Zinc-induced differential oxidative stress and antioxidant responses in **Chlorella sorokiniana** and **Scenedesmus acuminatus**

Zinc-induced differential oxidative stress and antioxidant responses in *Chlorella sorokiniana* and *Scenedesmus acuminatus*

Seham M. Hamed[1#*], Gaurav Zinta[2,3], Gerd Klöck[4], Han Asard[2], Samy Selim [5,6] Hamada AbdElgawad[2,7#*]

[1]Department of Soil Microbiology, Soils Water and Environment Research Institute, Agricultural Research Center, Giza, Egypt

[2]Integrated Molecular Plant Physiology Research, Department of Biology, University of Antwerp, Antwerp, Belgium

[3]Centre of Excellence PLECO (Plant and Vegetation Ecology), Department of Biology, University of Antwerp, Antwerp, Belgium

[4]Hochschule Bremen, University of Applied Sciences, Bremen, Germany

[5]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Aljouf University, Sakaka, P.O. 2014, Saudi Arabia

[6]Microbiology and Botany Department, Faculty of Science, Suez Canal University, Ismailia, P.O.Box 41522, Egypt

[7]Department of Botany & Microbiology, Faculty of Science, University of Beni-Suef, Beni-Suef 62511, Egypt

*Corresponding authors:

# These authors are equal contributors

seham_moussa939@yahoo.com

hamada.abdelgawad@uantwerpen.be

Abstract

Algae are frequently exposed to toxic metals, and zinc (Zn) is one of the major toxicants present. We exposed two green microalgae, *Chlorella sorokiniana* and *Scenedesmus acuminatus*, to sub-lethal concentrations (1.0 and 0.6 mM) of Zn for seven days. Algal responses were analysed at the level of growth, oxidative stress, and antioxidants. Growth parameters such as cell culture yield and pigment content were less affected by Zn in *C. sorokiniana,* despite the fact that this alga accumulated more zinc than *S. acuminatus.* Also, *C. sorokiniana*, but not *S. acuminatus,* was able to acclimatize during long-term exposure to toxic concentrations of the test metals (specific growth rate (μ) was 0.041/day and total chlorophyll was 14.6 mg/ml). Although, Zn induced oxidative stress in both species, C*. sorokiniana* experienced less stress than *S. acuminatus*. This could be explained by a higher accumulation of antioxidants in *C. sorokiniana*, where flavonoids, polyphenols, tocopherols, glutathione (GSH) and ascorbate (ASC) content increased. Moreover, antioxidant enzymes glutathione S transferase (GST), glutathione reductase (GR), superoxide dismutase (SOD), peroxidase (POX) and ascorbate peroxidase (APX), showed increased activities in *C. sorokiniana*. In addition to, and probably also underlying, the higher Zn tolerance in *C. sorokiniana,* this alga also showed higher Zn biosorption capacity. Use of *C. sorokiniana* as a bio-remediator, could be considered.

Keywords: Green microalgae; Zinc; Oxidative stress; Antioxidants; Zinc detoxification

1. Introduction

Heavy metals are a serious threat to soil and aquatic ecosystems. They are derived from various anthropogenic sources, including industrial effluents, urban runoff, sewage treatment and agricultural fungicide runoff (Pinto 2003). Heavy metals induce toxicity to algae primarily through binding sulfhydryl groups in proteins, the disruption of protein structure or element displacement (De Filippis and Pallaghy 1994). Zn is an important microelement, but high levels are strongly phytotoxic and cause growth inhibition (Omar 2002; Ouyang et al., 2012; Dinesh et al., 2014). Moreover, heavy metals can promote oxidative damage through elevated cellular concentrations of reactive oxygen species (ROS) (Dietz et al., 1999). Accumulated ROS oxidises macromolecules, leading to permanent metabolic dysfunction and cell death (Gill and Tuteja 2010). Several studies on the impact of Zn on microalgal growth have been performed, but its oxidative impact, and the role of antioxidants to provide Zn tolerance, is not well investigated. High Zn concentrations induce oxidative stress and elevated malondialdehyde (MDA) content of malondialdehyde (MDA), an indicator of oxidative damage in lipids in *Pavlova viridis, Spirulina platensis* and *Desmodesmus communis* (Li et al., 2006; Choudhary et al., 2007; Novák et al., 2014). Similarly, cell membrane damage of green algae *Pseudokirchneriella subcapitata* increased significantly, and chlorophyll *a* concentration was decreased after Zn exposure (Soto et al., 2011). As other plants, algae minimize free radical damage by enhanced antioxidant defences (Li et al., 2006). A wide array of antioxidants i.e., non-enzymatic (e.g., ascorbate, glutathione, tocopherols and carotenoids) and enzymatic (e.g., superoxide dismutase, catalase and ascorbate peroxidase) are known to involve in protecting plant and animal cells from oxidative stress induced by various stresses including heavy metal exposure (Pinto et al., 2003; Zouari et al., 2016). Accumulation of superoxide (SOD) and proline resulted in scavenging of ROS in *Spirulina platensis* under Zn and Cu stressed condition (Choudhary et al., 2007). SOD of *Scenedesmus* sp. was 30% higher during long-term exposure to Zn than during short-term (Tripathi et al., 2006). Cu and Zn stimulated the activities of CAT and GSH in the marine microalga *Pavlova viridis,* counteracting the oxidative stress (Li et al., 2006). Different species react differently to heavy metals. For

example, Dinesh et al., (2014) reported that addition of Zn brings about significant differences in the growth of microalgal species due to differences in their resistances where, *Tetraselmis* sp., having the most metal tolerating ability survived up to 3.8 mM of Zn, while, *Dunaliella salina, Chlorella marina*, *Isochrysis galbana* and *Nannochloropsis* sp., shows resistance to 0.8 mM Zn. *Scenedesmus obliquus* was more tolerant to Zn phytotoxicity than *Scenedesmus quadricauda* (Omar 2002). Moreover, time of exposure also impacts microalgae responses. *Scenedesmus* sp. experienced less metal stress in long-term exposure (7 d), versus short-term (6 h) exposure (Tripathi et al., 2006). The toxicity effect of Zn progressively decreased when the exposure time was increased, judged by an increasing growth rate of the cultures (Dinesh et al., 2014). Growth and photosynthesis of *Chlorella vulgaris* were dependent on both concentration and exposure time under the impact of sub-lethal concentrations of the heavy metals Cu, Cr, Zn, Cd and Pb (Ouyang et al., 2012).

Algae are generally exposed to elevated concentrations of heavy metals in polluted water bodies for a long period of time. Two species were selected for their high prevalence in industrial waste water showing high biosorption capacities for various heavy metals (Akhtar et al., 2008). The main objective was to provide a better understanding of the molecular mechanisms of cellular protection against oxidative stress induced by Zn in two green microalgae and providing a tolerant species more justifiable in bioremediation application.

