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Antioxidant modulation in response to selenium induced oxidative stress in unicellular cyanobacterium *Synechococcus elongatus* PCC 7942

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A unicellular cyanobacterium *Synechococcus elongatus* PCC 7942 was exposed to various concentrations of SeO_4^{2-}. A growth supportive effect was seen at 50 µM concentration of SeO_4^{2-} whereas 50% growth retardation was observed at 200 µM of SeO_4^{2-}. Selenium (Se) stress at 200 µM SeO_4^{2-} induced the formation of reactive oxygen species such as superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA). Modifications in antioxidant enzyme levels, namely, superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione reductase (GR), glutathione peroxidase (GPX) and isozyme patterns were also examined. This article outlines the synergistic action of antioxidant components on Se toxicity in *S. elongatus* PCC 7942.

Key words: Antioxidant, reactive oxygen species, selenium, *Synechococcus elongatus* PCC 7942, toxicity.

INTRODUCTION

In freshwaters, selenium (Se) entering primarily as selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) oxyanions has high bioavailability and therefore easily exert their toxicities. Planktonic microalgae and cyanobacteria are directly exposed to Se pollution in aquatic environments due to a variety of natural and anthropogenic causes (Chavan and Bhattacharjee, 2016). Se act as beneficial element by boosting normal cell growth and function as observed in plants at low doses (Feng and Wei, 2012). Se is considered as an essential micronutrient in human, animals and microorganisms including the green alga *Chlamydomonas reinhardtii* (Vriens et al., 2016). However, at high doses Se is toxic to algae, leading to reduction of growth rate or alterations in the levels of reactive oxygen species (ROS) (Arauz et al., 2016). These ROS involve superoxide radical ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) that may cause oxidation of nucleic acids and proteins as well as lipid peroxidation (LPO), leading further to inactive enzymes, disrupted membranes, mutations, and ultimately causing cell death (Schiavon et al., 2012).

To eliminate excess of ROS and protect cellular membranes and organelles from its damaging effects many reducers are synthesized in algae and plants as a part of the antioxidative defense system, which include non enzymatic low molecular weight antioxidants (for

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example, GSH) and enzymatic antioxidants such as superoxide dismutase (SOD, EC 1.15.1.11), catalase (CAT, EC 1.11.1.6) and peroxidase (POX, EC 1.11.1.7) (Wu et al., 2012). These antioxidants can counter ROS directly or via enzyme catalysis (Schiavon et al., 2012). SOD is considered as the first line of cell defense against free radicals that can catalyze the one-electron dismutation of superoxide ($O2^{\bullet-}$) into O_2 and hydrogen peroxide (H_2O_2) (Ruiz-Litago et al., 2012; Feng and Wei, 2012). Algae *Ulva* species and *Chlorella vulgaris* showed induced expression of SOD enzyme activity in response to ROS production caused by different Se compounds (Schiavon et al., 2012; Sun et al., 2014). The production of H_2O_2 can be reduced to water by catalase, ascorbate peroxidase and peroxidase or through the ascorbate (AsA)-GSH cycle (Feng and Wei, 2012). As the machinery of antioxidant enzyme, Se is a key component in Se-dependent glutathione peroxidases that plays a central role in maintaining redox potential in both prokaryotic and eukaryotic systems (Kieliszek and Blazejak, 2013). In plant, glutathione reductase (GR), which catalyses the NADPH-dependent reduction of oxidized glutathione, was induced by Se application (Jozefczak et al., 2012; Feng et al., 2013). High capacity of scavenging ROS, indicating high activities of antioxidative enzymes and high contents of low molecular weight antioxidants, is thought to be one mechanism of high Se tolerance in *Ulva* spp. (Schiavon et al., 2012).

Algae and cyanobacteria are reported to provoke resistance to a variety of metal and transition metal oxyanions, including Se (Huang et al., 2013; Dixit and Singh, 2014). The resistance is attributed to the capacity of these organisms to modify their antioxidative machinery in response to toxic ROS generated under stressful condition which in turn can damage the cellular compartments. *Synechococcus elongatus* is reported to be involved in the protection against the toxic effects of ROS produced as a result of temperature, light intensities and heavy metal stress by the enhanced production of stress proteins. These stress proteins may stamp out or reduce cellular damage (Ludwig and Bryant, 2012; Xiong et al., 2015). However, not much is known regarding the biochemical modulation of the antioxidant defense system of cyanobacteria under Se stress. Thus, to study the tolerance mechanism through stress induction in cyanobacteria, *S. elongatus* PCC 7942 has been chosen as a test organism to study the changes associated with Se stress on antioxidative system.

