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SELENIUM AMELIORATES CADMIUM STRESS-INDUCED DAMAGE BY IMPROVING ANTIOXIDANT DEFENSE SYSTEM IN *CHLAMYDOMONAS REINHARDTII*

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Abstract

Present study was aimed to investigate the role of selenium in amelioration of cadmium stress in *Chlamydomonas reinhardtii*. Cadmium treatment growth declined by reducing the pigment synthesis and increasing the oxidative damage to membranes. Selenium supplementation caused a significant decline in hydrogen peroxide production and hence the rate of lipid peroxidation which was well correlated with the increase in the activities of antioxidant enzymes including superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase. Proline increased due to supplementation of selenium and optimised the synthesis of nitric oxide for better counteracting the cadmium stress. Supplementation of selenium protected membrane structures in *C. reinhardtii* from the cadmium stress by maintaining the higher contents of polyunsaturated fatty acids.

Key words: Antioxidants, Lipid peroxidation, Fatty acids, Cadmium, *Chlamydomonasreinhardtii*, Selenium.

Introduction

The contamination resources i.e., soil and water with pollutants like heavy metals has been accepted as a serious issue and threat to biodiversity. Regarding the water quality, with the concentration of pollutants exceeding to the WHO drinking water guidelines in many regions of the world, the biodiversity has been affected to a considerable extent. There has been an increase of research reports available discussing the impact of heavy metals, metalloids on the growth of higher plants. However regarding their multifaceted effect on the metabolism of lower plants particularly the ones growing in marine ecosystem are scanty. Marine ecosystems are the ultimate sinks of any type of pollution leading to disturbances in the existing biodiversity (Carfagna *et al.,* 2013; Piotrowska-Niczyporuk *et al.,* 2015).

Cadmium (Cd) is one of the non-essential toxic metals known to impart photosynthetic arrest in photosynthesising organisms (Kucera *et al.,* 2008). Cd has high mobility between the environment-plant system and is continuously added to environment from both natural and anthropogenic sources like weathering of metal rich rocks, power stations, excessive fertilizer usage, mining, use of waste water and sewage sludge for irrigation purposes (McLauglin *et al*., 2000; Zoffoli *et al.,* 2013). In humans consumption of Cd containing foods causes renal disorders and the development of weak bones (Horiguchi *et al.,* 2010). At very low concentrations, the living systems exposed to Cd treatment start showing toxicity symptoms like necrosis, reduction in root and shoot growth leading to phytotoxicity (Schutzendubel *et al.,* 2001). Cd has the potential to restrict the enzyme activity through its greater affinity to bind with the cysteine sulfhydryl groups of the enzymes (Mendoza-Cozatl *et al.*, 2005). Key physiological and biochemical pathways affected by Cd include photosynthetic and respiratory electron transport and the mineral nutrition and water uptake (Ahmad *et al.,* 2015; Khan *et al.,* 2015).

Its effects on the electron transport systems consequently provoke generation of toxic reactive oxygen species by leaking electrons to molecular oxygen and hence leading to oxidative stress (Abd_Allah *et al.,* 2015; Ahanger & Agarwal, 2016). Behaving as non-redox active metal Cd generates ROS through interference with the enzymes involved in the maintenance of redox homeostasis thereby altering the physiological and biochemical stability of the affected organism (Ahmad *et al.,* 2015). Cd can affect photosynthetic process at multiple sites that are associated with the electron transport like the primary photochemistry of photosystem II (PSII) which has been observed to be very sensitive for Cd causing interference by interacting with its electron donors and acceptors (Khan *et al.,* 2015).

Several ingenuously accepted intricate cellular protective mechanisms for example extracellular detoxification, restricted uptake and transport of toxic metals, sequestration through phytochelatins etc. are involved in enhancing the algal tolerance to naturally occurring heavy metals in aquatic environment (Tripathi *et al.,* 2006). In addition to the above referred tolerance mechanisms, algae also contain the enzymatic antioxidant defence system that includes superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and non-enzymatic antioxidant defence system like glutathione, ascorbate, leading to protection of cells from the ROS induced oxidative damage (Piotrowska-Niczyporuk *et al.,* 2012; Polonini *et al.,* 2015). Such type of defence systems becomes highly operative generally at the elevated levels of metals or any other stress components in the environment. Antioxidants work in close coordination with enzymatic components depending on the non-enzymatic one for the quick removal of ROS and the

maintenance of the redox buffer for optimal functioning of the cells (Radotic *et al*., 2000). In the current era analysing the changes in the enzymatic and non-enzymatic antioxidant system has remained under intense focus with regard of the algal species under stress (Tripathi & Gaur, 2006; Bajguz, 2011; Piotrowska-Niczyporuk *et al.,* 2012). Increased activity of APX, SOD and glutathione peroxidase (GPX) in copper stressed *Scenedesmusbijugatus* has been demonstrated by Nagalakshmi & Prasad (2001). In addition it has been also observed that glutathione content in the stressed cells gets easily depleted with parallel increase in concentrations of stress. Such alterations in the equilibrium of synthesis and the subsequent utilization of redox buffers like glutathione is attributed to its role as antioxidant and as precursor phytochelatin synthesis (Nagalakshmi & Prasad, 2001). In Ectocarpus siliculosus, filamentous brown seaweed model organism of class *Phaeophyceae* has also been reported to exhibit oxidative stress when exposed to increased copper treatment resulting in significant reduction in the concentrations of photosynthetic pigments and the efficiency, however antioxidant system was induced (Saez *et al*., 2015).