2. Materials and Methods

2.1. Algal strains

Two green microalgal species *Chlorella sorokiniana* and *Scenedesmus acuminatus* were obtained from the Culture Collection of Algae, Hochschule Bremen University, Germany, and were cultivated axenically in 200 ml of 1% Wuxal medium with 0.05% (w/v) $MgSO_4.7H_2O$, in 500 ml Erlenmeyer flasks. Wuxal medium is a commercially available liquid plant fertilizer consisting of 8% N, 8% P_2O_5, 6% K_2O, 0.01% B, 0.004% Cu, 0.02% Fe, 0.012% Mn and 0.004% Zn (Wilhelm Haug GmbH & Co.KG Germany).

2.2. Zinc treatment and microalgae growth

The algae were grown under laboratory conditions in 100 ml sterile optimized culture medium (OCM) medium as described by Arroyo et al., (2011). The culture media were spiked with different concentrations of Zn i.e. control (0.77×10^{-3}), 0.4, 0.6 and 1.0 mM in the form of $ZnCl_2$. The cconcentration of total and dissolved Zn and it's chemical speciation in experimental media of the study were calculated using Visual MINTEQ. Growth conditions were: 100 µmol photons m^{-2} s^{-1} of light intensity with a photoperiod of 16/8 h (light/dark) at 25 ± 2°C, shaken at 120 rpm in an orbital incubator shaker (GFL-Gesellschaft fur labortechnikmb H, D-30938 Burgwedel, Germany). The growth performance of *C. sorokiniana* and *S. acuminatus* were determined in terms of cell count and optical density at 700 nm at each concentration. All reported data are the mean of three biological replicates.

2.3. Total zinc accumulation

In the first phase of culture, *C. sorokiniana* and *S. acuminatus* were grown photoautotrophically in a 2 L glass vessel containing 1.3 L OCM medium, inoculated by 130 mL of an axenic culture, in the exponential growth phase. The culture was kept at the same condition as previously mentioned for 10 days. 250 mL of culture suspension was collected from the culture vessels and centrifuged at 5000 rpm for 15 min under sterilized conditions. Microalgal pellets were placed into 500 mL culture flasks units of Sixfors photobioreactor INFORSAG-CH4103 (Bottmingen, Switzerland), filled with OCM medium treated with sub-lethal concentration of heavy metal for each organism [control (0.77×10^{-3} mM), Zn (1.0 and 0.6 mM)]. Cell count at zero time was normalized in all treatment to 46×10^5 and 25×10^5 for *C. sorokiniana*, *S. acuminatus* respectively. All were illuminated by tubular fluorescent lamps (PHILIPS Master TL-D 85 W/840). The light intensity at the surface of the culturing vessels was 100 µmol photons $m^{-2}.s^{-1}$ with a photoperiod of 16/8 h (light/dark). Stirring speed, internal temperature, pH and dissolved oxygen were kept constant in all bioreactor units and was adjusted automatically (150 rpm, 25 ±1°C, 7 and 90-100% dissolved oxygen).

2.4. Specific growth rate

After 7 days of incubation, cell density (cells mL^{-1}) was determined by using Zeiss Axiostar Plus light microscope (Carl Zeiss, Germany) and a Thoma Neu chamber with a depth of 0.1 mm (Paul Marienfeld GmbH & Co. KG, Germany). Cell suspension was diluted with OCM medium to give an appropriate cell concentration. Counting of at least 25 squares, of three biological replicate, ensured an error less than 10% (Venrick, 1978). Specific growth rate μ were calculated using the equation $\mu = \ln (N_{(t)} / N_0)(t - t_0)^{-1}$ as described by Guillard (1973), where N_t and N_0 are defined as the cell density (cells ml^{-1}) at time t (7 days) and t_0 (zero time), respectively. Values obtained were expressed as average ± S.E.

2.5. Total zinc content

Algal pellets were freeze-dried in a low freeze-drier (Leybold, Belgium) and digested in HNO_3/HCL in a microwave oven and determined by mass spectrometry (ICP-MS, Finnigan Element XR, Scientific, Bremen, Germany). Acid conditions of digestion were: 10 mL of HNO3 (10 $mol \cdot L^{-1}$) and 2 ml of HCl (10 $mol \cdot L^{-1}$). Lutetium (Lu) was added as internal standard. The dissolutions were cooled, and then the samples were filtered, transferred to 25 mL volumetric flasks and filled up with Milli-Q water. Before the ICPSFMS measurements, the samples were diluted 1:50 with Milli-Q water and in tracer were added as external standard. Optimization of the method was made with a standard solution of approximately 10 $\mu g \cdot L^{-1}$ of Zn, coming from a standard dissolution that contained nearly 1 $mg \cdot L^{-1}$ (Merck, Germany). Analysis of each sample was carried out in triplicate. Spike recovery measurements of total zinc (%) (Supplementary Table 1).

2.6. Chlorophyll content

Chlorophyll (*a* and *b*) and total chlorophyll content in the algal cultures was determined spectrophotometrically according to method based on MacKinney (1941). Five mL of

algal culture was centrifuged at 5000 g for 10 minutes, the supernatant was drained off and re-suspended in 1 mL distilled water and 4 mL acetone. Samples were centrifuged at 5000 g for 5 minutes. The optical density was measured at 645 nm and 663 nm using 80% acetone as a blank. The chlorophyll concentration ($mg.L^{-1}$) of each algal suspension fraction was calculated using the equations described by Mackinney (1941), and analysis of each sample was carried out in triplicate.

2.7. Oxidative stress markers

TBARS content, was assayed according to Hodges et al. (1999). Ten mg of freeze dried algae cells were homogenized in 1 mL of 80% ethanol using MagNALyser (Roche, Vilvoorde, Belgium; 1 min, 7000 rpm) and reacted with thiobarbituric acid to produce pinkish red chromogen thiobarbituric acid-malondialdehyde (MDA). Absorbance was measured at 440, 532 and 600 nm by using a micro-plate reader (Synergy Mx, Biotek Instruments Inc., Vermont, VT, USA). TBARS content was calculated and expressed as nmol/g wet weight. Hydrogen peroxide (H_2O_2) concentration was measured in 100 mg frozen algal pellets by using the FOX1 method (Jiang et al., 1990), based on the peroxide-mediated oxidation of Fe^{2+}, followed by reaction of Fe^{3+} with xylenol orange. Absorbance was read at 560 nm using a micro-plate reader (Synergy Mx, Biotek Instruments Inc., Vermont, VT, USA) after incubating the samples for 45 min at room temperature. The extinction coefficient of the Fe3+-xylenol orange complex at 560 nm was determined 1.5 x 104 M-1.cm-1 in 25 mM H2SO4. Specificity for H_2O_2 was confirmed by eliminating H_2O_2 from the reaction mixture with catalase. Analysis of each sample was carried out in triplicate.