MATERIALS AND METHODS

Cyanobacterial strain and growth conditions

The test cyanobacterium *S. elongatus* PCC 7942 (Pasteur Culture Collection of cyanobacteria, Institute Pasteur, Paris, France) were grown in BG-11 medium, under constant illumination of 60 µE photons/m^2s using cool fluorescent lamps (Philips, India) as previously mentioned (Rippka et al., 1979). The purity of the culture was routinely tested by plating onto Luria-Bertani medium and under bright field microscope (Sambrook et al., 1989). Initial optical density was set at 0.2 at 750 nm (OD_{750}) at the time of inoculation, and cultures were allowed to grow for 12 d in Erlenmeyer flasks.

Selenium treatment

The Erlenmeyer flasks (100 ml) containing 50 ml of BG-11 media were used for all treatments. Growth response in the presence of SeO_4^{2-} was examined in BG-11 media containing increasing concentration of SeO_4^{2-}. Exponentially grown cells were harvested, washed twice with SO_4^{2-} free medium and 10% v/v inoculum was resuspended in fresh media with absence (control) and presence of different concentration of SeO_4^{2-} (50 - 500 µM). Effect of Se on growth of *S. elongatus* PCC 7942 was investigated and measured by the absorbance at 750 nm which was determined spectrophotometrically. Absolute growth was monitored as ΔOD_{750} nm ($0 \rightarrow 6$ days).

Preparation of cell free extract

Cyanobacterial cells were grown without or with added SeO_4^{2-} (50, 100, 150, and 200 µM) in the BG-11 medium for 72 h. Cells were collected by centrifugation at 5,000 × *g*, and the pellet was washed and re-suspended in 20 mM Tris-HCl buffer, pH 8.0 to reach a final density of 1: 3 (w/v). The cell suspension was sonicated at 4°C for 10 min by two cycles of 5 min each and interval of 5 min between the cycles. The sonicated cells were centrifuged at 12,000 × *g* for 10 min at 4°C; the resulting supernatant was dialyzed using 15 kDa cut-off membrane against the extraction buffer (Bagchi et al., 2012). Protein content in the cell free extracts was estimated according to Lowry et al. (1951).

Measurement of ROS generation

Cellular ROS levels (MDA content, $O2^{\bullet-}$ generation and H_2O_2 level) were measured under various SeO_4^{2-} concentrations. *S. elongatus* PCC 7942 cells were harvested by centrifugation at 5,000 × *g* for 10 min and incubated with or without SeO_4^{2-}. Lipid peroxidation level was measured in terms of 2-thiobarbituric acid (TBA) reactive metabolite, mainly MDA content (Ribeiro de Souza et al., 2012). $O^{2\bullet-}$ were measured by monitoring the nitrite formation from hydroxyl amine (Yang et al., 2008). H_2O_2 content was evaluated according to Lee and Shin (2003). ROS contents were measured as µmol g^{-1} fresh weight (FW).

Determination of enzymatic antioxidants

The SOD activity was assayed according to Saeed et al. (2012) by monitoring the inhibition of reduction of nitro blue tetrazolium (NBT). CAT activity was determined by monitoring the decrease in absorbance due to consumption of H_2O_2 at 240 nm (Heikal et al., 2012). POX activity was measured according to Hirsch et al. (1961). GPX activity was assayed by coupling the oxidation of glutathione to NADPH oxidation in the presence of t-butyl H_2O_2 as following the decrease in absorbance at 340 nm (Bagchi et al., 2012). The GR activity was determined according to Sukkhaeng et al. (2015) by measuring the reduction of oxidized glutathione by quantifying NADPH oxidation at 340 nm.

Cell free extract equivalent to 75 µg protein was used as the enzymatic source for all the aforementioned enzyme assays. For CAT, POX, SOD and GPX, blank set was reaction mixture omitting H_2O_2 while in case of GR, it was reaction mixture omitting NADPH. Activity of all enzymes were measured as µmol product min^{-1} mg^{-1} protein.