C. reinhardtii, a single celled photosynthetic green alga of about 10 µM diameter showing flagellated movements and its cell wall is mainly composed of hydroxyproline rich glycoproteins with cup shaped chloroplasts, a large pyrenoid and an eyespot. Itis a well-studied biological model organism due to its ease of culturing and the ability to manipulate its genetics. *C. reinhardtii* can grow photo-autotrophically or in dark if supplied with organic carbon. Commercially, *C. reinhardtii* is being promoted as biopharmaceutical and biofuel agent in addition of its well-being use as a valuable research tool in making hydrogen. With this perusal present study was undertaken to investigate the role of selenium in mitigating the impact of Cd polluted growth medium in *C. reinhardtii* focussing the impact on fatty acid composition and the antioxidant system.

Materials and Methods

The experimental algae, treatments and growth conditions: The experimental algal (*Chlamydomonas reinhardtii*) was isolated from Wadi Hanifa, Riyadh, Saudi Arabia. The algal cells were grown in Erlenmeyer flasks (250 in capacity) containing 100 ml of TAP (Tris-acetate-phophate) medium (Gorman & Levine 1965). Selenium was used as sodium selenite (Na_2SeO_3) at concentration (0.1 mg/100 culture medium). Cd added as 0.2 mM $CdCl_2$ at the exponentially phase of growth culture. The concentration of cadmium used in the current experiment produced 50% growth inhibition (EC_{50}) and selected based on preliminary experiment was carried out to determine the suitable concentration used in the current experiment. Control flasks used as references for each treatment. The flasks were inoculated with one ml (1×10^5 cells ml^{-1}) of *C. reinhardtii* at logarithmic growth phase and incubated at shaking state (100 rpm) and temperature 30°C under white-light illumination (100 µmol m^2s^{-1}, 16 h/ day) for 6 days. The initial hydrogen ion concentration (pH) of the growth medium was adjusted to be neutral (pH 7.0) using dilute HCl and $CaCO_3$. The treatments had five replicates. At end of the experiment, the cells were counted with a Levy-Hausser hemacytometer with each value being the means of ten repeats. The algae growth was centrifuged at 10,000 g for 10 min at 4◦C in a refrigerated centrifuge. The algal cell pellets were harvested, air dried and grinded in hand mortar to be fine powder hence stored at -80°C until used for chemical and biochemical analysis.

Estimation of Chlorophylls content: Known volume (5 ml) of algal growth culture medium used for estimation of chlorophylls content adopting method of Wellburn (1994). Chlorophylls content was expressed as µg/3 × 10^{*6} cells.

Estimation of proline: Proline was estimated in accordance with the method of Bates *et al*. (1973). 0.1g of air powder samples were extracted in sulphosalicylic acid and supernatant was reacted with acid ninhydrin solution and glacial acetic acid. Proline was separated using toluene and optical density was taken at 520 nm against toluene.

Estimation of hydrogen peroxide (H_2O_2): Known weight of algal growth (air dry) was homogenised in 5 ml of tri-chloroacetic acid (0.1%, TCA) and followed by centrifugation at 12,000g for 15 min. There after 0.5 ml of the supernatant was mixed with equal volume of potassium phosphate buffer (pH 7.0) and potassium iodide. Samples were vortexed and optical density was read at 390 nm (Velikova *et al*., 2000).

Estimation of lipid peroxidation (malonaldehyde): Amount of malonaldehyde (MDA) formation after reaction with thiobarbituric acid was considered as rate of lipid peroxidation (Heath & Packer, 1968). Extraction was carried in TCA and aliquot was boiled with thiobarbituric acid and the absorbance was recorded at 600 nm and 532 nm. Extinction coefficient of 155 mM cm^{-1} was used for calculation.

Estimation of nitric oxide (NO): Method of Zhou *et al*. (2005) was followed for determining the nitric oxide (NO) content by estimating the nitrite (NO_2^-) formation. Air dried algal growth (0.5 g) was macerated in chilled mortar and pestle in 50 mM ice-cold acetic acid buffer (pH 3.6) containing 4% zinc diacetate. Extract was centrifuged at 11,500 g at 4 °C for 15 min. Pellet was washed using 1 mL extraction buffer and was again centrifuged. Supernatants were pooled together and neutralised with charcoal (0.1 g) followed by vortexing and filtration. Thereafter 1 mL of filtrate was mixed with 1 mL of the Greiss reagent and left for 30 min at room temperature. Thereafter the absorbance was recorded at 540 nm and content of NO was calculated the standard curve of sodium nitrite ($NaNO_2$).

Extraction and estimation of antioxidant enzymes: Known weight of air dried algal growth (500 mg) was extracted in 10 mL of chilled phosphate buffer (50 mM; pH 7.8) and extract was centrifuged at 15,000g for 15 min at 4 °C. Supernatant was used as enzyme source.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by measuring rate of photoreduction of nitrobluetetrazolium at 560 nm after incubating the assay mixture for 15 minutes under fluorescent light. Amount of protein causing 50% reduction in SOD-inhibitable NBT reduction was considered as one SOD unit and activity was expressed as Unit mg-1 protein (Beauchamp & Fridovich, 1971).

