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Antioxidant response of *Chlamydomonas reinhardtii* grown under different element regimes

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SUMMARY

Nutrient stress is one of the most favorable ways of increasing neutral lipid and high value-added output production by microalgae. However, little is known about the level of the oxidative damage caused by nutrient stress for obtaining an optimal stress level for maximum production of specific molecules. In this study, the antioxidant response of *Chlamydomonas reinhardtii* grown under element deprivation (nitrogen, sulfur, phosphorus and magnesium) and supplementation (nitrogen and zinc) was investigated. All element regimes caused a decrease in growth, which was most pronounced under N deprivation. Element deprivation and Zn supplementation caused significant increases in H_2O_2 and lipid peroxidation levels of *C. reinhardtii*. Decrease in total chlorophyll level was followed by an increase of total carotenoid levels in *C. reinhardtii* under N and S deprivation while both increased under N supplementation. Confocal imaging of live cells revealed dramatic changes of cell shape and production of neutral lipid bodies accompanied by a decrease of chlorophyll clusters. Antioxidant capacity of cells decreased under N, S and P deprivation while it increased under N and Zn supplementation. Fluctuation of antioxidant enzyme activities in *C. reinhardtii* grown under different element regimes refers to different metabolic sources of reactive oxygen species production triggered by a specific element absence or overabundance.

Key words: element deprivation, element supplementation, oxidative stress, reactive oxygen species.

INTRODUCTION

Elevated or depleted micro- and macronutrient levels are often a direct cause of oxidative stress in plants (Reitan *et al*. 1994). Depletion of essential elements such as nitrogen, sulfur, manganese and iron results in major disruptions in the photosynthetic machinery in particular, while overabundance of trace elements is associated with a response similar to that of heavy metal toxicity (Szivak *et al*. 2009). In a general sense, various cellular mechanisms are affected by changes in existence or overabundance of essential elements, and cells re-organize the production level of their defensive metabolites in order to compensate for the damage associated with various types of reactive oxygen species (ROS) formed during the process. Antioxidant defense mechanisms of living cells include several enzymatic antioxidant molecules such as catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7), superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (AP, EC 1.11.1.11), glutathione reductase (GR, EC 1.6.4.2), as well as non-enzymatic scavengers such as ascorbate, glutathione, carotenoids and flavonoids. Various antioxidative enzymes mentioned above have been implicated or demonstrated to function as effective quenchers of ROS in algae (Ledford & Niyogi 2005). Some microalgae and a variety of plants have been reported to accumulate proline in the presence of overabundance of some metals such as copper, zinc and cadmium, and proline pretreatment was reported to help overcoming potential metal stress and to sustain osmotic balance (Tripathi *et al*. 2006; Verbruggen & Hermans 2008). The putative role of proline in overcoming metal stress or osmotic imbalance has been basically discussed in the scope of a potential role of proline as metal chelator in the cell cytoplasm (Verbruggen & Hermans 2008). Recently, changes in element concentration of *Chlamydomonas reinhardtii* P.A. Dangeard CC-124 wild type mt- [137c] growth medium was shown to cause dramatic changes in cellular metal content (Cakmak *et al*. 2014). Thus, changes in proline content, ROS accumulation and related antioxidant responses are evaluated in the present study.

Apart from molecular and biochemical changes, element stress induces accumulation of lipids, carbohydrates or proteins as a source of energy for further use by microalgae. In cases where element starvation- or overexposure-related metabolic effects are desirable, such as when the stimulus in question results in increased production of a commercially important metabolite, nutrient-mediated changes in enzyme expression levels have also been used to generate higher yields either alone or in tandem with genetic manipulation of relevant synthesis pathway (Rodríguez-Sáiz *et al*. 2010). As such, investigation into the microalgal metabolic response to element depletion or overabundance is of considerable academic and commercial importance.

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Chlamydomonas reinhardtii is a unicellular bifilagellar microalga common in both soil and freshwater. It is considered as a model organism in microalgal research, with well-characterized metabolic pathways and a published genome sequence (Merchant *et al*. 2007). There is an extensive record in the literature concerning the metabolic response of *C. reinhardtii* to depleted concentrations of various elements, with genome-wide microarray studies conducted on nitrogen and sulfur deprivation in particular (Bölling & Fiehn 2005; Wang *et al*. 2009). Element over-nutrition studies are generally conducted on wastewater systems which contain various elements in high concentrations, and these studies generally consider the removal of toxic compounds by bioremediation (Kong *et al*. 2010). However, to our knowledge, a comprehensive study comparing the effects of overexposure to or absence of various elements on oxidative stress-related tolerance and compensation mechanisms has yet to be attempted for *C. reinhardtii*. In our recent study, we evaluated growth and triacylglycerol production related parameters of *C. reinhardtii* grown under N, S, P, Mg, K, Ca, Fe or Zn deprived Tris-Acetate-Phosphate (TAP) media, or in TAP media supplemented with each of these elements in concentrations five times that of the base medium. All nutrient limitations studied, except those of K and Zn had a negative impact on growth, while only Zn and N over-supplementations elicited a significant decrease in the growth rate (Cakmak *et al*. 2014). Moreover, the increase in triacylglycerol levels were in most cases associated with a decrease in cell growth; thus, N, S, P, Mg deprived, and N and Zn supplemented media were selected for further analysis in the scope of biodiesel feedstock production and the level of the oxidative stress caused by the element regimes applied in this study. Hence, the present study examines antioxidant response of *C. reinhardtii* in response to the abovementioned element manipulations and discusses the level of oxidative stress caused by element manipulation for production of neutral lipids by *C. reinhardtii*.