2.8. Determination of antioxidant molecules

Total antioxidant capacity (ferric reducing/antioxidant power assay, FRAP) was determined by grinding 30 mg of the freeze dried algal cells in 2 ml 80% ice cold ethanol in liquid nitrogen. FRAP (ferric reducing/antioxidant power assay) reagent (0.3 M acetate buffer (pH3.6), 0.01 mM TPTZ (2,4,6-tripyridyl-striazine) in 0.04 mM HCl and

0.02 M $FeCl_3.6H_2O$) was mixed with 2 ml of the prepared extract for 30 min and measured at 600 nm using a micro-plate reader (Synergy Mx, Biotek Instruments Inc., Vermont, VT, USA) (Benzie and Strain, 1996). Trolox was used as standard. Polyphenols and flavonoids were extracted in 80% ethanol (v/v) and determined according to Zhang et al. (2006), and Chang et al. (2002), with gallic acid and quercetin as standards respectively. Ascorbate (ASC), glutathione (GSH) were determined by HPLC by using the method of Potters et al (2004). The redox status (ASC/tASC) was calculated as the ratio of the reduced form to the total concentration of the antioxidant. Tocopherols were extracted in hexane and quantified by HPLC (Shimadzu‘s Hertogenbosch, The Netherlands) by using dimethyl tocol (DMT) as internal standard (5 ppm) (Abd Elgawad et al. 2015a). Proline content was extracted with toluene and the ninhydrin acid reagent was added to the supernatant, and the product was measured calorimetrically at 520 nm by using a microplate reader (Synergy Mx, Biotek Instruments Inc., Vermont, VT, USA) (Abd Elgawad et al. 2015b). Analyses of all metabolites were carried out in triplicates.

2.9. Antioxidant enzymes assays

Peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione-s-transferase (GST) were determined by homogenizing (MagNALyser, Synergy Mx, Biotek Instruments Inc., Vermont, USA) 100 mg of freeze dried algal cells in 1 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 10% polyvinyl pyrrolidone (PVP), 0.25% Triton X-100, 0.001 M polymethyl sulfonyl fluoride (PMSF) and 0.001 M ascorbate. All enzyme activities were determined in 200µl volume reactions buffer at 25 0C. APX and GR activities were measured by the method of Murshed et al. (2008). APX and GR activates were measured in phosphate buffer and HEPES buffer (100 mM, pH 7), respectively. The decrease in ascorbic (A_{290} = 2.8 $mM^{-1}.cm^{-1}$) acid and NADPH (A340 = 6.22 $mM^{-1}.cm^{-1}$) were used to measure the activities of APX and GR, respectively. GST enzyme activity was calculated by measuring the conjugation of GSH with excess 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig et al., 1974). Peroxidase (POX) activity was determined by the oxidation of pyrogallol

252 (A_{430} = 2.47 $mM^{-1}.cm^{-1}$) (Kumar and Khan, 1982). Superoxide dismutase (SOD) activity
253 was determined according to Dhindsa et al. (1981) by measuring the inhibition of NBT
254 reduction in 0.5 mL of reaction mixture at 560 nm. All activity measurements were
255 scaled down for semi-high throughput using a micro-plate reader (Synergy Mx, Biotek
256 Instruments Inc., Vermont, USA). Protein concentration was determined by the method
257 of Lowry et al. (1951). All enzyme analyses were carried out in triplicate.
258
259 **2.10. Statistical analyses**
260
261 The results of the measured parameters are expressed as mean of three biological
262 replicates. Statistical analysis was performed using one way ANOVA and two way
263 ANOVA in the SPSS 20 software (IBM Corporation, Armonk, New York, USA) and
264 means were compared using Duncan's multiple range tests at 5%. Heat maps were
265 generated with Multi Experiment Viewer (MeV) in the TM4 software package (Dana-
266 Farber Cancer Institute, Boston, USA) by using Euclidian distance metric. PCA was
267 performed with Origin Lab 9 software (Origin Lab, Northampton, MA, U.S.A by using the
268 average of the measured oxidative and antioxidant parameters for both the algal
269 species, and the first two components were used to make biplots. The PCA allowed for
270 the detection of similarities between samples and for identifying the main associations
271 among variables that are responsible for the total variability of the studied data.
272
273 **3. Results**
274
275 **3.1. Selection of sub-lethal zinc concentration.**
276
277 The algal species *C. sorokiniana* and *S. acuminatus,* showed different susceptibility to
278 the applied Zn concentrations, where highest Zn concentration (1 mM) inhibited growth
279 of both the species as observed by decrease in the cell number on 7^{th} day (Fig 1A,B). At
280 the lower Zn concentrations (0.6 mM and 0.4 mM), *C. sorokiniana* showed increase in
281 the cell number (Fig 1A), but *S. acuminatus* showed a decrease (Fig 1B), when
282 compared to their respective controls. Similar to cell number, optical density

283 measurements at 700 nm, reflecting population density, confirmed the differential
284 susceptibility of these algal species to Zn (data not shown). Since, we observed that 1
285 mM Zn exposure had a severe effect on *S. acuminatus* growth, where its culture
286 contained cellular debris and dead cells. Therefore, 1 and 0.6 mM Zn were selected as
287 sub-lethal concentrations for *C. sorokiniana* and *S. acuminatus* respectively, because
288 they were the highest Zn concentration at which the microorganism shows tolerance
289 and remains alive to study the oxidative stress response.
290
291 **3.2. Photosynthetic pigment and specific growth rate**
292
293 The chlorophyll (*a*/*b*) ratio decreased by 29% in *C. sorokiniana* at 1 mM, after 1 week Zn
294 exposure, whereas *S. acuminatus* showed a decrease by 59% at 0.6 mM. *C.*
295 *sorokiniana* showed a significant decrease in the chlorophyll (*a/b*) ratio compared to *S.*
296 *acuminatus* ($P \leq 0.05$) at their respective Zn sub-lethal concentrations (Fig.2). Specific
297 growth rate was significantly reduced in both species at the tested sub-lethal Zn
298 concentrations. *C. sorokiniana* exhibited marked reduction by 58%, while *S. acuminatus*
299 showed 70% of reduction with respect to controls.
300
301 **3.3. Total zinc content**
302
303 Increasing concentration of Zn in the culture media resulted in a significant increase in
304 total Zn content but to variable extents. Under the Zn stressed condition C. sorokiniana
305 showed 9.2-fold increase of the total Zn content, while it was only 3.1-fold in *S.*
306 *acuminatus* when compared to their respective controls (Fig. 3). Although the total Zn
307 content was about 2-fold higher in *S. acuminatus* than *C. sorokiniana* in the absence of
308 externally added Zn, at sub-lethal Zn concentration *C. sorokiniana* showed a significant
309 increase of 1.5 fold over *S. acuminatus*, indicating that *C. sorokiniana* accumulated
310 more Zn under Zn stress. The Data of Visual MINTEQ revealed that, Zn remained
311 mostly as dissolved Zn, but formed a myriad of complexes with the anions in the media.
312 Thus, the predicted free Zn^{+2} activity were $7.8E^{-14}$ % at control and 7.9 E^{-10} % under
313 both 0.6 mM and 1.0 mM Zn stressed.