For estimation of ascorbate peroxidase (APX, EC 1.11.1.11) activity method of Chance & Maehly (1955) was employed and decrease in absorbance was monitored at 290 nm for 3 min. Activity was expressed as EU mg^{-1} protein.

Activity of catalase (CAT, EC1.11.1.6) was estimated spectrophotometrically in a reaction mixture of 2 mL containing phosphate buffer (50 mM; pH 7.0), H_2O_2 (5.9 mM) and 100 µL enzyme extract and decrease in absorbance was measured at 240 nm for 2 min. Activity was expressed as EU mg^{-1} protein (Chance & Maehly, 1955).

Method of Carlberg & Mannervik (1985) was used for estimation of glutathione reductase activity (GR, EC1.6.4.2). Decrease in absorbance was recorded at 340 nm for 2 min and activity was calculated using the extinction coefficient of 6.2 mM^{-1} cm^{-1} for NADPH. Activity was expressed as EU mg^{-1} protein.

Estimation of soluble protein: Protein in the enzyme extract was estimated according to Lowry *et al*. (1951).

Extraction, separation and estimation of fatty acids: Method described by Metcalfe *et al*. (1966) was employed for the preparation of fatty acid methyl esters and were subsequently separated and quantified using gas liquid chromatography (GLC) [Perkin-Elmer Model 910, Perkin Elmer, Shelton, CT, USA] equipped with a flame ionization detector following Johnson & Stocks (1971). For recordings GLC was connected to a dual-open recorder and a computing integrator (Perkin-Elmer Model M1). The temperature of both the injector and detector was maintained at 230 and 250 ^{o}C respectively with nitrogen as the carrier gas at the rate of 1 mL/min with split injector system (split ratio maintained at 1:100). Methyl ester standards (Sigma Co., St. Louis, USA) were used to separate and quantify the peak fatty acid methyl esters by comparing their retention times.

Statistical analysis: Data presented is mean of five replicates. Treatment means were compared using Least Significant Difference (LSD) analysis using SPSS 22 software for statistical analysis.

Results

To characterize the impact of selenium (Na_2SeO_3) in presence and absence of Cd ($CdCl_2$) on *C. reinhardtii*, we firstly examined how (EC_{50}) of Cd influenced *C. reinhardtii* growth in presence of selenium at different incubation periods. The control cells (not subjected to any Cd stress or selenium) were bright green, whereas those subjected to Cd stress seen as pale green. The impact of selenium was stimulatory to increase the greenish of cultural growth to dark green in control flask whereas in presence of Cd decrease its adverse impact hence the treatment remained green better than only Cd (Fig. 1). *C. reinhardtii* exhibited increased growth with extension of incubation period to sixth day and the selenium supplemented growth medium showed more 19.27% increase over control. On sixth day of incubation percentage reduction in growth of *C. reinhardtii* due to Cd was 47.90% which was observed only 26.51% in selenium treated ones (Table 1). Cd treatment resulted in considerable reduction in the synthesis of pigments. Percent reduction in chlorophyll a, chlorophyll b and total chlorophylls was 42.18%, 46.86% and 43.53%, respectively in Cd supplemented *C. reinhardtii* which was observed to increase by 23.36%, 31.40% and 25.99%, respectively due to selenium supplementation. Selenium supplemented Cd treated *C. reinhardtii* showed only 21.04%, 23.54% and 21.80% reduction in chlorophyll a, chlorophyll b and total chlorophylls, respectively (Table 2). Relative to control, treatment of Cd resulted in increase of 66.66% and 43.41% in H_2O_2 and lipid peroxidation (measured in terms of MDA formation), respectively which were declined by 43.88% and 24.42% in the selenium supplemented counterparts over the Cd stressed ones (Figs. 2A, B).

Activity of antioxidants SOD, CAT, APX, and GR was enhanced by 48.73%, 70.21%, 47.29% and 53.38% due to Cd treatment and selenium supplementation further stimulated the activity of SOD by 21.83%, CAT by 17.76%, APX by 27.92% and GR by 16.25% over Cd treated ones (Figs. 3A-D). Under normal growth conditions selenium improved the activity of SOD, CAT, APX, and GR was enhanced by 21.69%, 34.09%, 19.19% and 16.12% (Figs. 3A-D). Proline and protein contents were increased by 23.99% and 23.68% due to selenium supplementation in *C. reinhardtii*. Relative to control, an increase in proline and protein content was observed to be 49.88% and 54.23% due to Cd stress and selenium supplementation to Cd treated *C. reinhardtii* further improved proline and protein by 51.09% and 13.04% respectively (Figs. 4A and B). Relative to control, selenium enhanced the endogenous concentrations of nitric oxide by 39.37% under normal conditions and by 50.41% when supplemented to Cd treated *C. reinhardtii*. Alone Cd treatment resulted in 75.77% increase in nitric oxide (Fig. 5).