MATERIALS AND METHODS

Strain and culturing conditions

The wild type *C. reinhardtii* mt (-) 137c strain CC-124 was obtained from the Chlamydomonas Resource Center (http://www.chlamy.org). Cells were grown at 23°C under continuous light (150 μmol photons m^{-2} s^{-1}) in liquid cultures on a rotary shaker (120 rpm). Standard TAP medium, which uses acetate (17.4 mM) as the carbon source and Tris-base (20 mM) as a buffer, was prepared as described previously (Harris 1989) and slightly modified for element deprivation and overabundance applications. Cell number in stock *C. reinhardtii* culture was determined by immobilizing cells with lugol solution and using Neubauer hemocytometer. A 20 μL cell suspension was spread on Neubauer hemocytometer slide and cells were counted under microscope (Carl Zeiss-Axio A.1, Jena, Germany). Stock cell culture was grown until late log phase (when daily cell count revealed a less than 20% increase) with around 1.2×10^7 cells mL^{-1} and the respective volume of cell suspension for 3×10^4 cells mL^{-1} was inoculated into 100 mL new culture medium in 250 mL flasks for experimental analysis during 10 days of incubation period.

For N starvation studies, cells were centrifuged at 2000 *g* for 5 min under room temperature and the pellets were washed twice in TAP medium without N (TAP-N). The pellets were then re-suspended and the cells were grown under constant light exposure on a rotary shaker. The same procedure was followed for S, P and Mg starvation studies, using TAP medium without S, P and Mg (TAP-S, TAP-P, and TAP-Mg, respectively). Each treatment was performed in triplicate. Initial pH values in all media were set to seven prior to algal cell inoculation, pH values of the media were checked every 24 h and found not to deviate more than 8% from the initial throughout the 10-day incubation period. Cell number in different growth media was calculated on first 12th, 24th and then every 24 h of incubation as described above. Average doubling time ($D.T_{Ave}$) was calculated using the equation $T = (t_y\text{-}t_x)/\log_2 (N_y/N_x)$ with Ny and Nx being the number of cells at the start (t_x) and end (t_y) over 10 days of growth (Lim *et al*. 2012). Exponential doubling time was calculated between 12–48 h where the maximum slope on the average cell density *x*-axis time plot was registered. The cell aliquots (100 mg) were prepared by using harvested cell suspensions on 1st, 3rd, 5th, 7th and 10th days of element manipulation, centrifuged at 3000 *g* for 5 min at 4°C, cell pellets were frozen in liquid nitrogen and then stored at –86°C in a freezer for further experimental analyses.

Chlorophyll and carotenoid content

Chlorophyll and carotenoids were determined using a spectrophotometric method modified from Jeffrey and Humphrey (1975). A 300 mg frozen microalgal pellet was re-suspended with 500 mL of 90% acetone, incubated by mixing for 15 min and centrifuged at 15 000 *g* for 5 min at room temperature. The supernatant was then loaded in a 96-well plate. The absorbances were collected at 470, 630, 647, 664, and 750 nm and ratios were calculated using the equations of Jeffrey and Humphrey (1975). Total chlorophyll results were presented as a sum of chlorophyll *a*, *b*, and *c*.

Confocal laser scanning microscopy

The absorbance of 1-mL cell suspension was measured at 750 nm and the suspension was either condensed or diluted with respective media to 0.2 absorbance at 750 nm. Next, 1 mL of cell suspension was stained with 15 μL of 1 mM Nile Red (Invitrogen, Carlsbad, CA, USA) dissolved in pure acetone (final concentration 15 μM) on a rotator for 15 min under dark conditions. After incubation, slides were prepared to acquire images by using an LSM 510 confocal microscope (Carl Zeiss) and a Plan Apo 63 oil immersion objective lens with a numerical aperture of 1.40–0.60. The Nile Red signal was captured using a laser excitation line at 488 nm, and the emission was collected between 560 and 600 nm. Chlorophyll fluorescence was captured using a laser excitation line at 633 nm, and the emission was collected at 650 nm. As Nile Red stains lipids as yellow-orange color that overlaps with the red autofluorescence of chlorophyll, we changed chlorophyll signal to green for better visualization of merged images.

Hydrogen peroxide measurement

Hydrogen peroxide levels were determined in cell extracts using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen). For the extraction, 200 mg cell pellet was homogenized in 120 μL potassium phosphate buffer (20 mM, pH 7.4). After centrifugation at 5000 *g* for 5 min at 4°C, 50 μL supernatant was incubated with an equal volume of reaction solution for 30 min in the dark. Relative fluorescence intensity of H_2O_2 was quantified by a fluorescence spectrometer (SpectraMax M5, MDS Analytical Technologies, Sunnyvale, CA, USA) using 544 nm excitation and 585 nm emission wavelengths.

Lipid peroxidation

The thiobarbituric acid (TBA) test, which determines malonyldialdehyde (MDA) as an end product of lipid peroxidation, was used to measure lipid peroxidation in the cells. Briefly, 0.5 g of algal sample was homogenized in 2 mL 80% ethanol solution. The homogenate was centrifuged at 3000 *g* for 5 min under room temperature and 1 mL of supernatant was aliquoted. Then, 20% trichloroacetic acid (w/v) solution including 0.01% butylated hydroxytoluene (w/v) with or without 0.65% TBA (w/v) was added into these aliquots and incubated at 95°C. The reaction was stopped by placing the reaction tubes in an ice bath for 5 min and then the samples were centrifuged at 3000 *g* for 5 min at 4°C. The absorbance of the supernatants was monitored at 532 nm for MDA compounds, 440 nm and 600 nm for correction of anthocyanin and sugar absorbance. The MDA equivalents were calculated using an extinction coefficient of 157 $mM^{-1}\,cm^{-1}$ as described previously (Hodges *et al*. 1999).