3.4. Oxidative stress

3.4.1. Oxidative damage markers

Induction of oxidative stress by Zn was assessed by measuring H_2O_2 levels and lipid peroxidation. As compared to the non-stressed controls, the content of H_2O_2 increased by 2.1 and 1.7-fold in *C. sorokiniana* and *S. acuminatus* respectively (Fig. 4A). In parallel with the increase in H_2O_2 levels, lipid peroxidation (TBARS) was also induced by the Zn exposure (Fig. 4B). However, these differences were not statistically significant.

3.4.2. ROS scavenging enzymes

The responses of GR, GST, SOD, APX and POX scavenging enzymes to stress conditions varied between species at the applied sub-lethal concentrations of Zn (Fig. 5). Overall, the activities of ROS scavenging enzymes were increased under Zn stress in both species, but to variable extents. GR and GST activities were induced by Zn stress in *C. sorokiniana* only (1.8 and 3-fold, respectively) (Fig. 5A,B). Significant increase in SOD and APX activities were observed in both strains. however, *C. sorokiniana* achieved higher level under Zn stress by 2.2 fold of control (Fig. 5C, D). No significant changes in POX activities were observed in either species (Fig. 5E). Among species, *C. sorokiniana* exhibited a significant increase ($P \leq 0.05$) in the activity of GR, SOD and APX enzymes compared to *S. acuminatus* under Zn stress.

3.4.3. Molecular antioxidants

GSH levels were significantly increased by Zn stress in the two strains (Fig. 6A). Similarly, ASC content was induced in *C. sorokiniana* (Fig. 6B) however, a lower ASC redox status was observed in both strains with 54% and 26% decrease for *C. sorokiniana* and *S. acuminatus* respectively (Fig. 6C). Considerable changes were recorded in some antioxidant metabolites under Zn stress. A marked increase was observed in flavonoid and polyphenol content in *C. sorokiniana* by 1.9 and 2.5-fold (Fig

6D,E). Interestingly, *S. acuminatus* showed higher proline content by 2-fold compared to *C. sorokiniana* under Zn stress. In the absence of stress, proline level was higher in *S. acuminatus* with 1.8 fold as compared to *C. sorokiniana*) (Fig. 6F). Tocopherol levels were considerably high in *C. sorokiniana*, than in *S. acuminatus* (Fig. 5G), in the absence and presence of added Zn. In both strains, Zn stress increased the levels of total tocopherols by 3.2- and 1.9-fold in *S. acuminatus* and *C. sorokiniana* respectively, and the stress effect was higher in *C. sorokiniana* (Supplementary Table 2). In both strains, β tocopherol constituted the largest fraction, followed by α tocopherol. Notably, the ratio α/β is dissimilar in both species, but increases in both species under Zn exposure. The total antioxidant capacity (FRAP) increased in *C. sorokiniana* and *S. acuminatus* by 3.4- and 3-folds respectively (Fig. 6H).

3.4.4 Clustering and principal component analysis (PCA)

Hierarchical Clustering separated all measured components mainly into three clusters (Fig. 7a). The first cluster consisted of ASC/ tASC, GSH/tGSH and Chl a/b. The second cluster consisted of FRAP, proline, H_2O_2, Flavonoids, TBARS and GST. The third cluster consisted of peroxidase, glutathione, ascorbate peroxidase, glutathione reductase, Zn content, polyphenols, ascorbate and tocopherol. To further substantiate the outcome of our clustering data, we performed principal component analysis (PCA). PCA confirmed most of the observations (Fig 7b). The PCA bi-plot separated both species and also grouped them separated accordingly to their stress response. Principal component 1 (PC 1) explained 67% of the variance and PC 2 explained 27% of the variance (Fig. 7b). Overall, our clustering and PCA indicate the specificity of oxidative and antioxidant responses to Zn stress in the two algal species.

4. Discussion

4.1. Zn stress inhibits growth and induces oxidative stress

375 Zn exposure-induced growth inhibition was less prominent in *C. sorokiniana,* whereas
376 the growth of *S. acuminatus* was severely affected. Similarly, in *C. sorokiniana* the
377 chlorophyll *a/b* ratio and cell counts were less affected by Zn toxicity. In our study, we
378 reported a significant decrease in chlorophyll a/b ratio in S. acuminatus, C. sorokiniana
379 under their respective sub-lethal Zn concentration by 59 and 29%. These findings could
380 indicate that *C. sorokiniana* is more Zn tolerant than *C. sorokiniana*. It has been well
381 documented that exposure to heavy metals inhibits photosynthesis by replacing
382 magnesium (Mg^{2+}) in chlorophyll molecules, and inhibit the synthesis of the D1 reaction
383 centre protein (Soto et al., 2011).
384
385 Studies investigating microalgae responses to Zn stress, have so far mostly addressed
386 changes in growth and metal accumulation (Omar 2002; Bácsi et al., 2014; Dinesh, et
387 al., 2014; Novák et al., 2014). Comparatively, less is known on the effects of Zn in *C.*
388 *sorokiniana* and *S. acuminatus*, at the biochemical level. Although *C. sorokiniana*
389 showed higher total Zn content than *S. acuminatus,* it appeared *C. sorokiniana* was
390 more Zn stress tolerant than *S. acuminatus.* Green algae showed differential Zn
391 tolerance and Zn binding abilities not only in different species, but also at strain level of
392 the same species (Bácsi et al., 2014; Novák et al., 2014), and in a wide range of Zn
393 concentrations on *Desmodesmus communis, Monoraphidium pusillum and*
394 *Monoraphidium griffithii*. As far as we know, these are the first data about zinc tolerance
395 of *C. sorokiniana* and *S. acuminatus.* It seems that the zinc tolerance of these species is
396 in the medium range, as concentrations of 0.6-1 mM were not yet lethal. Growth rates of
397 extremely zinc-tolerant species, such as a *Chlorella vulgaris* and *Scenedesmus acutus*
398 strain, exhibited similar growth in control as well as medium in 1.5–4.6 and 1.9 mM zinc,
399 respectively (Travieso et al., 1999). EC_{50} was 1.2 mM zinc in the case of a
400 *Chlamydomonas acidophila* strain (Nishikawa and Tominaga 2001). On the other hand,
401 there are algal species that show hypersensitivity to Zn. Tripathi et al., (2006) observed
402 that 0.025 mM Zn inhibited growth of a *Scenedesmus* strain within 48h. Zn
403 concentration of 0.1 mM caused growth inhibition and oxidative stress in a *Pavlova*
404 *viridis* strain (Li et al. 2006). Heavy metal induces ROS accumulation (Tripathi et al.,
405 2006; Murugan and Harish 2007; Choudhary et al., 2007; Sabatini et al. 2009). Over-