In the present study *C. reinhardtii* subjected to Cd stress showed a marked increase in saturated fatty acid content with concomitant reduction in unsaturated component (Table 3). Selenium supplementation resulted in improvement in the contents of unsaturated fatty acids and also ameliorated the negative effects of Cd on them. Individually among the unsaturated fatty acids, selenium increased palmitoleic ($C_{16:1}$) by 43.33%,α-linolenic ($C_{16:3}$) by 45.46%,oleic ($C_{18:1}$) by 22.39%, linoleic ($C_{18:2}$) by 21.47%,α-linolenic ($C_{18:3}$) by 24.29% and 11-eicosenoic ($C_{20:1}$) by 25.71% over control while as a reduction of 34.32% in palmitic ($C_{16:0}$),40.44% in stearic (C_{18}),25.47% in arachidic (C_{20}), 44.73% in eicosadienoic ($C_{20:2}$), 51.42% in behenic ($C_{22:0}$),and59.99% in lignoceric ($C_{20:4}$) was observed (Table 3).

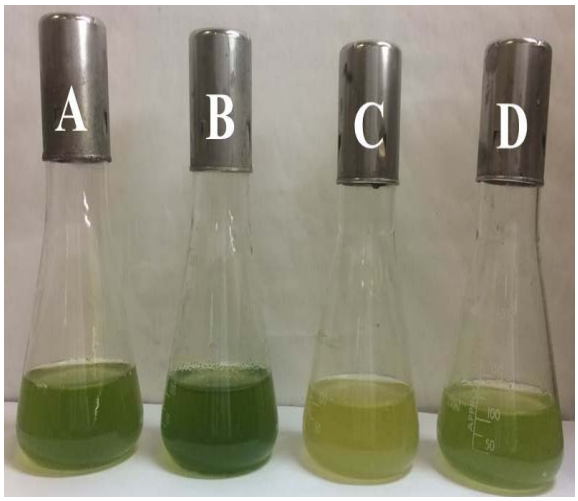

Fig. 1. The effects of salt on *C. reinhardtii* cell growth in TAP (Tris-acetate-phophate) medium after 6 days of incubation. **A**, Control; **B**, selenium (as 0.1 mg/100 culture medium of Na_2SeO_3); **C**, Cadmium (as 0.2 mM of $CdCl_2$); **D**, Both Na_2SeO_3 and $CdCl_2$.

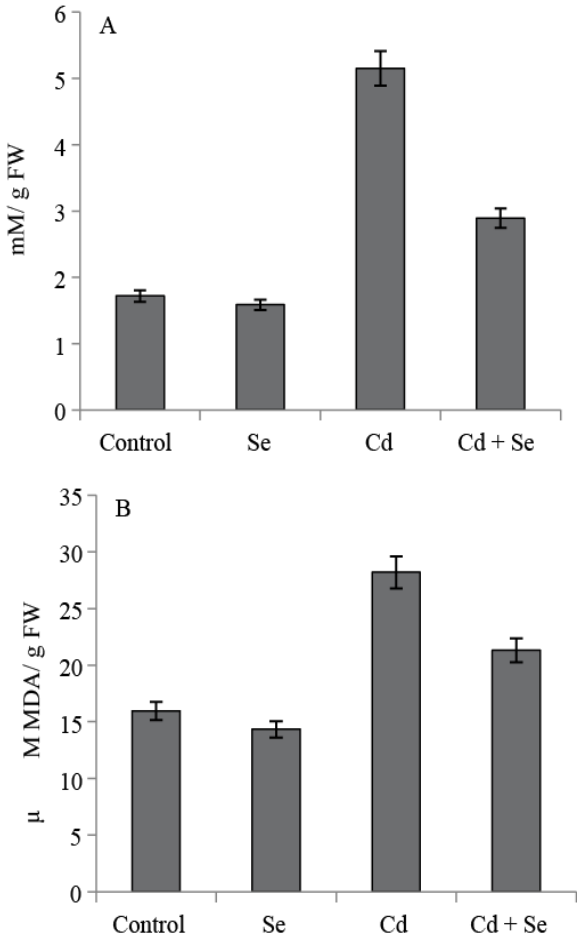

Fig. 2. Effect of cadmium on (**A**), hydrogen peroxide (mM/ g FW) and (**B**), lipid peroxidation (µM MDA/ g FW) in *C. reinhardtii* with and without selenium supplementation. Data presented is mean of five replicates.