Determination of proline content and oxygen radical absorbance capacity

Proline was extracted with 3% sulphosalicylic acid and estimated by the method of Bates *et al*. (1973) using L-proline as a standard. Oxygen radical absorbance capacity (ORAC) assay was performed based on the procedure of Alberto *et al*. (2004). A 200 mg cell sample was mixed with extraction solution (80% acetone in 0.2% formic acid) and incubated for 1 h. Then samples were centrifuged at 6400 *g*, the supernatant was filtered and diluted 10 times with phosphate buffer (75 mM, pH = 7.4). This solution was used as the sample solution. The sample solution (20 μL) and fluorescein (150 μL, 96 nM) were pre-incubated at 25°C and the ORAC analysis was initiated by adding 30 μL of 320 mM AAPH (2,2′-Azobis [2 amidinopropene] dihydrochloride). Trolox equivalents of each sample were determined from the fluorescein decay curves. Butylated hydroxytoluene and ascorbic acid were used as positive controls.

Antioxidant enzyme activities

For the preparation of enzyme extracts, 300 mg of cells were harvested by centrifugation and were suspended in 3 mL ice-cold potassium phosphate buffer (50 mM, pH 6.5) containing 0.2 mM EDTA, 1.0% polyvinylpyrrolidone (w/v), with the addition of 1 mM ascorbate in the case of the AP assay. The homogenate was centrifuged at 13 000 *g* at 4°C. The supernatant was used as the crude extract for enzyme activity assays. Protein content of the extracts was measured (Bradford 1976) for the calculation of enzyme activities.

Catalase activity was assayed by the method of Claiborne (1985). Activity of SOD was measured spectrophotometrically (absorbance at 530 nm) by a superoxide radical dependent reaction in which hydroxylamine is oxidized to nitrite at 25°C (Elstner & Heupel 1976). GR and AP activities were studied according to Schaedle and Bassham (1977) and Nakano and Asada (1989), respectively.

The final data of each experimental group in this article are mean values represented by at least six replicate samples. Standard errors and *t*-tests (two tails, pair type) with significance criteria of 0.05, 0.01, or 0.001 are used to assess significance.

RESULTS AND DISCUSSION

Reorganization of growth under element stress

Depending on level and duration of the stress conditions, formation of gametes, accumulation of lipids or carbohydrates, induction of autophagy or degradation of various cellular elements are reflected by changes in growth efficiency of a microalga (Li *et al*. 2012). To determine effects of N, S, P, Mg deprivation and N, Zn overabundance on growth of *C. reinhardtii*, cells were counted every 24 h during the 10 day incubation period. Initial lag phase was not observed in the controlled and experimental groups during the first 24 h of incubation; however, cells were also counted for the first 12 h of incubation and the cell number was registered as lag phase considering the slope obtained during the first 12 h of incubation (Fig. 1a). Calculation for exponential doubling time was made over 12–48 h time plot where the highest slope on average cell density versus time was registered. Average doubling time was also calculated (0–240 h) to evaluate overall effects of element manipulation on *C. reinhardtii* growth (Fig. 1b).

Exponential growth of *C. reinhardtii* grown in control, N and Zn oversupplemented media occurred until the 5th day of incubation and ended up with approximately 11.2×10^6, 8.72×10^6 and 6.81×10^6 cells mL^{-1}, respectively (Fig. 1a). On the other hand, maximum cell number was determined on the last day of incubation with a value of 13.78×10^6, 10.48×10^6 and 9.82×10^6 cells mL^{-1} in controlled, N and Zn oversupplemented media, respectively, and the decrease in cell density was found statistically significant ($P < 0.05$). An increase in N or Zn level in growth media was recently reported to have adverse effects on *C. reinhardtii* growth (Cakmak *et al*. 2014; Mikulic & Beardall 2014). Despite its role as cofactor for many biochemical processes, at high concentrations, zinc damages photosystem II (Spijkerman *et al*. 2007) and decreases efficiency of Calvin cycle (Bertnard & Poirrier 2005) resulting in reduction of growth accompanied by several metabolic responses. We used moderately high levels (5×) of Zn and N in the growth media to analyze the antioxidant response of *C. reinhardtii*. Slight

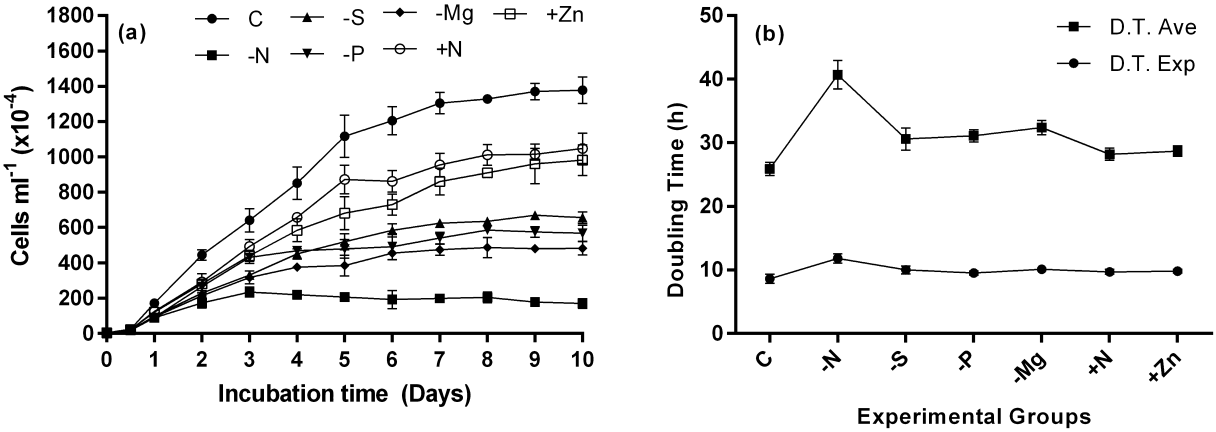

Fig. 1. Changes in the amount (a) and doubling time (b) of *Clamydomonas reinhardtii* grown under different element regimes. Doubling time (D.T.) for exponential (D.T. Exp.) and average growth (D.T. Ave) was calculated for each group. For all data sets, each point represents mean (± SD) of at least six replicate flasks.

extension of the exponential and average doubling time of *C. reinhardtii* under N or Zn supplementation supports this evaluation (Fig. 1b).