Activity staining of antioxidant enzymes

The crude enzyme samples were subjected to non-denaturing native PAGE according to Kumar et al. (2012) with a uniform amount of protein (75 µg). Native-PAGE was carried out on a mighty small SE 250 vertical slab gel electrophoretic apparatus (Hoeffer, USA) at 4 ± 1°C with Tris-glycine buffer pH 8.3 described by Laemmli (1970), at 50 V through 5% stacking gel and at 80 V through 7.5% resolving gel, except for GPX and GR which was run in 10% resolving gel. After electrophoresis separation of antioxidant enzyme, the gels were stained for individual enzyme activity. Activity staining for SOD was carried out by the method of Yen et al. (1996). Activity of POX was described by Mittler and Zilinskas (1993). CAT activity was determined by Srivastava et al. (2012). Staining of GPX was performed by the method described by Das and Bagchi (2010). Activity staining for GR was depicted according to Scott et al. (1963).

Statistical analysis

A one-way analysis of variance (one-way ANOVA) with multiple comparisons by the Duncan's test at $p < 0.05$ was applied to compare means among different treatments. Data are presented as mean±standard deviation (SD) from three replicates and statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, USA). The correlation analysis was carried out using Microsoft Excel, Office 2007.

RESULTS

Growth kinetics

In order to study the effect of Se on the growth of unicellular cyanobacterium *S. elongatus* PCC 7942, culture was grown in BG-11 medium in the absence (control) and presence of various concentration SeO_4^{2-} (50 to 500 µM). SeO_4^{2-} has either stimulating or toxic effects on the culture of *S. elongatus* PCC 7942 depending on the Se levels in BG-11 medium. As shown in Figure 1a and b, the biomass concentration was significantly enhanced upon exposure to 50 µM SeO_4^{2-}, while upon increasing Se concentration in the culture medium (beyond 250 µM) growth ceased. Growth was observed to be repressed by 50% upon incubation with 200 µM SeO_4^{2-}.

Determination of ROS generation

In our study, effect of Se on cellular ROS level was investigated in cyanobacterium. Se stress caused an elevation in the level of ROS, especially with exposure to 200 µM SeO_4^{2-} concentration. Lipid peroxidation, $O2^{\bullet-}$ and H_2O_2 are important stress indicators, were assayed with TCA extracted samples. An accumulation of lipid peroxide is an indicative of enhanced production of ROS. At higher concentration of Se, the MDA content increased by around 9 folds for 200 µM SeO_4^{2-} (Table 1). The same tendency also appeared at the change of $O2^{\bullet-}$ activities and H_2O_2 levels. The addition of SeO_4^{2-} at 200 µM, $O2^{\bullet-}$ levels and H_2O_2 content increased significantly by around 7 and 9 folds, respectively in treated cells compared to untreated cells (Table 1). Furthermore, at 50 µM SeO_4^{2-} concentrations, there was no significant difference in contents of oxidative radicals and products between control and treated cells (Table 1).

Antioxidant activity measurement

The antioxidant enzyme activities were measured in the absence and presence of 50-200 µM SeO_4^{2-} treated cyanobacterial crude extracts. It became important to determine the counteractive role of antioxidant which may suppress the Se caused oxidative damage. Table 2 shows the activities of several antioxidant enzymes, namely, SOD, POX, CAT, GPX and GR following Se application. Changes were observed in the activities of all the enzymes tested. All enzymes showed enhanced activities in lower concentrations (50 µM) of SeO_4^{2-}, although following an exposure to higher Se concentration upto 200 µM SeO_4^{2-}, the enzyme activities reached much higher values than the untreated controls except CAT. There was a 6 fold enhancement of total SOD activity compared to the control (Table 2). The activity of POX was found to show 4 fold increase at 200 µM SeO_4^{2-} (Table 2). However, activity of CAT (Table 2), showed no significant difference without/with SeO_4^{2-} present in the media. GPX and GR values were significantly increased with increasing Se concentration. The activities of GPX and GR at 200 µM SeO_4^{2-} registered as 4 and 5 fold increased over control activities (Table 2). A direct correlation between antioxidant enzymes and SeO_4^{2-} concentration was obtained by linear regression analysis (Table 3).