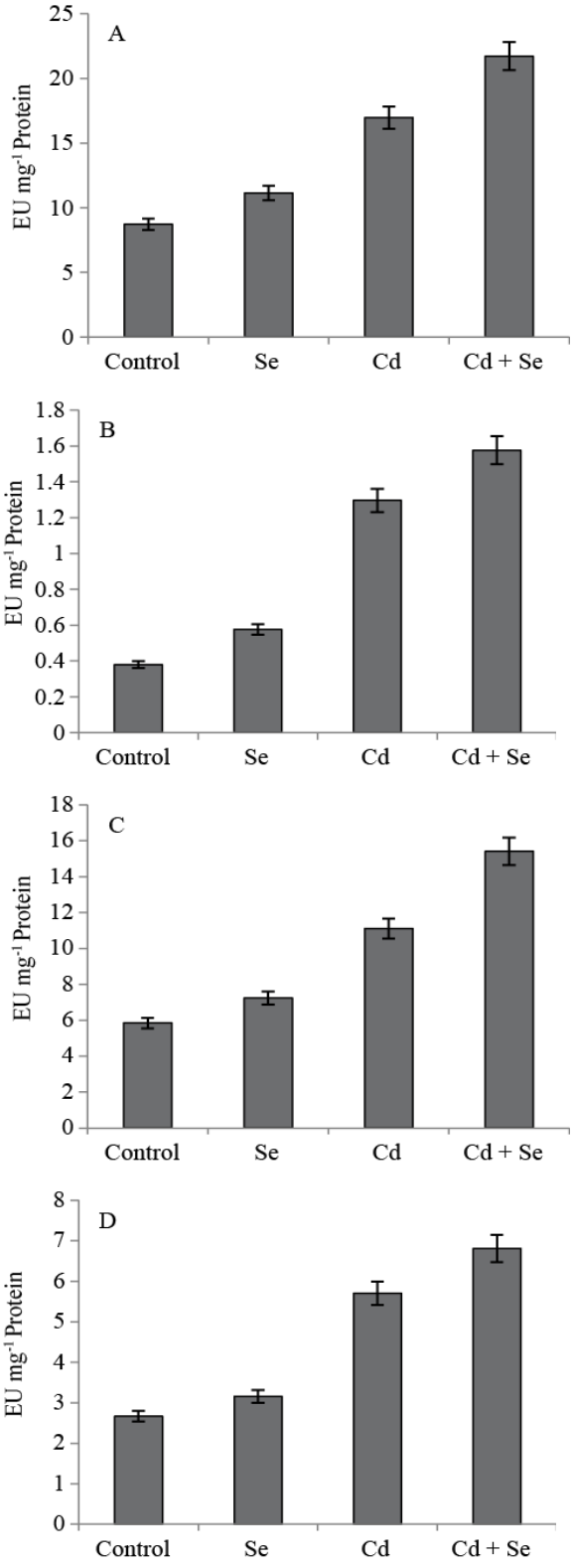

Fig. 3. Effect of cadmium on (**A**), superoxide dismutase (SOD, EC 1.15.1.1); (**B**), catalase (CAT, EC1.11.1.6); (**C**), ascorbate peroxidase (APX, EC 1.11.1.11) and (**D**), glutathione reductase (GR, EC1.6.4.2) in *C. reinhardtii* with and without selenium supplementation. Data presented is mean of five replicates.

Table 1. Effect of cadmium on growth of *C. reinhardtii* with and without selenium supplementation. Data presented is mean of five replicates.

Treatments	Algal growth (cell x 10^6 / mL)					
Control	2.87	7.86	15.34	19.48	24.19	28.89
Selenium	3.16	11.12	19.72	23.97	28.27	35.79
Cadmium	1.04	2.58	5.17	9.10	16.01	15.05
Cadmium + selenium	1.85	4.15	9.87	13.43	19.79	21.23
LSD at: 0.05	0.14	0.72	3.06	1.43	1.87	2.35

Table 2. Effect of cadmium on chlorophylls content in *C. reinhardtii* with and without selenium supplementation. Data presented is mean of five replicates.

Treatments	Chlorophylls content (µg/3 × 10*6 cells)			
	Ch a	Ch b	a/b	Total Chl
Control	10.69	4.63	2.31	15.32
Selenium	13.95	6.75	2.07	20.70
Cadmium	6.18	2.46	2.52	8.65
Cadmium + selenium	8.44	3.54	2.40	11.98
LSD at: 0.05	1.07	0.82	0.05	2.36

Ch a: Chlorophyll a; Ch b: Chlorophyll b; Total Chl: Total Chlorophylls

Table 3. Effect of cadmium on saturated and unsaturated fatty acids (%) in *C. reinhardtii* with and without selenium supplementation. Data presented is mean of five replicates.

Treatments	Palmitic ($C_{16:0}$)	Palmitoleic ($C_{16:1}$)	α-Linolenic ($C_{16:3}$)	Stearic (C_{18})	Oleic ($C_{18:1}$)	Linoleic ($C_{18:2}$)	α-Linolenic ($C_{18:3}$)	Arachidic (C_{20})	11-Eicosenoic ($C_{20:1}$)	Eicosadienoic ($C_{20:2}$)	Behenic ($C_{22:0}$)	Lignoceric ($C_{20:4}$)	Total SFA	Total USFA
Control	31.75	4.046	3.35	16.8	14.52	17.22	9.91	1.04	0.780	0.38	0.116	0.067	50.16	49.83
Selenium	20.85	7.136	6.14	10.0	18.71	21.93	13.09	0.78	1.050	0.21	0.056	0.026	31.93	68.06
Cadmium	46.79	2.133	1.57	19.2	8.01	12.03	5.45	2.46	0.616	0.913	0.376	0.333	70.17	29.82
Cadmium + selenium	36.99	3.203	2.45	18.0	11.89	14.99	6.98	1.41	0.676	0.646	2.506	0.190	59.79	40.20
LSD at: 0.05	3.18	0.45	0.31	0.38	1.79	1.82	0.21	0.11	0.03	0.07	0.02	0.01	4.97	5.02

Total SFA: Total saturated fatty acids; Total USFA: Total unsaturated fatty acids