accumulation of ROS can lead to alterations in cell structure and mutagenesis (Halliwell and Gutteridge 1999). In our study, inductions of H_2O_2 accumulation, as well as, oxidative damage (TBARS) were more pronounced in *S. acuminatus* (exposed to 0.6 mM) than in *C. sorokiniana* (exposed to1 mM) (Fig. 7). Therefore it can be concluded that Zn induced oxidative damage differently in the two algal strains, and *S. acuminatus* exhibited more oxidative damage. Zn stimulated lipoxygenase activity and consequently increases lipid peroxidation (Weckx and Clijsters, 1997). Consistent with this finding, lipid peroxidation was enhanced at increased Zn concentrations in *Pavlova viridis and Chlorella vulgaris* (Li et al., 2006, Suman et al., 2015). Increased amount of MDA was also indicative of increased ROS formation in *Spirulina* sp., *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* under Zn, Cu and Cr treatment (Choudhary et al., 2007, Soto et al., 2011,Shilip et al., 2014).

4.2. Zn stress up-regulates antioxidant defense system

Consistent with the changes observed in the oxidative stress parameters (lipid peroxidation, H_2O_2 levels), antioxidant enzymes, such as GR, SOD, GST and APX increased in both algae, and this effect was somewhat more pronounced, and more often statistically significant in *C. sorokiniana*. Maintenance of a high antioxidant capacity in cells has been linked to increased tolerance against environmental stress (Nowicka et al., 2016). The increase in the activity of antioxidant enzymes (SOD, CAT and APX) might reduce concentration of $O_2^{\cdot-}$ and H_2O_2, thereby minimizing the risk of oxidative stress. Consistently, *Scenedesmus* sp and *Pavlova viridis* exposed to Zn and Cu stress, showed enhanced antioxidant defences (Tripathi et al., 2006; Li et al., 2006). Similar findings were reported in *Cladophora glomerata* under heavy metal exposure (Murugan and Harish 2007). Moreover, we observed that exposure to Zn stress enhanced GST activity, which could be related to alleviating metal-induced oxidative stress in *C. sorokiniana* and *S. acuminatus*. Similarly, Pb stress in *Porphyra vietnamensis* (Pise et al., 2013) and Cu stress in *Scenedesmus bijugatus* (Nagalakshmi and Prasad 2001) led to increased GST activity.

C. sorokiniana showed higher tolerance to applied Zn stress, possibly through accumulating higher amounts of antioxidant molecules such as flavonoids, polyphenols, tocopherols, glutathione (GSH) and ascorbate (ASC) (Fig. 7). Similarly, exposure to heavy metals evoked oxidative stress and an increase in the antioxidant defenses (GSH level) responses in *S. vacuolatus*, *Gonyaulax polyedra*, *Scenedesmus* sp. and *S. acuminatus* (Sabatini et al., 2009, Okamoto et al., 2001, Suman et al., 2015). We also observed increases in the levels of proline in both species under Zn stress. Tripathi et al., (2006) indicated that accumulation of proline by *S. acuminatus* could be an important strategy for alleviating metal-induced oxidative stress in *Scenedesmus*. Also, proline levels were increased in *Spirulina* species in the presence of different heavy metals. Proline not only acts as an osmoprotectant, but plays an important role as a protein stabilizer and a ROS scavenger (Shilip et al., 2014).

4.3. Zn metal biosorption capacity

Algal species can be used to assist in the control of heavy metal pollution in natural water sources, and to detoxify metal toxicants. *C. sorokiniana* exhibited high Zn tolerance, and higher total Zn content, probably because of its high Zn absorption capacity. The biosorption capacity of *C. sorokiniana* appears to be much higher than that of *D. communis* (Novák et al., 2014), *M. pusillum* and *M. griffithii* (Bácsi et al., 2014), which are reported to be good Zn biosorbents. Therefore, application of a resistant algal species for the purification of wastewater offers a high potential and a cheap strategy for removal of toxic metal ions from polluted water bodies. For instance, Wang et al., (1995) reported that *Phormidium* sp has a good potential for removal of heavy metals and has a wide range of metal biosorption capacity.

5. Conclusion

The ability of two green algal species viz. *C. sorokiniana* and *S. acuminatus* to grow and absorb Zn from the media supplemented with high Zn, was studied. *C. sorokiniana* showed higher absorption of Zn than *S. acuminatus*. *C. sorokiniana* also showed less

growth inhibition and oxidative stress response, which could possibly be caused by a more efficient antioxidant defense system, alleviating the stress induced by Zn exposure (Fig. 7). These properties make *C. sorokiniana* a potentially interesting microalga for the treatment of waste water contaminated with Zn. It can be concluded that introducing metal tolerant algal species as a bio-remediator could be a good alternative strategy for remediation of water bodies contaminated with toxic metals.

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Figures and tables legends

Fig.1. Culture density (cells count /ml) of (A) *C. sorokiniana* and (B) *S. acuminatus* grown in OCM medium under control or spiked with different initial zinc concentrations (0.4-1.0 mM) after 7 days of exposure. Data are expressed as means of 3 biological replicates ± standard error (SE).

Fig.2. Chlorophyll *a*/*b* ratio after 1 week Zn exposure of *C. sorokiniana* and *S. acuminatus* cultured in OCM medium under control or at sub-lethal Zn concentrations (1.0 and 0.6mM) respectively. Different letters denote significant differences at $P < 0.05$: allow comparisons between treatments within the same species and between contents within different species. Data are expressed as means of 3 biological replicates ± standard error (SE).

Fig.3. Total zinc content of *C. sorokiniana* and *S. acuminatus* cultured in OCM medium under control or at sub-lethal Zn concentrations (1.0 and 0.6mM) respectively, after1 week exposure. Different letters denote significant differences at $P < 0.05$: allow comparisons between treatments within the same species and between contents within different species. Data are expressed as means of 3 biological replicates ± standard error (SE).