Effect of selenium on isozyme patterns of antioxidant enzymes

The changes of antioxidant isozyme patterns in response to Se were also analyzed by means of activity staining of the native-PAGE gel. SOD (Figure 2a) showed consistently increased band intensity with increasing Se concentration (50 to 200 µM SeO_4^{2-}). The strongest activity of SOD was detected at 200 µM SeO_4^{2-}. In case of POX, there is one high molecular weight band of its isozyme which appeared in the presence of 50 to 200 µM SeO_4^{2-}. However the intensity of POX bands were increased with increasing Se concentration (Figure 2b). In order to examine whether CAT isozyme profiles might be affected by Se, cyanobacterial extract was subjected to native-PAGE and stained for CAT activity (Figure 2c). There was only a single enzyme band for CAT activity in the control and all Se treated (50 to 200 µM SeO_4^{2-}) cyanobacterial samples. As shown in Figure 2d, GPX in *S. elongatus* PCC 7942, after Se treatment existed as one high molecular isozyme under 50 µM SeO_4^{2-}. Whereas under the higher Se concentration, two GPX

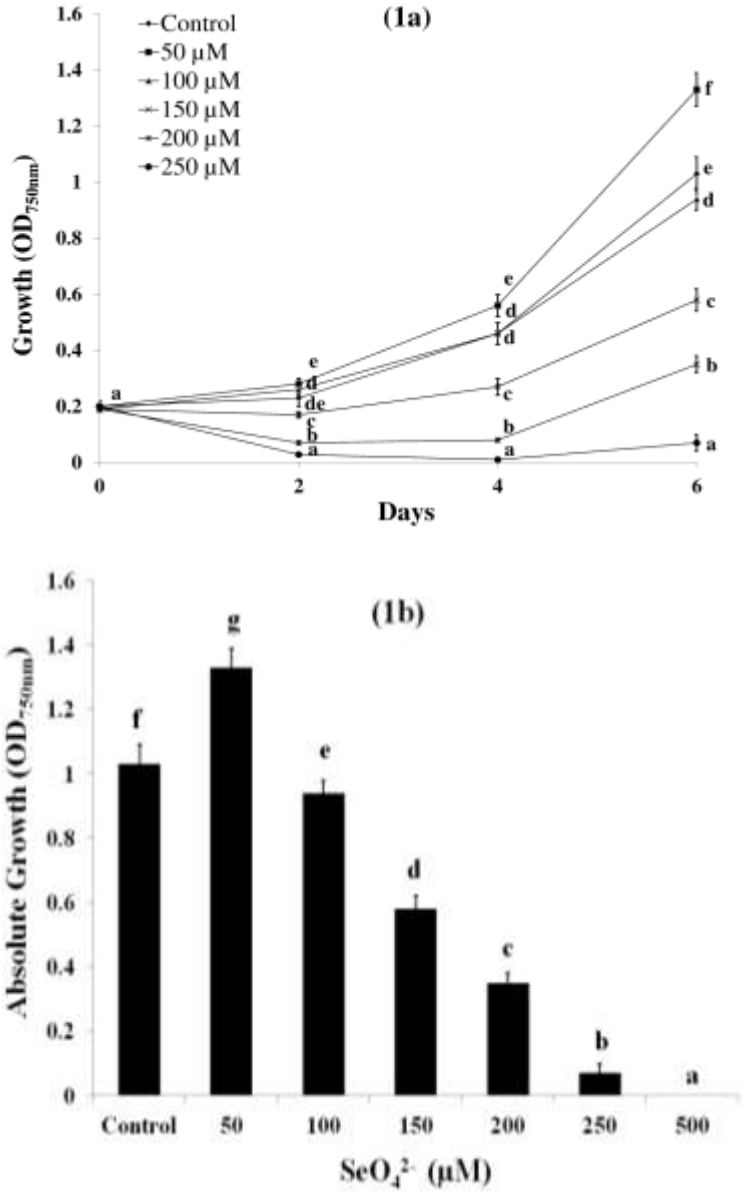

Figure 1. Growth of *S. elongatus* PCC 7942 in BG-11medium with varying SeO_4^{2-} concentrations **(a),** column represents the absolute growth during 6 days **(b)**. Values expressed are means ± SD of three replicates, Significant differences were calculated at $p<0.05$ by one way ANOVA followed by Duncan's post hoc test. Different letters above lines and bars indicate significant differences between treatments.

