Zn^{2+} on hydrogen peroxide (H_2O_2), superoxide radical (SOR) and lipid peroxidation (MDA equivalents) in *S. maxima* and *S. platensis*. Data are mean ± standard error of three independent experiments. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

Similar trend was observed in case of SOR (O^{2-}) and the results are shown in Fig. 6C and 6D. The SOR significantly(P<0.05) increased by 8.0, 43.5 and 81.6 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* while in *S. platensis* it increased by 8.1, 38.8 and 67.8 % at low, middle and high dose of Zn^{2+}, respectively after 72 h of treatment as compare to control (100 %). After 144 h of treatment The SOR (O^{2-}) content showed a significant (P<0.05) increase by 8.4, 34.6 and 68.1 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* while in *S. platensis* it increased by 6.2, 45.6 and 76.0 % at low, middle and high dose of Zn^{2+}, respectively after 144 h of treatment as compare to control (100 %).

To examine ROS induced oxidative damage to the cells of both species of *Spirulina*, lipid peroxidation (as malondialdehyde; MDA) was determined which is shown in Figure 6E and 6F. The levels of MDA were significantly (P<0.05) modified by the Zn^{2+} treatment (Fig. 6E and 6F). MDA production was highest at the high dose, followed by middle, and then low dose of Zn^{2+}. The MDA content significantly (P<0.05) increased by 8.7, 17.9 and 28.4 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* while in *S. platensis* it increased by 4.7, 12.6 and 25.5 % at low, middle and high dose of Zn^{2+}, respectively after 72 h of treatment as compare to control (100 %). Further after 144 h of treatment MDA significantly (P<0.05) increased by 2.3, 13.5 and 25.7 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* and in case of *S. platensis* it increased by 2.6, 7.0 and 17.1 % at low, middle and high dose of Zn^{2+}, respectively as compare to control (100 %).

Enzyme activities

Results showed that the POD (Fig.7A and 7B) significantly (P<0.05) increased by 13.1, 19.2 and 26.1 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* while in *S. platensis* POD increased by 8.9, 13.0 and 26.8 % at low, middle and high dose of Zn^{2+}, respectively after 72 h of treatment as compare to control (100 %). The results also showed that there was increase in POD content at 72 h as compare to144 h of treatment where it significantly (P<0.05) increased by 7.3, 14.0 and 19.7 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* while in *S. platensis* the POD activity increased by 6.0, 9.4 and 16.1 % at low, middle and high dose of Zn^{2+}, respectively after 144 h of treatment as compare to control (100 %).

Similar trend was also observed in case of SOD (Fig. 7C and 7D) which significantly (P<0.05) increased by 2.5, 6.1 and 20.6 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* while in *S. platensis* it increased by 6.4, 9.8 and 25.6 % at low, middle and high dose of Zn^{2+}, respectively after 72 h of treatment as compare to control (100 %). There was less SOD activity observed after 144 h as compare to 72 h of treatment and significantly (P<0.05) increased by 1.6, 5.3 and 15.6 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* and in case of *S. platensis* it increased by 4.0, 8.2 and 18.4 % at low, middle and high dose of Zn^{2+}, respectively after 144 h of treatment as compare to control (100%). Results revealed that Zn^{2+} treatments significantly accelerated SOD (Fig. 7A, 7B) and POD (Fig. 7C, 7D) activity in both the species of *Spirulina*. As SOD belonging to family of metallo enzymes that catalyzes the destruction of O^{2-} free radical andit protects oxygen metabolizing cells against harmful effect of

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superoxide free radicals. Increase in SOD and POD activity after the commencement of 72 h of experiment compared to 144 h of experiment might have supported appreciably the organisms to scavenge SOR and H_2O_2 respectively. It was reported in different studies that under different oxidative stress such as metal (lead, copper and zinc) and pesticides (endosulfan) *Spirulina platensis* and other *cyanobacteria* like *Aulosira fertilissima*, *Anabaena variabilis* and *Nostoc muscorum* showed reduction in growth and increased level of total protein MDA, SOD and proline contents[45]. The enhanced total peroxide production may be due to increased cellular metabolism rate to combat stress or may be produced as the resultant of protection for cells against stress.