Element deprivation caused significant decreases in growth of *C. reinhardtii*. Exponential growth phase ended up on the 3rd day of incubation with a number of 2.34×10^6 cells mL^{-1}, which was also registered as the maximum cell density grown under N deprivation (Fig. 1a). *Chlamydomonas reinhardtii* is thought to have entered death phase after 3 days of N deprivation because there was a gradual decrease in cell number resulting with 1.7×10^6 cells mL^{-1} at the end of the incubation period. Retardation of doubling time was most pronounced in N-deprived *C. reinhardtii* cells (Fig. 1b). Our results are in concert with previously published studies where *C. reinhardtii* CC-124, CC-125 (James *et al*. 2011) and a clone of *C. reinhardtii* CC-124 (Gopaul *et al*. 2012) strains were reported to reach maximum cell density on the 3rd day of N deprivation. Similar to N-starvation, exponential growth phase lasted in 3 days of incubation with a cell number of 4.31×10^6 cells mL^{-1} under P deficiency (Fig. 1a). However, unlike the N-starvation case, death phase was registered after 8 days of incubation where the highest cell density was recorded with a number of 5.96×10^6 cells mL^{-1} followed by decreases in cell density lasting with a value of 5.68×10^6 cells mL^{-1} at the end of 10 days incubation. In their valuable contribution to understanding P deficiency on biohydrogen production by *C. reinhardtii*, Batyrova *et al*. (2012) reported a similar decrease in growth accompanied by a decrease of chlorophyll levels upon 7 days of P deprivation.

The cells entered stationary phase on the 4th day of S or Mg deprivation with a value of 4.5×10^6 and 3.75×10^6 cells mL^{-1}, respectively. In fact, doubling time of S and Mg starved samples are recorded as approximately 10 h during 12–48 h of incubation while it was measured as 8.6 h in the control group (Fig 1b). This alga was able to increase its cell number up to 6.69×10^6 cells mL^{-1} on the 9th day under S deficiency while maximum cell number was recorded with 4.87×10^6 cells mL^{-1} on the 8th day of incubation under Mg deficiency (Fig. 1a). Restricted ability to maintain the photosynthetic functions resulting with decreases in growth in response to S and Mg starvation was previously reported (Finkle & Appleman 1953; Zhang *et al*. 2002).

Chlorophyll and carotenoid levels under element stress

Photosynthesis is of vital importance for microalgae in order to supply energy demand to sustain metabolism. Photosynthetic efficiency of microalgae is predominantly reflected by the chlorophyll and carotenoid levels and microalgae need to keep their chlorophyll and carotenoid levels in a balance for an efficient utilization of carbon sources (Zhang *et al*. 2002). Our results showed that N, S, and Mg deprivation caused a decrease in total chlorophyll content while decrease in chlorophyll content was found to be significant only after the 7th day of incubation under P deprivation (Fig. 2a). A decrease in chlorophyll content under N and S deprivation was reported previously (Young & Beardal 2003; Cakmak *et al*. 2012). Zinc supplementation caused a considerable decrease on the first day and no change was observed later while N supplementation caused a linear increase in total chlorophyll levels (Fig. 2a). On the other hand, Chlorophyll *a*/*b* ratio also dramatically decreased over time under N, S, P deprivation, and N or Zn overabundance while there was not a consistent change under Mg deprivation (Fig. 2). A decrease in the Chlorophyll *a*/*b* ratio reflects changes in the size of the antenna complex and PSII/PSI (Melis 1991). Except for Mg deprivation, total carotenoid content of the cells increased under all element regimes studied (Fig 2c). Increase in carotenoid content of algal cells was previously reported as part of a defense mechanism against photo-damage (Ledford & Niyogi 2005).

Confocal imaging of live cells showed that lipid body formation is most pronounced when cells were grown under element starvation while production of lipid bodies are also noticeable in cells grown under N and Zn supplementation (Fig. 3). Besides, degradation of chlorophyll clusters is noticeable in element deprived group, N and S deprived ones in