Table 1. Effect of SeO_4^{2-} incubation on ROS (µmol g^{-1} FW) level in *S. elongatus* PCC 7942: Lipid peroxidation as MDA content, $O2^{\bullet-}$ and H_2O_2.

Parameter	SeO_4^{2-}				
	Control	50 µM	100 µM	150 µM	200 µM
MDA	3.9±0.1	13.77±0.2	34±0.6	46.9±0.1	55.3±1.1
$O2^{\bullet-}$	12±0.4	25±0.7	49±0.8	55±0.1	67±0.3
H_2O_2	29.73±0.2	55.87±1.7	120.67±0.5	170.33±0.5	211±1.7

Values are expressed as means ± SD of three replicates. Significant differences were calculated at $p<0.05$ by one way ANOVA followed by Duncan's post hoc test.

Table 2. Effect of SeO_4^{2-} on activities (µmol product min^{-1} mg^{-1} protein) of antioxidant enzymes of *S. elongatus* PCC 7942.

Parameter	SeO_4^{2-}				
	Control	50 µM	100 µM	150 µM	200 µM
SOD	24.57±0.5	42±1	67.23±1.3	102.3±0.6	143.67±1.5
POX	47±1	58.89±0.8	67.69±1.5	89.8±0.7	103.67±1.5
CAT	51.2±1.5	47±1	46.53±0.4	44.1±0.3	41.87±0.1
GPX	41.16±0.7	64.77±0.6	98.43±0.3	121.33±1.5	156±1
GR	80.4±1.4	161.57±1.5	220.6±0.5	277.53±1.4	358.53±1.5

Values are expressed as means ± SD of three replicates. Significant differences were calculated at $p<0.05$ by one way ANOVA followed by Duncan's post hoc test.

Table 3. Correlation analysis between antioxidant enzymes under different SeO_4^{2-} (0-200 µM) concentration.

Parameter	Equation	R^2
SOD	y = 0.597x + 16.253	R^2 = 0.9735
POX	y = 0.2885x + 44.547	R^2 = 0.9768
CAT	y = -0.0431x + 50.453	R^2 = 0.9031
GPX	y = 1.3443x + 85.3	R^2 = 0.9952
GR	y = 1.3443x + 85.3	R^2 = 0.9952

isozymes were obtained with higher band intensity at 200 µM SeO_4^{2-}. Analyses of the pattern of GR isozyme by native PAGE and activity staining revealed two low molecular bands under the presence of Se whereas enhanced intensity of bands of GR was found only in case of 200 µM SeO_4^{2-} incubation (Figure 2e).

DISCUSSION

Se has dual effect on the growth of unicellular green alga *C. vulgaris* depending on the Se level in the medium, that is, minimal concentration (≤75 mg l^{-1}) of Se considerably improve the growth whereas high Se concentration (≥100 mg l^{-1}) lead to toxicity, resulted in lowering of biomass concentrations and growth rate (Sun et al., 2014). Babaei et al. (2017), have seen the synergistic effect of Se and irradiance intensity in *Chlorella* cultures. At low irradiation (250 µmol photons m^{-2} s^{-1}), the daily dose of Se below 8.5 mg per g of biomass (<20 µM) partially induced the growth and photosynthetic activity of *Chlorella* culture. In contrast, at daily doses of Se and increased irradiance (750 µmol photons m^{-2} s^{-1}) the synergistic effect manifested by significant inhibition of growth, photosynthesis and lowered Se incorporation to biomass was seen. In our study, cells of the wild type *S. elongatus* PCC 7942 were grown in the absence (control) and presence of increasing concentration (50 to 500 µM) of SeO_4^{2-}.

As illustrated in Figure 1, trace amount of Se was found to be growth supportive for *S. elongatus* PCC 7942 and higher Se (up to 200 µM SeO_4^{2-}) concentration showed 50% inhibition in growth (Figure 1).