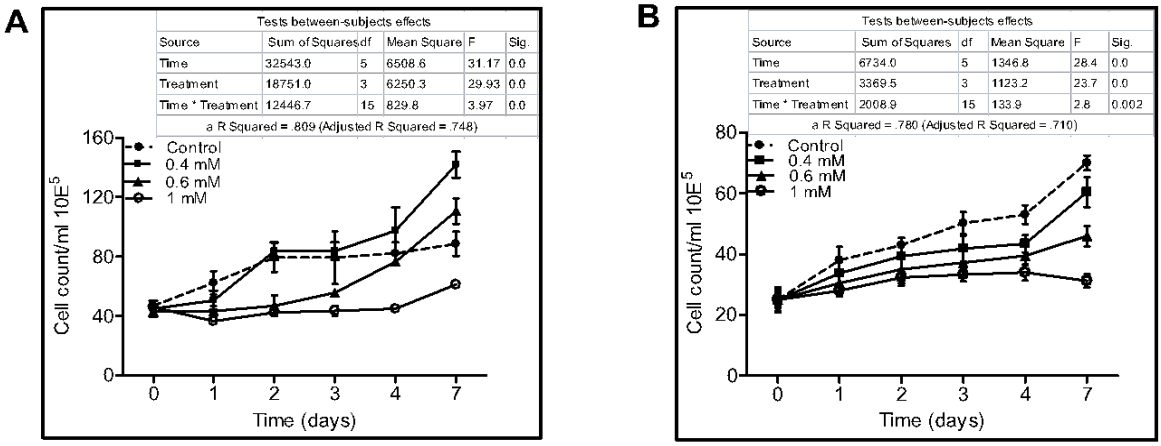
683 Fig.4. Oxidative damage markers (A) H_2O_2 and (B) TBARS in *C. sorokiniana* and *S.*
684 *acuminatus* cultured in OCM medium under control or at sub-lethal Zn concentrations
685 (1.0 and 0.6mM) respectively, after 1 week exposure. Different letters denote significant
686 differences at $P < 0.05$: allow comparisons between treatments within the same species
687 and between contents within different species. Data are expressed as means of 3
688 biological replicates ± standard error (SE).
689
690 Fig.5. Changes in antioxidant enzyme activities in *C. sorokiniana* and *S. acuminatus*
691 cultured in OCM medium under control or at sub-lethal Zn concentrations (1.0 and
692 0.6mM) respectively after 1week exposure. (A) Glutathione reductase, (B) Glutathione-
693 s-transferase, (C) Superoxide dismutase, (D) Ascorbate peroxidase and (E) Peroxidase.
694 Different letters denote significant differences at $P < 0.05$: allow comparisons between
695 treatments within the same species and between contents within different species. Data
696 are expressed as means of 3 biological replicates ± standard error (SE).
697
698 Fig.6. Changes in antioxidant molecules, (A) GSH, (B) GSH/tGSH, (C) ASC, (D)
699 ASC/tASC (E) Flavenoids, (F) Polyphenols, (G) Proline (H) Tocopherols and (I) Total
700 antioxidant capacity (FRAP) in *C. sorokiniana* and *S. acuminatus* cultured in OCM
701 medium under control or at sub-lethal Zn concentrations (1.0 and 0.6mM) respectively
702 after 1 week exposure. Different letters denote significant differences at $P < 0.05$: allow
703 comparisons between treatments within the same species and between contents within
704 different species. Data are expressed as means of 3 biological replicates ± standard
705 error (SE).
706
707 Fig 7. Hierarchical clustering and principal component analysis (PCA), (A) Heat map
708 and cluster tree representation, (B) PCA plot showing the loading of all the measured
709 metabolites/enzymes on the axes of plot.
710
711 Supplementary Fig 1. Total zinc content (µg/g DW) of *C. sorokiniana* and *S. acuminatus*
712 cultured in OCM medium under control or at sub-lethal Zn concentrations (1.0 and
713 0.6mM) respectively, after1 week exposure. Different letters denote significant

differences at P < 0.05: allow comparisons between treatments within the same species and between contents within different species. Data are expressed as means of 3 biological replicates ± standard error (SE).

Supplementary Table 1. Spike recovery of total zinc (%) measured in C. sorokiniana and S. acuminatus cultured in OCM medium under control or at sub-lethal Zn concentrations (1.0 and 0.6mM) respectively after 1 week exposure.

Supplementary Table 2. Tocopherol profile of *C. sorokiniana* and *S. acuminatus* cultured in OCM medium under control or at sub-lethal Zn concentrations (1.0 and 0.6mM) respectively after 1 week exposure. Different letters denote significant differences at P < 0.05: allow comparisons between treatments within the same species and between contents within different species. Data are expressed as means of 3 biological replicates ± standard error (SE).

745 **Fig.1**



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748 **Fig. 2**

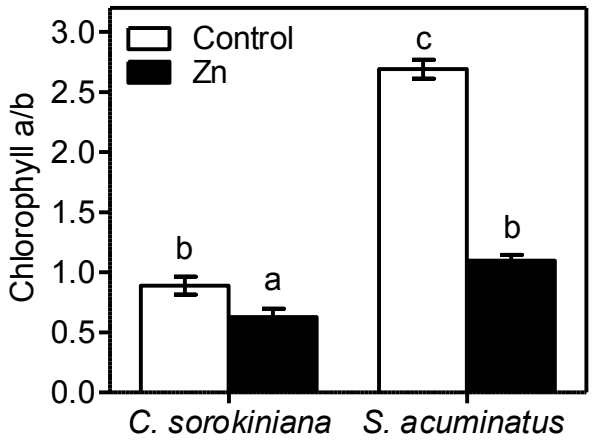

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Fig. 3

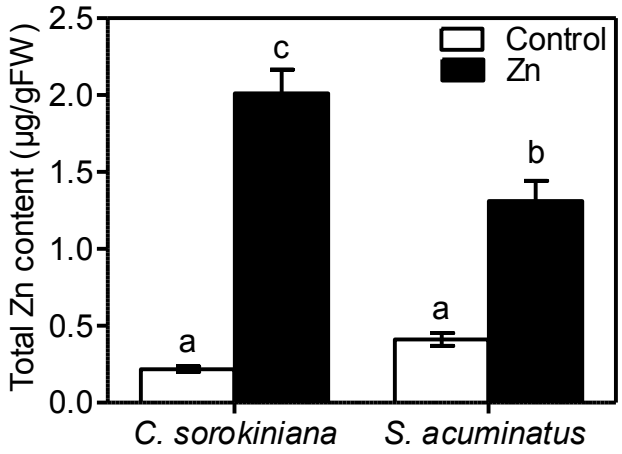

Total Zn content (µg/gFW)
2.5
2.0
1.5
1.0
0.5
0.0
Control
Zn
a
c
a
b
C. sorokiniana
S. acuminatus