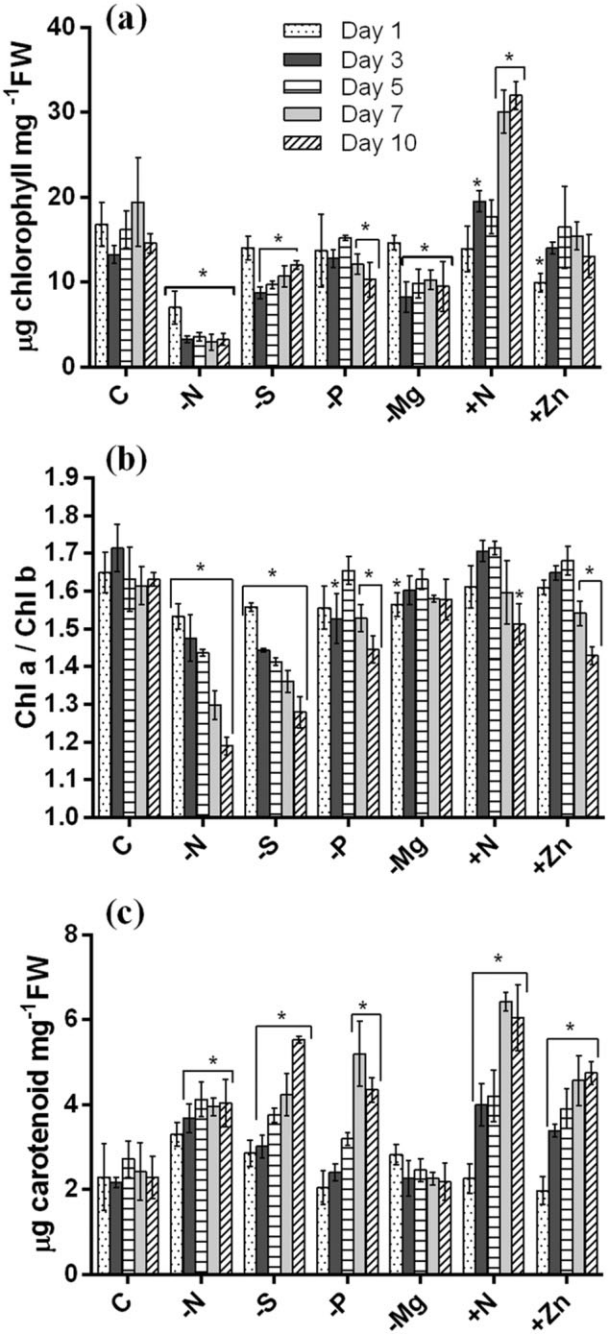

Fig. 2. Changes in (a) total chlorophyll, (b) chlorophyll *a*/*b* and (c) total carotenoid content of *C. reinhardtii* in response to different element manipulations. For all data sets, each point represents mean (± SD) of at least six replicate samples. Asterisks represent statistically significant difference ($P < 0.05$). C, control; –N, –S, –P and –Mg represents N, S, P and Mg deprived cells; + N and +Zn represents cells grown in 5 × N and 5 × Zn media, respectively.

particular. Altogether, images show that *C. reinhardtii* accumulates lipid droplets in response to element stress, and degradation of chlorophyll clusters is a major cause of oxidative stress giving rise to accumulation of storage material (Fig. 3).

Level of the cellular oxidative stress of *C. reainhardtii* under different element regimes

Abiotic stress responses are characterized by an increase in reactive oxygen species (ROS) levels as excess ROS production would cause oxidation of different biomolecules such as lipids, proteins, and nucleic acids leading to the disruption of cellular redox homeostasis (Torres & Dangl 2005). In this study, H_2O_2 content of the cells increased rapidly under N deprivation starting from the first day of incubation (Fig. 3a). Besides this, changes in H_2O_2 content were not found statistically significant on the first days of S, P and Mg deprivation, and Zn supplementation while important increases were recorded especially after the 5th day (Fig. 4a). On the other hand, N supplementation did not cause an important change in H_2O_2 levels (Fig. 4a) which may refer that oxidative stress induced by all element regimes is the main cause of decrease in cell growth except for N supplementation in which the cellular antioxidant response is thought to be efficient in getting rid of potential overproduction of ROS. Increase of intracellular ROS levels induced by H_2O_2 exposure or N deprivation was reported to be directly associated with rapid decrease in growth of *C. reinhardtii* (Yilancioglu *et al*. 2014). Data obtained from chlorophyll, carotenoid content, and lipid peroxidation levels also support these aforementioned trends. Lipid peroxidation level of the cells increased under element deprivation and Zn supplementation while it did not change under N supplementation (Fig 4b). The increase in lipid peroxidation level was most pronounced under N deprivation where highest H_2O_2 levels were recorded. Rapid decrease in growth accompanied by the dramatic degradation of chlorophyll clusters support this outcome. Of the mineral nutrients, N is required in the largest amount and is most often limiting as it is a necessary part of all proteins, enzymes, and metabolic processes involved in the synthesis and transfer of energy (Patterson *et al*. 2010).

Induction of neutral lipid body formation of microalgal cells under element starvation and overabundance (Fig. 3) was an anticipated result of abiotic stress. Change in the lipid content of microalgae was reported to be related to regulation of osmolyte production in microalgae (Pancha *et al*. 2014). In algae, proline was reported to act as an effective osmolyte for maintaining water balance (Wegmann 1986), a chelator of heavy metals in the cytoplasm, and a scavenger of hydroxyl radicals (Smirnoff & Cumbes 1989). In our recent study, we showed that macro- and microelement composition of *C. reinhardtii* greatly varied when cells were grown under different element regimes (Cakmak *et al*. 2014). Thus, in this study, proline content of cells was determined in order to have a better evaluation of the level of oxidative stress in *C. reinhardtii* grown under different element deprivation or overabundance. A dramatic decrease of proline content was determined with a value of 45.6% on the first day and ended up with approximately 70% decrease in *C. reinhardtii* cells grown under N deprivation (Fig. 4c). A similar trend was observed in S, P and Mg deprived cells to a lesser extent. In microalgae, proline level is mostly associated with osmoregulation and oxidative stress caused by metal toxicity (Siripornadulsil *et al*. 2002). A decrease in proline level in our study suggests that defined as a universal stress signal in