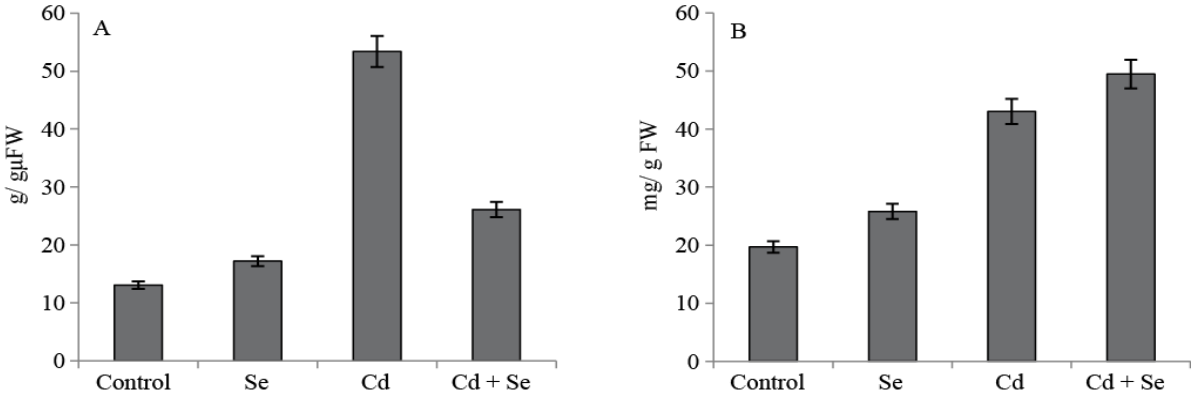

Fig. 4. Effect of cadmium on (**A**), proline (µg/ g FW) and (**B**) soluble protein (mg/ g FW) in *C. reinhardtii* with and without selenium supplementation. Data presented is mean of five replicates.

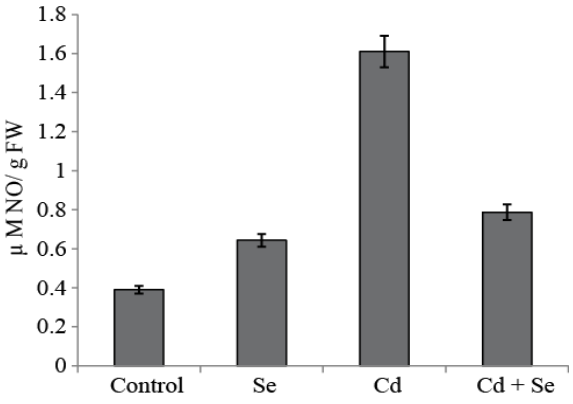

Fig. 5. Effect of cadmium on nitric oxide (µM NO/ g FW) in *C. reinhardtii* with and without selenium supplementation. Data presented is mean of four replicates.

Discussion

In the present study, we used a comparative physiological approach to investigate the mode of action underlying the impact of selenium to alleviate the adverse impact of Cd in the unicellular alga *C. reinhardtii*. Cd caused a considerable decline in the growth patterns of the microalgae *C. reinhardtii*. Pollutants reduce the physiocochemical properties of water including dissolved oxygen, pH, temperature as well as irradiance posing a direct impact on the growth of microflora including the algae (Chen*et al*., 2008; Bajguz, 2011; Carfagna *et al*., 2013). Due to the possible alterations in physiocochemical properties of the growth medium induced by Cd*C. reinhardtii* exhibited declined growth. Similar to our findings, Stoiber *et al*. (2010) have also observed a strong reduction in relative growth rate in *C. reinhardtii* due to

Cdand chromium treatments.Cd hampers the cell cycle progression by affecting the synthesis phase through reduction in the rate of DNA synthesis (Sobkowiak & Deckert 2004; Bajguz 2011; Aksmann *et al*., 2014). Selenium promoted growth of *C. reinhardtii* with parallel amelioration of the Cd induced growth inhibition which reported in our results may be attributed to the improved metabolic rate in them (Huang *et al*., 2007; Chen *et al*., 2008). Selenium resulted in improved growth as it is a fundamental microelement important for microalgae organisms like *Spirulina platensis* and has been found to impart tolerance to stresses and also enhances the pharmacological attributes of the microalgae (Huang *et al*., 2007). However higher concentrations may prove toxic so care must be taken to optimise the selenium concentrations for a particular algal species (Chen *et al*., 2008). Supplementation of selenium has been reported to improve the biomass accumulation therefore the pharmacological output of *Spirulina platensis* (Mosulishvili *et al*., 2002).