Fig. 4

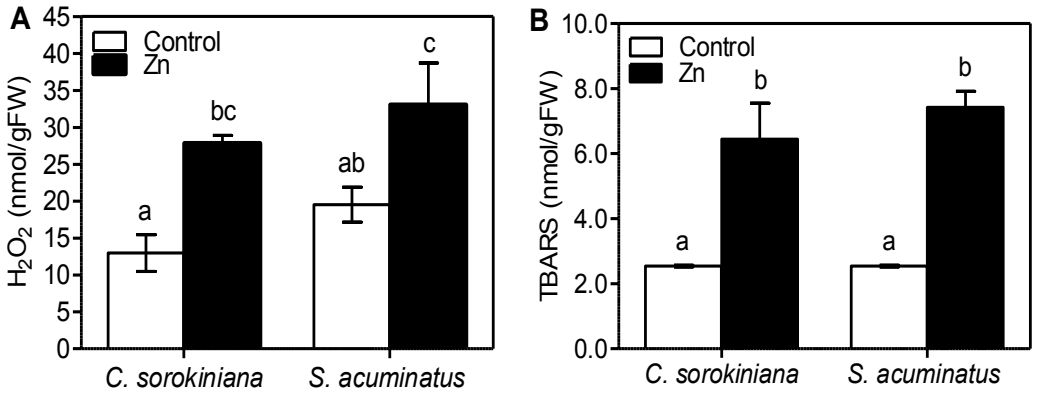

A
H_2O_2 (nmol/gFW)
45
40
35
30
25
20
15
10
5
0
Control
Zn
a
bc
ab
c
C. sorokiniana
S. acuminatus
B
TBARS (nmol/gFW)
10.0
8.0
6.0
4.0
2.0
0.0
Control
Zn
a
b
a
b
C. sorokiniana
S. acuminatus

Fig. 5

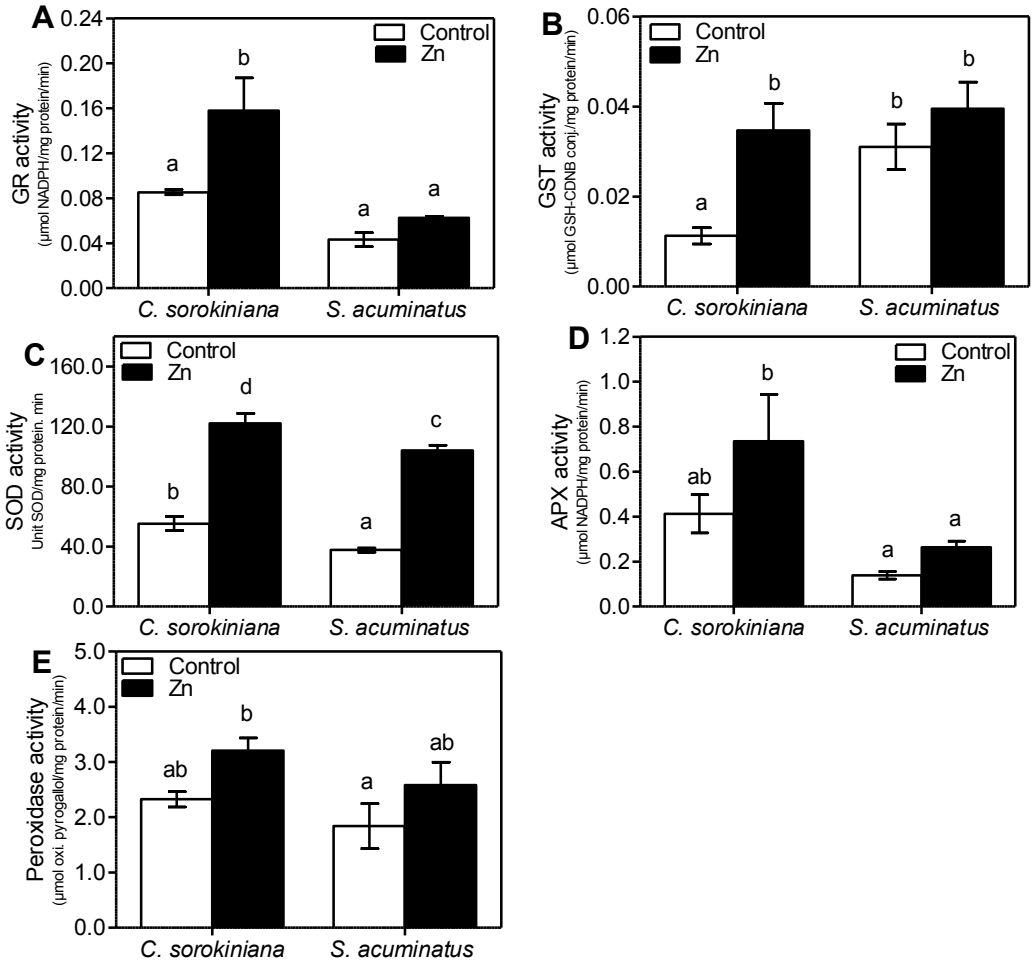
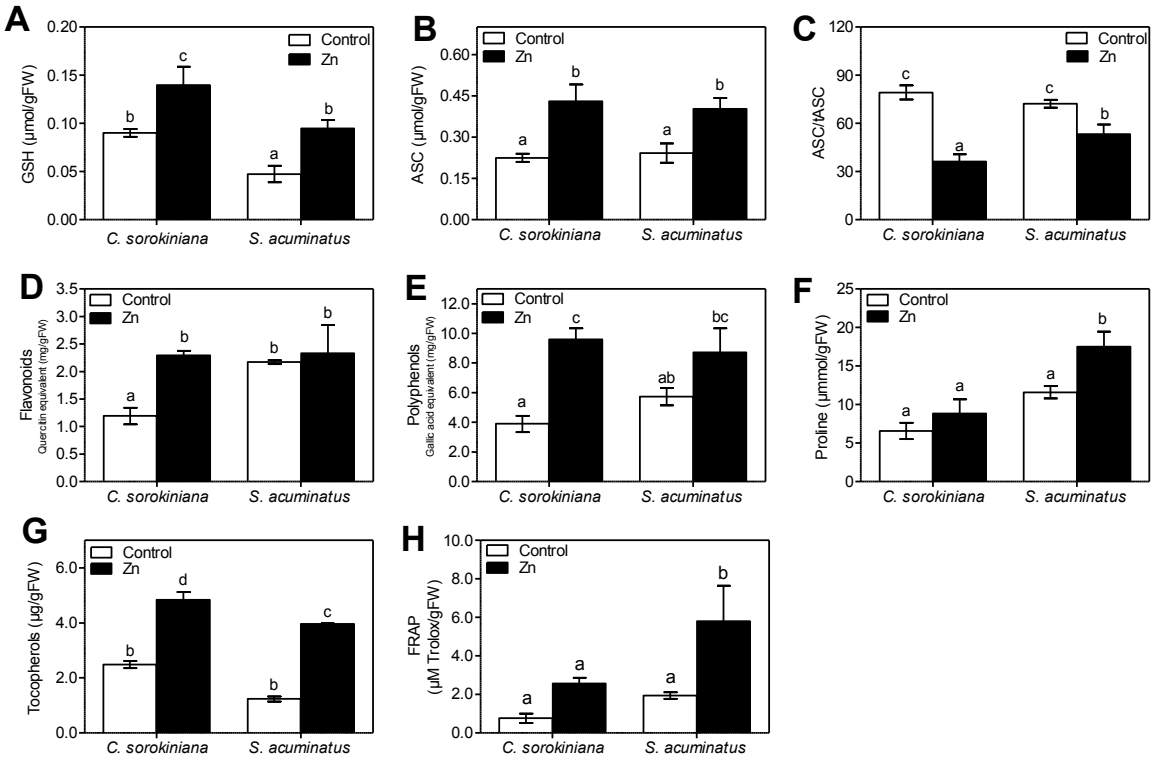
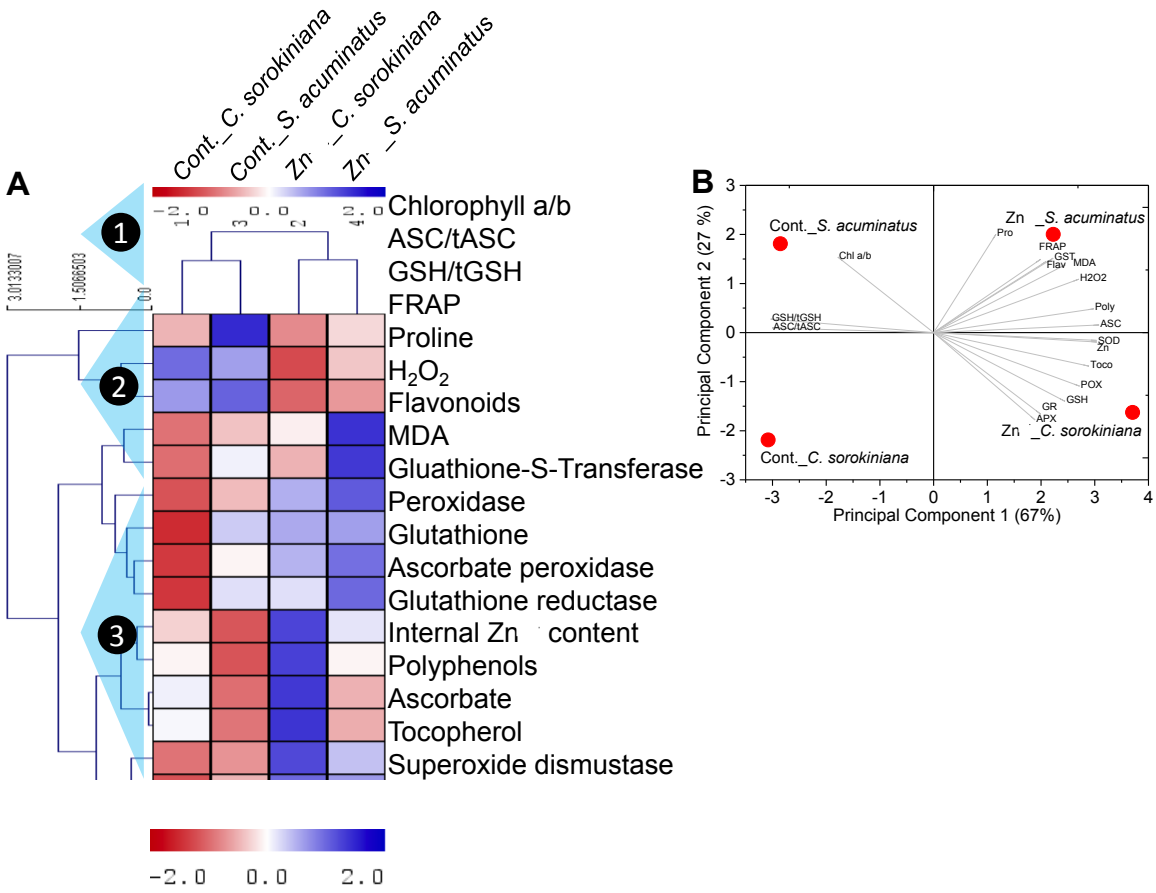