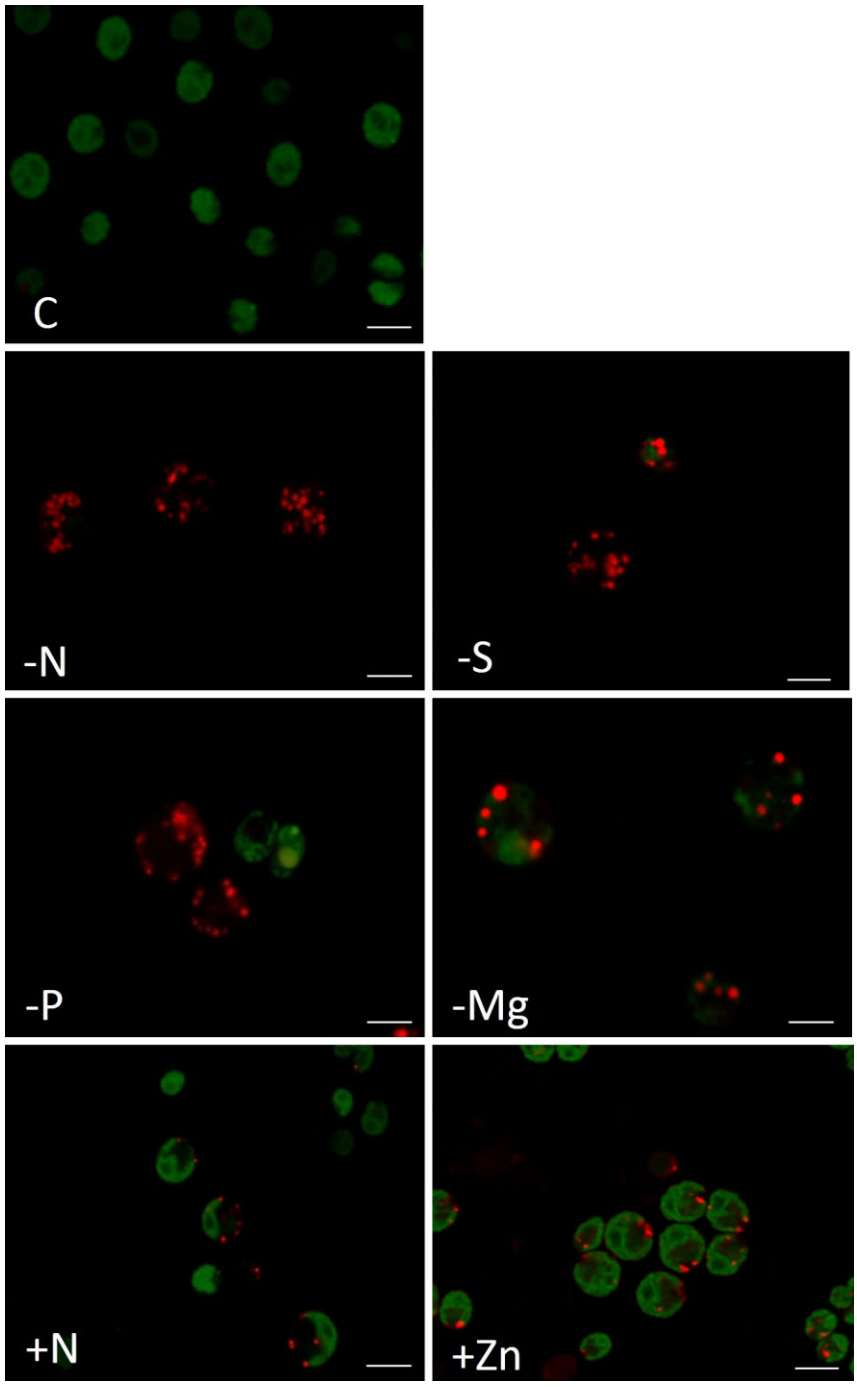

Fig. 3. Confocal fluorescence microscopy images of *C. reinhardtii* cells grown under different element regimes for 10-days. Orange, Nile Red fluorescence; green, chlorophyll fluorescence. Scale = 10 μm. This figure is available in colour online at wileyonlinelibrary.com/journal/pre.

higher plants, changes in proline level would be more pronounced for redox imbalance caused mainly by metal toxicity rather than element deprivation in *C. reinhardtii*. Proline was reported to facilitate cellular detoxification in response to mercury stress in *C. reinhardtii* (Elbaz *et al*. 2010). On the other hand, more recent studies showed that a decrease in proline level is associated with an increase in neutral lipid content and a decrease in growth of *Scenedesmus sp* CCNM and *Nannochloropsis oceanica* Suda et Miyashita IMET1 grown under N-deprivation (Xiao *et al*. 2013; Pancha *et al*. 2014). Our data also supports the findings that increased neutral lipid content might be associated with a decrease of osmolyte content and degradation of chlorophyll clusters resulting in increased ROS production and decreased growth in *C. reinhardtii* grown under macroelement deprivation.

Antioxidant response

In order to keep ROS levels in a balance photosynthetic organisms have evolved antioxidant defense mechanisms involving enzymatic antioxidant molecules such as SOD, CAT,