Our results found that Cd caused significant decline in the synthesis of photosynthetic pigments of *C. reinhardtii*. Cd has been observed to affect the photosynthetic processes through its ability to affect the multiple sites directly linked with the photosynthetic electron transport (Gorman & Levine, 1965). In the same context, it has been reported that, Cd affects the PSII electron transport by hampering its electron accepting potential at the ubiquinone binding sites which reduce photosynthetic pigments (Sigfridsson *et al*., 2004). Cd displaces the important cofactors involved in photolysis of water and also affects the oxygen-evolving complex by showing direct competition with Ca^{2+} ions on the donor side of PSII (Faller *et al*., 2005). In the present study, selenium maintained its promotory effect on the protection of photosynthetic apparatus through reductions in the Cd induced alterations. In *C. reinhardtii*,Perreault *et al*. (2011) have demonstrated Cd induced reduction in the photosynthetic attributes including electron transport potential of PSI and PSII, and the oxygen evolution reflecting in obvious growth retardations. Cd stress disrupts chloroplast ultrastructure and chlorophyll in *Chlorella sorokiniana* (Carfagna *et al*., 2013). Selenium maintained the growth promotor impact when supplemented along with the Cd. In *Spirulina platensis* supplementation of selenium has been reported to improve the phycocyanin synthesis that serve the important light harvesting function resulting in optimal photosynthetic potential and the growth maintenance (Huang *et al*., 2007). Improved synthesis of chlorophyll and carotenoids in selenium supplemented *C. reinhardtii* may contribute to the efficient management of the excitation energy and its subsequent integration of photosynthetic functions in addition of the efficient biogenesis of chloroplast membranes. Carotenoids share antioxidant property and protect the photo damage by mediating the quenching of ROS like singlet oxygen and the triplet chlorophyll species by mediating the maintenance of redox state (Bohne & Linden, 2002; Baroli *et al*., 2003). Increased carotenoid synthesis in selenium supplemented cultures in the present study advocates the use of selenium in protecting *C. reinhardtii*from the ill effects of Cd pollution as accumulation of accessory pigment molecules protect cells from the photodamage (Baroli *et al*., 2003).

C. reinhardtii cultured in medium containing Cd exhibited a considerable enhancement in the production and accumulation of ROS including hydrogen peroxide which was reflected in the enhanced peroxidation of lipids. However selenium supplementation was observed to bring down the rate of ROS production hence the stability of membranes was restored. Production of ROS has been accepted as the exclusively general response of living cells to heavy metal exposure which unbalances the cellular redox homeostasis (Hegedus *et al*., 2001). At the acute increase in the concentration of metals in the medium algal cell stability is hampered due to the ROS levels exceeding the potentiality of antioxidant machinery. Selenium protected *C. reinhardtii* cells from the ROS-induced membrane damage and hence contributing to the improved stability of important cellular molecules like lipids, proteins, nucleic acids and the pigments. ROS deactivate enzymes and damage membranes resulting in leakage of cellular components (Sofo *et al*.,2016). Increased production of H_2O_2 in Cd treated *C. reinhardtii* was observed to impart direct influence in MDA production. Production of MDA, a measure of the extent of lipid peroxidation rate, indicates the higher free radical formation in Cd treated ones as compared to selenium supplemented counterparts (Heath & Packer, 1968). Earlier many reports coincide with our findings of improved lipid peroxidation and the ROS production with concentration-dependent increase in metals e.g., *Bacopa monnieri* (Singh *et al.,* 2006), *Wolffia arrhiza* (Piotrowska-Niczyporuk *et al*., 2010) and green alga *Chlorella vulgaris* (Piotrowska-Niczyporuk *et al*., 2012). In green algae *Acutodesmus obliquus*, Piotrowska-Niczyporuk *et al*. (2015) have reported increased production of ROS resulting in lipid peroxidation due to lead treatments. More importantly increased production of ROS like H_2O_2 due to Cd treatment can accelerate the production of more toxic hydroxyl radicals through induction of Haber-Weiss reaction (Verma & Dubey, 2003) and such an observation which was clear in our study. H_2O_2 may be also produced through its activity of SOD which after dismutation of superoxide provide H_2O_2 and water which is acted upon by either CAT or APX in the ascorbate-glutathione pathway (Ahanger & Agarwal, 2016). During long and short term exposure of Scenedesmus sp. to Cu and Zn enhanced the production of H_2O_2 and the activity of SOD has been reported by (Tripathi *et al*., 2006). However increase in SOD and the efficient dismutation of superoxide due to selenium supplementation in *C. reinhardtii* can substantiate growth by protecting the photosynthetic electron transport and hence leading to the improved production of photo assimilates and the precursors for important metabolic pathways like amino acid synthesis. Our results indicate that significant increase in the activity of SOD was linked with the up-regulated activities of CAT and the APX and GR leading to quick elimination of H_2O_2 generated after

dismutation of superoxide by SOD (Khan *et al.,* 2015; Ahanger & Agarwal, 2016). Both APX and GR form the important components of ascorbate-glutathione pathway resulting in neutralisation of H_2O_2 and maintaining the optimal levels of redox buffers for protecting the cellular functioning (Mittler, 2002). Selenium induced enhancement in the activities of APX and GR may have contributed to maintenance of the NADP/NADPH ratio so that electron transfer gets least affected. In ascorbate-glutathione cycle GR reduces glutathione disulphide to its sulfhydryl form, glutathione that forms a key molecule in depicting the oxidative stress withstanding potential by bringing the redox environment of the cell (Sofo *et al*., 2016). Reports describing the selenium mediated Cd tolerance through modulation of the antioxidant components are scanty. Cd induced increase in antioxidant enzyme activity has been reported by Piotrowska-Niczyporuk *et al*. (2015) in *Acutodesmus obliquus*. Microalgae species maintaining higher activities of antioxidant enzymes have been reported to tolerate the metal induced oxidative damage more efficiently contrary to those exhibiting relatively lower activities (Bajguz, 2011; Piotrowska-Niczyporuk *et al.,* 2012). Exposure of *C. reinhardtii* to Cd treatments induced the activities of antioxidant enzymes and caused manifold enhancement in their expression levels (Aksmann *et al*., 2014).