A
GR activity
(μmol NADPH/mg protein/min)
0.24
0.20
0.16
0.12
0.08
0.04
0.00
Control
Zn
a
b
a
a
C. sorokiniana
S. acuminatus
B
GST activity
(μmol GSH-CDNB conj./mg protein/min)
0.06
0.04
0.02
0.00
Control
Zn
a
b
b
b
C. sorokiniana
S. acuminatus
C
SOD activity
Unit SOD/mg protein. min
160.0
120.0
80.0
40.0
0.0
Control
Zn
b
d
a
c
C. sorokiniana
S. acuminatus
D
APX activity
(μmol NADPH/mg protein/min)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Zn
ab
b
a
a
C. sorokiniana
S. acuminatus
E
Peroxidase activity
(μmol oxi. pyrogallol/mg protein/min)
5.0
4.0
3.0
2.0
1.0
0.0
Control
Zn
ab
b
a
ab
C. sorokiniana
S. acuminatus

Fig. 6


A
GSH (µmol/gFW)
Control
Zn
C. sorokiniana
S. acuminatus
B
ASC (µmol/gFW)
C
ASC/tASC
D
Flavonoids
Quercitin equivalent (mg/gFW)
E
Polyphenols
Gallic acid equivalent (mg/gFW)
F
Proline (µmmol/gFW)
G
Tocopherols (µg/gFW)
H
FRAP
(µM Trolox/gFW)

803 **Fig. 7**



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817

Supplementary Table 1

Control	Zn treated
C.sorokiniana	
72.47	76.24
79.32	69.34
77.25	71.14
S.acuminatus	
71.25	67.54
66.67	76.35
80.64	71.03

842 **Supplementary Table 2.**

	Alfa	*Beta*	*Gamma*	Total Tocopherols
Control				
C. sorokiniana	0.66±0.02[b]	1.77±0.11[b]	0.05±0.01[b]	2.48±0.13[b]
S. acuminatus	0.08±0.01 [a]	1.14±0.10[a]	0.02±0.00[a]	1.23±0.09[a]
Zn stressed condition				
C. sorokiniana	1.59±0.13[c]	3.07±0.15[c]	0.18±0.01[d]	4.84±0.28[d]
S. acuminatus	0.50±0.02 [b]	3.37±0.04[c]	0.09±0.00[c]	3.96±0.03[c]

843

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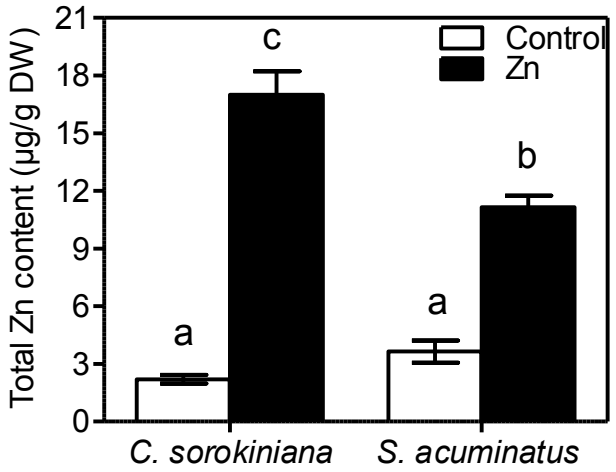
847

848 **Supplementary Fig.1**

849



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