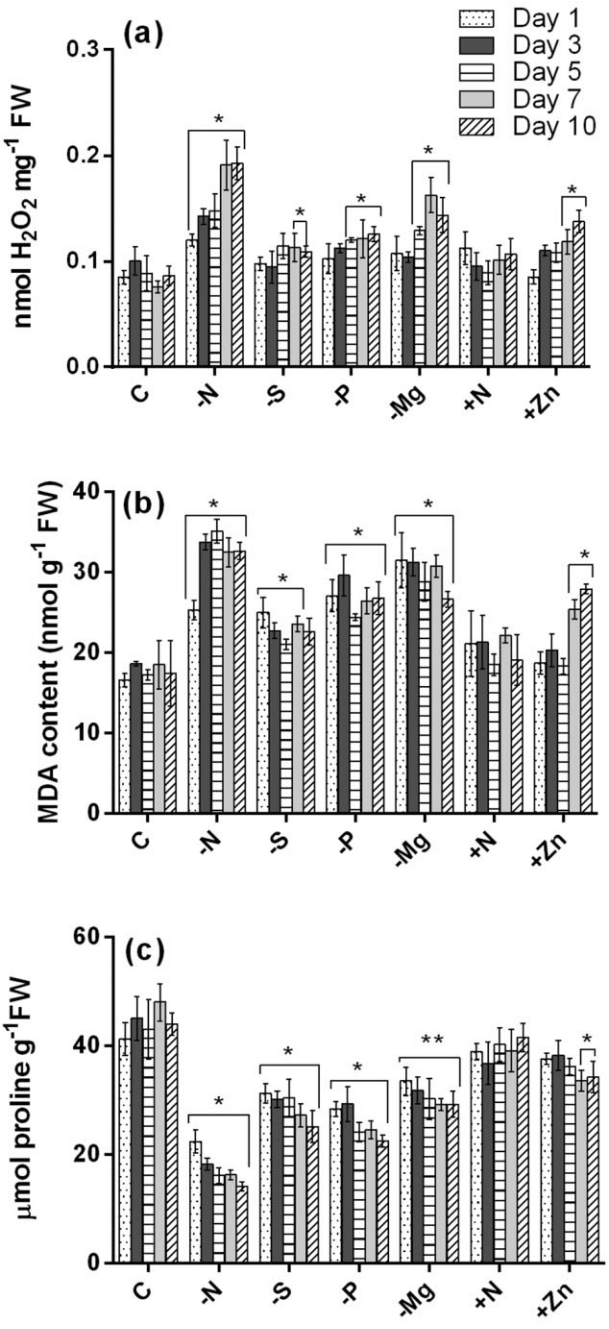

Fig. 4. Changes in (a) H_2O_2 levels, (b) malonyldialdehyte (MDA), and (c) proline content of *C. reinhardtii* cells in response to different element manipulations. The details are shown in Fig. 2.

AP and GR. The enzymatic reaction of SOD with superoxide radicals results in the formation of O_2 and H_2O_2. Hydrogen peroxide that has been produced is then scavenged by CAT, nonspecific peroxidases, and the ascorbate glutathione cycle where AP plays the first role of reducing H_2O_2 to H_2O and GR plays the final role of utilizing NADPH to reduce oxidized glutathione (Mittler 2002). To observe the general antioxidant response of *C. reinhardtii* grown under different element regimes, ORAC values were determined prior to determining enzymatic antioxidant response. Except for Mg deprivation, ORAC of the cells decreased under element deprivation while significant increments were recorded under N and Zn supplementation (see Appendix S1 in Supporting Information). Changes in the ORAC value of microalgae grown under element deprivation regimes (except for Mg deprivation) refer to a wide range of oxidative damage, whereas *C. reinhardtii* cells supplemented with extra N and Zn can sustain cellular homeostasis via increased production of antioxidants. In fact, morphologic traits of *C. reinhardtii* cells were better preserved in N and Zn supplemented media (Fig. 3).

The enzymatic antioxidant response of *C. reinhardtii* was found to vary under different element regimes. SOD activity exhibited marked increases on the first day in all conditions (Fig. 5). However, the activity either decreased (under N and S deprivation) or remained unaltered (under P and Mg deprivation) on day 10. While increases of 34.8% and 42% in the SOD activities occurred on the first day, significant decreases (58.2% and 10.3%) were recorded on day 10 in N and S deprivation studies, respectively (Fig. 5). Nitrogen and Zn supplementation caused remarkable increases in SOD activities on the first day. The increase in the activity was recorded as 33.3% and 83.4% on the first day and ended up with 43.3% and 29.5% under N and Zn supplementation, respectively (Fig. 5). Increased SOD activity of different microalgae under abiotic stress conditions was reported previously (Morelli & Scarano 2004; Li *et al*. 2006). However, our results showed that changes in SOD activity were not consistent in *C. reinhardtii* under element stress. Sabatini *et al*. (2009) reported that SOD activity increased in the BAFC CA4 strain of *Scenedesmus vacuolatus* Shihira and Krauss while it did not change in the BAFC CA10 strain of *Chlorella kessleri* Fott and Novakova under Cu stress. Moreover, Li *et al*. (2006) showed that Cu stress causes an increase in SOD activity while Zn stress does not affect the activity in S-3012 strain of microalga *Pavlova viridis* Tseng, Chen and Zhang.

Catalase activity decreased under element deprivation, while it showed significant increases under N and Zn supplementation (Fig. 5). The activity remained unaffected on the first day under S, P and Mg deprivation but decreased gradually to 19.7%, 64.6% and 81% on day 10, respectively. The activity under N deprivation decreased rapidly on the first day by 41.5% and resulted with a total decrease of 84.2%. Shao *et al*. (2008) reported that the activity of CAT in *C. reinhardtii* is regulated by the level of stress, and decrease in the activity was referred to the activation of ROS signaling. Despite a decrease in CAT activity under element deprivation, noticeable increments were recorded under N and Zn supplementation. The increase in the activity on day 10 was recorded as 87% and 84.6% under N and Zn supplementation, respectively. An increase in CAT activity in microalgae in response to element supplementation was reported previously (Morelli & Scarano 2004; Sabatini *et al*. 2009). Similar response in the case of SOD and CAT enzyme activity increments in *C. reinhardtii* under N or Zn supplementation may refer to increased superoxide levels, which are primarily scavenged by enzymatic action of SOD and CAT considering differential responses obtained from AP and GR measurements.