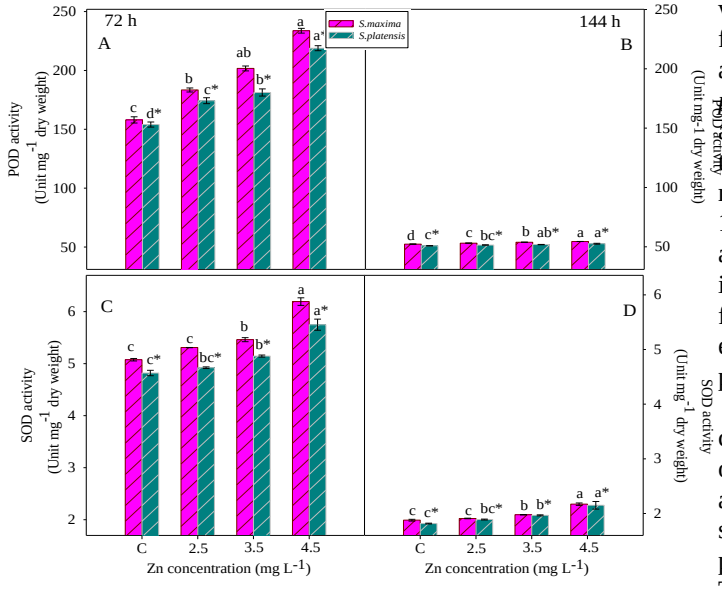

Fig. 7 Impact of precise/mild stress of Zn^{2+} on POD and SOD activity in *S. maxima* and *S. platensis*. Data are mean ± standard error of three independent experiments. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

Total phenolic content (TPC)

The TPC (Table 1 and 2) significantly (P<0.05) increased by 37.4, 19.2 and 15.6% at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* while in *S. platensis* it increased by 33.0, 9.8 and 7.4 % at low, middle and high dose of Zn^{2+}, respectively after 72 h of treatment as compare to control (100 %). After 144 h of treatment The TPC content showed a significant (P<0.05) increase by 35.1, 18.2 and 13.6 % at low, middle and high dose of Zn^{2+}, respectively in *S. maxima* and in case of *S. platensis* it increased by 33.0, 9.8 and 7.4 % at low, middle and high dose of Zn^{2+}, respectively after 144 h of treatment as compare to control (100%). Previous studies have emphasized that the phenolics are diverse secondary metabolites (flavonoids, tannins, hydroxycinnamate esters, and lignin) which possess antioxidant properties. Polyphenols can chelate transition metal ions, can directly scavenge molecular species of active oxygen, and can inhibit lipid peroxidation by trapping the lipid alkoxyl radical. Quantities of phenolics could be increased by altering the culture conditions, suggesting that it was possible to increase the antioxidant potential of *Spirulina* species biomass for use as a functional food supplement[46].

Non enzymatic antioxidants

Cysteine is an amino acid having thiol side chain, susceptible to oxidation to give disulfide derivative, which serves an important structural role in many proteins[47, 48]. The AsA (Table 1 and 2) showed a slight increase by 10.6 and 9.3 % at low dose in *S. maxima* after 72 and 144 h, respectively which significantly (P<0.05) decreased at middle dose followed by high dose of Zn^{2+} by 3.6 and 31.0 % after 72 h and 2.2 and 30.4 % after 144 h of treatment. In case of *S. platensis* there was also increment in AsA at low dose by 8.9 and 7.5 % after 72 and 144 h, respectively but at middle dose the activity reduced by 8.3 and 5.7 % with maximum reduction by 29.5 and 27.9 % at high dose of Zn^{2+} after 72 and 144 h of treatment, respectively. Besides this, cysteine may act as precursor in the food, pharmaceutical, and personal-care industries. One of the largest applications is the production of flavours together with chemical reaction of sugars and enhanced production of cysteine from cyanobacteria could provide a way to reduce costs[49].

Proline is an α-amino acid whose role under stress condition is well documented. Besides, acting as an excellent osmolyte, proline plays three major roles during stress, i.e., as a metal chelator, an antioxidative defense molecule, and a signaling molecule [50]. Due to its importance to humans, proline content in both the test organism was also investigated. The proline (Table 1 and 2) showed a slight increase by 15.4 and 13.1 % at low dose in *S. maxima* after 72 and 144 h, respectively which significantly (P<0.05) decreased at middle dose followed by high dose of Zn^{2+} by 19.6 and 33.2 % after 72 h and 14.4 and 32.3 % after 144 h of treatment. In case of *S. platensis* there was also increment in proline at low dose by 14.6 and 10.9 % after 72 and 144 h, respectively but at middle dose the activity reduced by 18.3 and 32.4 % with maximum reduction by 17.1 and 31.1 % at high dose of Zn^{2+} after 72 and 144 h of treatment, respectively. Proline mainly known for its osmoprotectant property also plays an important role in combating arteriosclerosis, therefore, decreases the pressure built up by the blockages and consequently the risk of heart disease; hence, it is being used in many pharmaceutical and biotechnological applications[51, 49].