ROS are generated by cellular exposure to heavy metals, these ROS are extremely reactive and rapidly disrupt normal cell metabolism (Sharma et al., 2012). Different environmental stresses lead to extreme production of ROS causing progressive oxidative damage. Among them is alteration in the cellular levels of ROS, which may cause cellular damage (Feng and Wei, 2012). In the present study enhancements of $O2^{\bullet-}$, H_2O_2 content and level of lipid peroxidation in terms of MDA content after SeO_4^{2-} treatment (Table 1) suggest that Se caused oxidative damage possibly by generating ROS. This is in accordance with the findings of Schiavon et al. (2012).

In plants, low doses of Se enhanced photosynthesis, primary metabolism, and antioxidative system, suggesting that these compounds were involved in the mechanisms of Se tolerance (Wang et al., 2012). The induction of antioxidants in response to enhanced ROS production is generally proportional to the duration and severity of the stress applied to algal cultures (Sun et al., 2014). In our study, it was observed that Se accumulation resulted in higher activities of SOD, POX, GPX and GR after 72 h (Table 2). The enzyme activities continued to augment with the increase in Se concentration in the BG-11 media except CAT. Additionally, the correlation analysis revealed that SOD, POX, CAT, GPX and GR activities were directly correlated with the different concentrations of SeO_4^{2-} in the medium (Table 3). In contrast, in an earlier report on green alga *Ulva fasciata*, Se remarkably