Metal induced abrupt rise in production of ROS brings alterations in the primary as well as secondary metabolism therefore hampering overall cellular metabolism. Increased ROS accumulation affects cellular membrane functioning by triggering alterations in the composition of fatty acids. Selenium supplementation to culture medium provided *C. reinhardtii* a strength against the Cd induced oxidative damage to membrane fatty acids. Guschina & Harwood (2006) are of the opinion that exposure to environmental extremes triggers the production of unusual biomolecules in microalgae. Fatty acids are synthesized as key building blocks for lipid formation and among which the long chain (C_{16} to C_{18}) are the most abundantly found (Petkov & Garcia, 2007). In the present study selenium supplementation provoked the synthesis of polyunsaturated fatty acids providing stability to the membrane fluidity. Lipid saturation has been reported to get affected by both physiological state of algae and the composition of growing medium. Cd treatment caused an increase in the saturated fatty acids with concomitant decline in the unsaturated ones and these findings are supported by Griffith *et al*.(2012) and Chia *et al*. (2013) for *Chlorella vulgaris*. In higher plants like *Ephedra alata* (Alqarawi *et al.,* 2014) and *Helianthus annuus* (Abd_Allah *et al*., 2015) have also demonstrated decrease in polyunsaturated fatty acids due to salinity and Cd stress. Selenium induced enhancement in the polyunsaturated fatty acids may help to maintain the membrane plasticity thereby growth under Cd exposure. In *Gracilaria tenuistipitata*, Pinto *et al*. (2011) have witnessed concentration dependent increase in saturated fatty acid synthesis due to Cd. Accumulation and production of membrane fatty acids has been found to show direct link with the production and accumulation of ROS leading to oxidative damage (Pinto *et al*., 2011; Chia *et al*., 2013). Degree of ratio between unsaturated and saturated fatty acids is an important physiological index to monitor stress (Abd_Allah *et al*., 2015). Supplementation of optimal levels of mineral elements promote growth and metal tolerance by improving the synthesis of polyunsaturated fatty acids like ω-3 fatty acids (Choi *et al.*, 2011; Chia *et al*., 2013) which however are reduced due to stress induced increase in expression and activity of ω-3 fatty acid desaturase genes. In *C. reinhardtii*, Nguyen *et al*. (2013) have demonstrated significant increase in the expression of ω-3 fatty acid desaturase coding gene due to stress resulting in considerable decline in the ω-3 polyunsaturated fatty acids and its subsequent over-expression causes stress tolerance induction by maintaining the structural integrity of plastidic and extraplastidic membranes. A present observation of enhanced polyunsaturated fatty acid content in *C. reinhardtii* under normal as well as Cd stress justifies the importance of selenium in membrane protection.

Selenium supplementation resulted in enhancement in the accumulation of proline and soluble protein content providing stability to important structures like membranes and enzymes by mediating the maintenance of water content and the protein turnover (Huang *et al*., 2007; Chen *et al*., 2008; Khan *et al*., 2015). Proline displays antioxidant activity and has been reported to accumulate in response to heavy metals (Hashem *et al*., 2016). Accumulation of proline improves the photochemical activity by preventing photo inhibition (Alia & Saradhi, 1991) and in addition has been observed to protect proteins, membranes functions and DNA by inhibiting the formation of conjugated dienes and malonaldehyde (Matysik *et al.,* 2002). From the observations of present study selenium caused a significant increase in the accumulation of proline leading to stability of cellular structures like proteins therefore contributing to regulation of metabolism under Cd stress in *C. reinhardtii*. In *Brassicajuncea*, Khan *et al.* (2015) have demonstrated selenium induced Cd stress tolerance through the accumulation of proline by way of enhancement in the activity of synthesising enzymes. Nitric oxide is considered as important signalling molecule mediating the regulation of several complex biological functions however higher concentration can be toxic because of its radical nature, short life and high diffusibility (Qiao *et al.,* 2014). Cd treatment to *C. reinhardtii* resulted in increased nitric oxide production which was however, reduced by selenium supplementation to considerable extent. However under control conditions selenium increased nitric oxide concentrations so leading to its optimization for eliciting the signalling responses. Higher endogenous levels of nitric oxide in Cd stressed *C. reinhardtii* may have affected photosynthetic electron transport, membrane stability, DNA structure leading to cell death and hence growth (Pedroso *et al.,* 2000). Nitric oxide shares crosstalk with different other signalling molecules like H_2O_2 upon exposure to environmental stimuli and in the present study selenium supplementation optimised nitric oxide and H_2O_2 levels to protect *C. reinhardtii* from Cd induced oxidative damage by eliciting signalling pathways for counteracting the stress and future research in this direction can be worthwhile.

Conclusion

From the present study it can be concluded that *C. reinhardtii* exhibited oxidative damage when exposed to Cd stress which was ameliorated by selenium supplementation. Selenium supplementation proved significantly affective in protecting ROS induced membrane damage by maintaining the antioxidant potential leading to increased ratio of polyunsaturated fatty acids.

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