Ascorbate (AsA) is the most abundant, low molecular weight antioxidant that has a key role in defence against oxidative stress caused by enhanced level of ROS. The AsA (Table 1 and 2) showed a slight increase by 10.6 and 9.3 % at low dose in *S. maxima* after 72 and 144 h, respectively which significantly (P<0.05) decreased at middle dose followed by high dose of Zn^{2+} by 3.6 and 31.0 % after 72 h and 2.2 and

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30.4 % after 144 h of treatment. In case of *S. platensis* there was also increment in AsA at low dose by 8.9 and 7.5 % after 72 and 144 h, respectively but at middle dose the activity reduced by 8.3 and 5.7 % with maximum reduction by 29.5 and 27.9 % at high dose of Zn^{2+} after 72 and 144 h of treatment, respectively. It was extensively documented that ascorbate (AsA- vitamin C) considered as a central antioxidant in plant metabolism, and involved in stress responses. This ascorbate is an essential nutrient for humans., as the diet deficient in vitamin C hinders the activity of a variety of enzymes, and might responsible for occurrence of ''scurvy'' in humans[52].

The overall results showed that the effect of tested doses of Zn^{2+} on physiological and oxidative biomarkers were more pronounced in *S. maxima* as compare to that of *S. platensis.*

Table- 1 Effect of Zn^{2+} on Total phenolic content (TPC), cystein, proline and ascorbate of *Spirulina maxima* and *Spirulina platensis* after 72 h of treatment. Data are mean ± standard error of three independent experiments. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test.

	Non-enzymatic antioxidants nmol (mg dry weight)$^{-1}$							
	TPC		Cysteine		Proline		Ascorbate	
Treatments	*S. maxima*	*S. platensis*	*S. maxima*	*S. platensis*	*S. maxima*	*S. platensis*	*S. maxima*	*S. platensis*
Control	4.38±1.21b	2.98±1.10b	10.45±1.01b	9.87±0.66b	10.28±0.26b	9.86±1.10b	13 8.37±1.33b	123±2.14b
2.5	6.16±1.61a	3.88±4.81a	11.97±1.12a	10.69±0.57a	11.83±0.34a	10.64±0.54a	15 2.25±1.77a	141.02±1.36a
3.5	5.49±1.46c	3.29±5.56c	9.96±0.98bc	9.04±0.83b	8.89±0.13c	8.07±0.46c	127.2±1.81b	129.65±2.64b
4.5	5.08±1.21d	3.06±1.23c	5.32±1.01c	4.78±0.16c	6.34±0.13d	5.47±0.12d	87.19±1.39c	95.11±1.51c

Table-2 Effect of Zn^{2+} on Total phenolic content (TPC), cystein, proline and ascorbate of *Spirulina maxima* and *Spirulina platensis* after 144 h of treatment. Data are mean ± standard error of three independent experiments. Bars followed by different letters show significant difference at P<0.05 significance level according to Duncan's multiple range test

	Non-enzymatic antioxidants nmol (mg dry weight)$^{-1}$							
	TPC		Cysteine		Proline		Ascorbate	
Treatments	*S. maxima*	*S. platensis*	*S. maxima*	*S. platensis*	*S. maxima*	*S. platensis*	*S. maxima*	*S. platensis*
Control	5.61±1.53b	3.36±1.10b	11.05±.30b	10.14±0.12b	11.22±0.26b	10.30±0.19b	143.79±1.13b	134.98±2.26b
2.5	7.01±2.80a	4.47±4.81a	12.23±.18a	11.20±0.29a	12.89±0.34a	11.81±0.24a	159.17±2.45a	141.02±1.85a
3.5	6.69±1.56bc	3.99±5.56c	10.45±.18c	9.53±0.26b	9.22±0.13c	8.21±0.23c	136.48±2.13c	129.65±2.03b
4.5	6.38±0.11c	3.91±1.23c	6.14±0.10ed	5.16±0.21c	7.49±0.13d	6.38±0.12d	99.16±1.26d	95.11±1.76c

IV.CONCLUSION

We can conclude that *S. maxima* and *S. platensis* could grow in zinc (Zn^{2+}) enriched nutrient medium at low concentration. Zinc (Zn^{2+}) at higher concentration decreased growth, photosynthetic pigments and photosynthetic activity of both the species of *Spirulina* and thereby causes oxidative stress which was tolerated by these spiral cyanobacteria through the hyperactivity of antioxidant defense system. On the other hand, increased photosynthetic pigments (chlorophyll, carotenoids and phycocyanin) and biomass production with enhanced protein content, photosynthetic activity and total antioxidant activity was observed in both the studied *Spirulina* species which was more pronounced in *S. maxima* as compare to *S. platensis* with increasing metal (Zn^{2+}) concentration of 2.5 mg L^{-1} in single metal systems. But there are many questions remain unanswered, including the interrelationship among them and the signal pathway under such low stress induce growth stimulation. However, this finding suggests that Zn^{2+} ions in 2.5 mg L^{-1}concentration may be added in the culture medium of *Spirulina* species for enhancing its biomass which can further utilized for production of value added products like functional foods and nutraceuticals. Through appropriate selection of mild stress condition and exploitation of such stress adapted strains with high productivity and enhanced nutrition would be an alternative approach may prove to be vital for *Spirulina* production at large scale through the improvement and innovation of technology with futuristic approach.

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