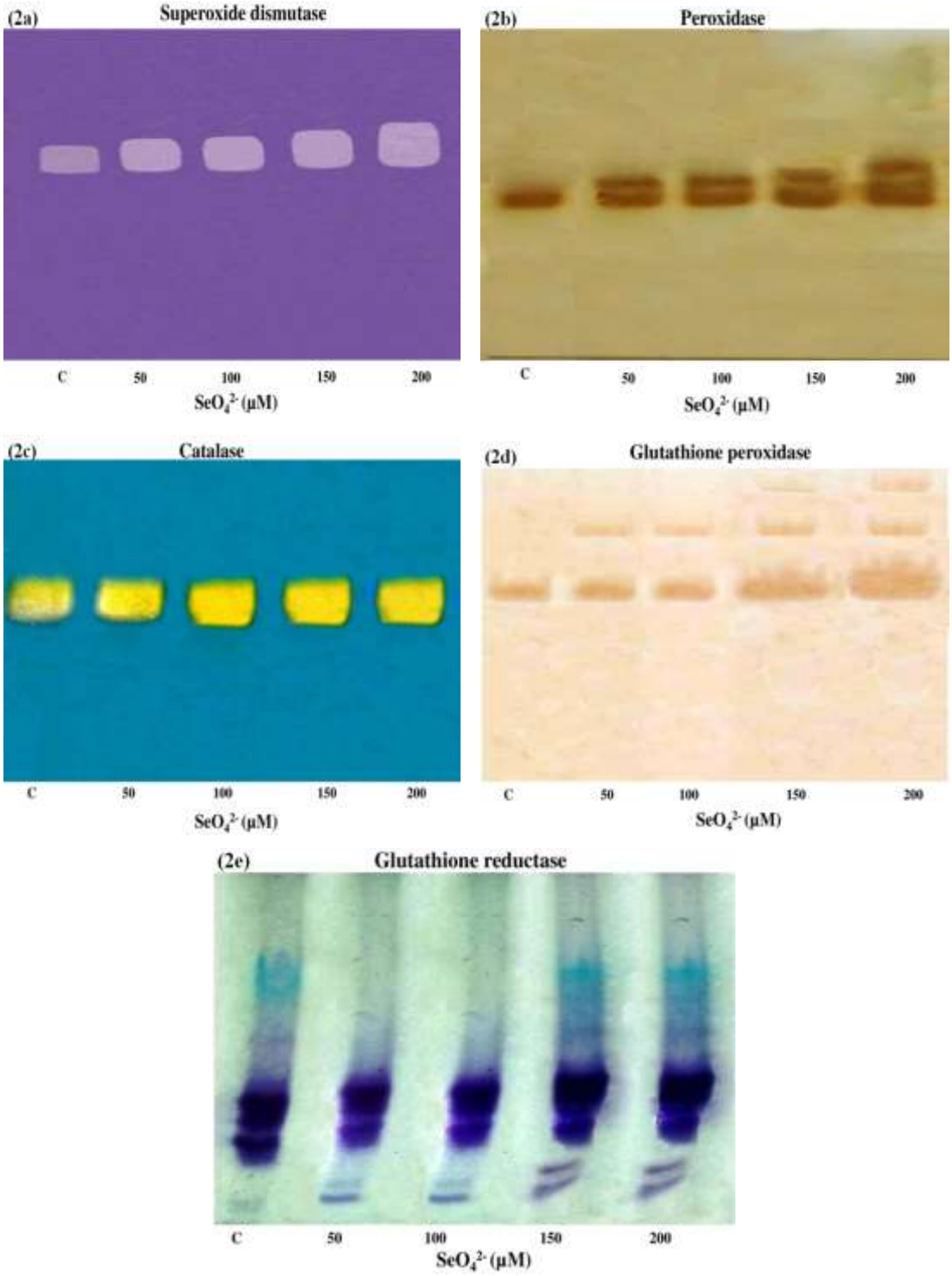

Figure 2. Changes in the isozyme patterns of antioxidative enzymes in *S. elongatus* PCC 7942 in the absence of $SeO4^{2-}$ (C=control); in the presence of 50-200 μM $SeO4^{2-}$: (a) superoxide dismutase (SOD), (b) peroxidase (POX), (c) catalase (CAT), (d) glutathione peroxidase (GPX), and (e) glutathione reductase (GR).

increased the activity of CAT, GPX, POD and SOD (Zhong et al., 2015). Se induced the expression of GPX and GR under UV stress in *Azolla caroliniana* (Mostafa and Hassan, 2015). In rice plant and sunflower (*Helianthus annus*) seedlings, Se alleviated Cd toxicity, and ROS by inducing SOD, POX and CAT (Lin et al.,

2012; Saidi et al., 2014). Malik et al. (2012) observed an increase in SOD, CAT and GR activities in Mungbean plant exposed to different Se concentration and antagonized toxic effect of As. Se treatment indicated the protective role of these antioxidative enzymes against Se-induced oxidative stress (Table 2).

In this work, alterations in zymograms of antioxidant enzymes was also found in the absence and presence of Se. Native PAGE study supported the spectrophotometric analysis of these enzyme activities (Figure 2). In case of SOD, the increment in band intensities at all concentrations of Se suggest that Se stress induces superoxide radicals in *S. elongatus* PCC 7942 (Figure 2a). Two major SOD isoforms have been reported in red seaweed *Gracilaria dura*, the protective role of exogenously supplied Se and polyamines (PAs) such as spermine (Spm) and putrescine (Put) in detoxifying the cadmium (Cd) induced toxicity in laboratory conditions has been suggested by Kumar et al. (2012). The rapid elevation in peroxidase and glutathione peroxidase activity in gel may be another mechanism of Se induced ROS protection and may be associated with the elevation of H_2O_2 level by the higher SOD activity (Shah and Nahakpam, 2012; Zhai et al., 2013). In this study, one isozyme of POX and two isozymes of GPX were observed in Se stress, suggesting that, these enzymes participated to remove H_2O_2 in *S. elongatus* PCC 7942 (Figure 2b and d). The catalase activity of *S. elongatus* PCC 7942 did not register wide variation over all Se concentrations used, both spectrophotometrically and in the native gel (Figure 2c). Several studies reveal, high level of GR induction as the result of ROS produced by different environmental stress in algae (Gill et al., 2013). In this study, GR showed two isozymes under 200 µM SeO_4^{2-} and is supposed to re-reduce the oxidized glutathione generated from GPX activity (Figure 2e), indicating that the oxidative protection mechanism was increased and that the ROS level in the *S. elongatus* PCC 7942 might be under control. Such maintainance of the ROS level is beneficial to this cyanobacterium.

Conclusion

Exposure of *S. elongatus* PCC 7942 to low concentration of Se revealed beneficial effect and promoted the growth. Se accumulation also triggered oxidative stress and provoked pronounce responses of antioxidant systems which protect the *S. elongatus* PCC 7942 to some extent against oxidative damage, but the direction of response was dependent on the intensity of the Se stress. The future line of research will focus on detection of intracellular accumulation of Se in *S. elongatus* PCC 7942.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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