Ascorbate peroxdase activity increased in response to N and Mg deprivation or Zn supplementation (Fig. 5). The

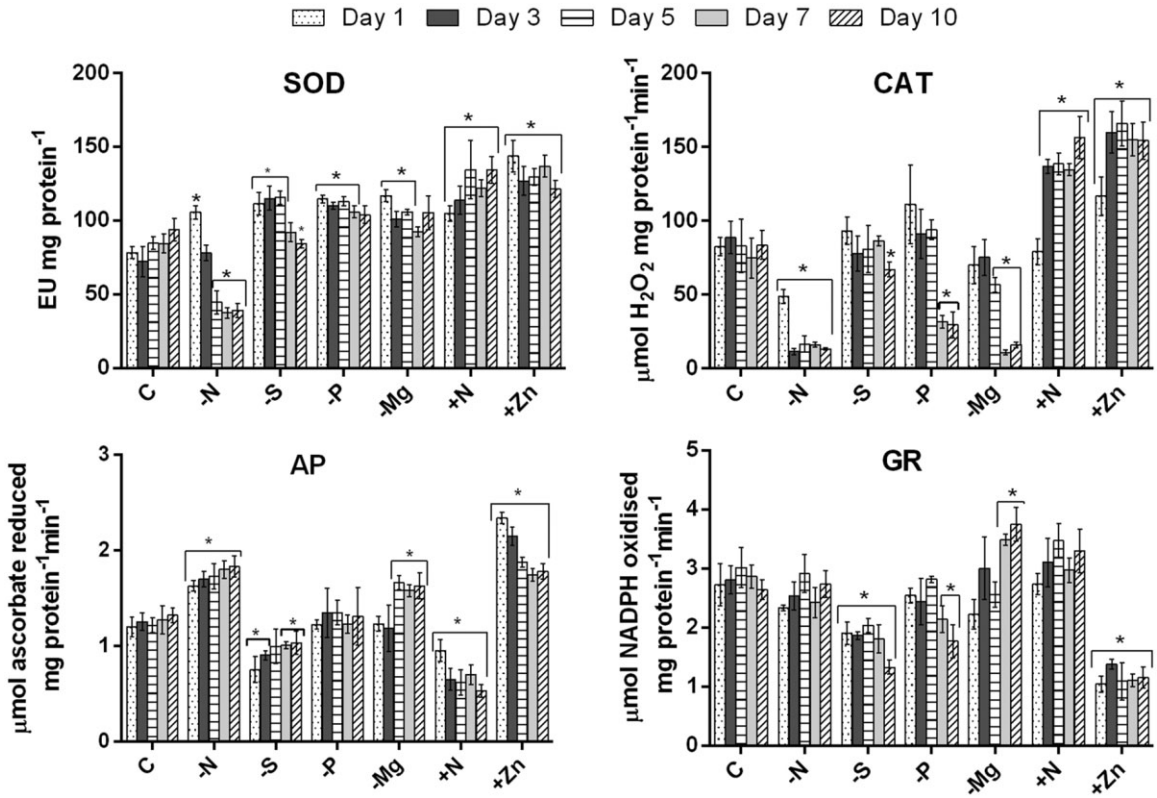

Fig. 5. Changes in catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (AP), and glutathione reductase (GR) enzyme activities in *C. reinhardtii* cells in response to different element manipulations. The details are shown in Figure 2.

increase in the case of N and Mg deprivation was consistent during the experiment; however, a rapid increase on the first day (95.2%) stopped and ended up at 34.4% under Zn supplementation. On the other hand, S deprivation and N supplementation caused a decrease while P deprivation did not lead any significant change in the AP activity of *C. reinhardtii*. The decrease in the activity of AP under N supplementation was most pronounced with a value of 20.5% on the first day and resulting in a 60% decrease on day 10. Different results were reported concerning a change in AP activity in response to a stress factor. Janknegt *et al*. (2009) applied high level of radiation on 15 different microalgal specimens and only two specimens increased their AP activity. Tripathi *et al*. (2006) reported that 2.5 µM Cu could induce prominent AP activity while a marked decrease was noted when the concentration was quadrupled.

Glutathione reductase activity decreased under S deprivation and Zn supplementation while it did not show significant differences under N deprivation and N overabundance (Fig. 5). On the other hand, GR activity did not differ during the first day under Mg and P deprivation; however, the activity decreased under P deprivation and increased under Mg deprivation after the 5th day. AP and GR are two important antioxidant enzymes in the ascorbate/glutathione cycle occurring in the cytoplasm, chloroplasts, mitochondria and peroxisomes (Del Rio *et al*. 2006). Thus, coherent changes in these two enzyme activities may reflect the level of oxidation in this cycle. The activity of these two enzymes decreased under S deprivation but increased under Mg deprivation. However, AP and GR enzyme activities were found to give opposite regulation patterns under Zn supplementation (Fig. 5). Additionally, dramatic increases in CAT and SOD activities were also noted in *C. reinhardtii* under Zn supplementation (Fig. 5). Considering this, cellular reductants are needed for the activation of GR; our data show that CAT and AP undertake direct roles for the scavenging ROS while cellular reductants are not primarily used for the activation of GR under Zn stress in *C. reinhardtii*. Our result is supported by the study of Tripathi *et al*. (2006) in which they reported significant decreases in GR activity under Cu and Zn supplementation in *Scenedesmus* sp.

CONCLUSION

Overall, our data show that oxidative stress caused by N deprivation is accompanied by a decrease in SOD and CAT levels while ascorbate/glutathione cycle would work as a primary ROS scavenging system in order to eliminate excess oxidants produced. In other words, antioxidant activity of enzymes is bypassed by ascorbate/glutathione cycle to invest more nitrogen to keep cellular dynamics optimal. On the other hand, fluctuation of antioxidant enzyme activities in *C. reinhardtii* grown under different element regimes refer to different metabolic source of ROS triggered by a specific element absence or overabundance. Lastly, decrease in proline content of *C. reinhardtii* would be associated with the increase in neutral lipid production rather than the oxidative stress caused primarily by macroelement deficiency.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. ORAC levels of *C. reinhardtii*.