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Isocitrate lyase activity and antioxidant responses in copper-stressed cultures of *Chlamydomonas reinhardtii* (Volvocales, Chlorophyceae)

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The effect of copper-imposed oxidative stress on isocitrate lyase (ICL) activity was studied in a *Chlamydomonas reinhardtii* wild type and a copper-tolerant pleiotropic mutant strain. Suspension batch bioassays were performed adding copper chloride under moderate irradiation. Two harvest points were selected to represent the acute and restoration phases and oxidative stress indicators were measured: antioxidative metabolites, phenolic compounds, ascorbic acid, abundance of thiobarbituric reactive substances, and activities of detoxifying enzymes such as catalase and ascorbate peroxidase. Copper stress under moderate irradiance favours acetate assimilation through enhanced ICL activity, especially in the acute phase. Though the mutant strain exhibited at least twofold ICL activity in the acute phase compared to the wild type, there was no significant difference in copper tolerance between strains. The higher ICL activity under copper stress may be an adaptation to promote mixotrophic organic carbon assimilation when photosynthetic carbon dioxide fixation is impaired. Thus, the higher ICL activity has the potential to be a useful indicator in acetate-flagellate bioassays.

Key Words: Ascorbate peroxidase, Catalase, *Chlamydomonas reinhardtii*, Copper, Isocitrate lyase, Lipid peroxidation, Oxidative stress

INTRODUCTION

The increase of environmental copper concentrations to hazardous levels is associated with aqueous discharges from mining, metal plating, power generation, manufacture of electrical equipment, the use of pesticides (including algaecides), and agrochemicals (Girling *et al.* 2000). Direct measurement of copper concentration in natural environments by analytical techniques such as anodic stripping voltammetry and ion-selective electrodes allow quantification of the different copper species, but they do not provide information of their deleterious effects on aquatic biota (Turner 1995). Bioassays enable the evaluation of the response to the metal, even when detailed specific knowledge of water quality parameters is lacking (Stauber & Davies 2000).

Several bioassays employing algae have been standardized and are used as diagnostic and predictive tools recommended by environmental protection organizations such as United Nations Environment Program, U.S. Environmental Protection Agency (EPA), and European Environment Agency (Batzias & Siontorou 2008). Algal based short-term bioassays are particularly useful for monitoring the aquatic environment because they are quick and inexpensive (Girling *et al.* 2000). Classical growth inhibition tests on unicellular algae cultures have been successfully complemented with, for example, measurements of oxidative stress parameters (Torres *et al.* 2008).

Acclimation to copper requires detailed temporal analyses because many of the changes occurring immediately after the heavy metal exposure may be transient and reflect metabolic changes related to transcription and/or mRNA turnover before the cells have become completely acclimatized.

In mixotrophic cultures of *Chlamydomonas,* light and acetate have an additive effect on growth promotion (Lalibertè & Noüie 1993). Acetate is metabolized in the glyoxylate cycle where isocitrate lyase (ICL) catalyses the formation of glyoxylate and succinate from isocitrate with ATP consumption.

Cultures grown with acetate as carbon source tend both to lower total chlorophyll content (Fischer *et al.* 2006; Sabatini *et al.* 2011) and reduce photosystem II (PSII) abundance (Kovács *et al.* 2000). Accordingly, a decrease in photosynthetic oxygen production is observed (Heifetz *et al.* 2000; Kovács *et al.* 2000). Photophosphorylation coupled to cyclic photosystem I (PSI) electron transport supplies ATP for acetate assimilation (Heifetz *et al.* 2000; Kovács *et al.* 2000; Fischer *et al.* 2006).

Additionally, light inhibits the expression of the ICL gene at the mRNA level (Petridou *et al.* 1997). Enzymatic activity peaks in the dark cycle and declines at the onset of illumination with moderate or high irradiance (Lalibertè & Noüie 1993; Paulson *et al.* 2010). On the other hand, acetate and darkness promote ICL activity (Lalibertè &

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Noüie 1993; Martínez-Rivas & Vega 1993; Petridou *et al.* 1997; Paulson *et al.* 2010).

Recently, Lemaire *et al.* (2004) and Bedhomme *et al.* (2009) have reported that in *Chlamydomonas*, the cellular redox state also regulates ICL activity through glutathionylation.

Isocitrate represents a key branching point in plant metabolism (Bedhomme *et al.* 2009). Oxidative decarboxylation by isocitrate dehydrogenase (IDH) leads to the energy-yielding tricarboxylic acid (TCA) cycle in the mitochondria. On the other hand, ICL cleaves isocitrate into glyoxylate and succinate in the glyoxylate cycle, supporting biosynthetic processes. Thus, through the regulation of the activities of IDH or ICL the cellular metabolism gains flexibility in the utilization of isocitrate. It has been suggested that under abiotic stress, acetate stimulates mitochondrial O_2 consumption by opening the gateway to the TCA cycle through IDH (Felitti *et al.* 2000). Increasing respiratory energy supply would allow recovery from oxidative stress (Fischer *et al.* 2006). Accordingly, we reported the inhibition of ICL activity under copper-induced oxidative stress (Sabatini *et al.* 2011) in cultures incubated under low light irradiance. In contrast, there have been some recent reports of abiotic stress triggering transcriptional up-regulation of ICL (Dittami *et al.* 2009).

In the present work we study the effect of copper-imposed oxidative stress on ICL activity, using two strains of *Chlamydomonas reinhardtii* (the wild type *4A+* and the pleiotropic mutant *cur1*, originally selected as a copper-tolerant phenotype in mixotrophic conditions). The metal effect on ICL activity was analyzed under moderate light intensity. In order to assess copper-induced oxidative stress in the acute and acclimation phases, several variables were measured: lipid peroxidation, abundance of antioxidant metabolites [carotenes, phenolic compounds, and ascorbic (AA) and monodehydroascorbic (DHA) acids], and the activity of the detoxifying enzymes catalase (CAT) and ascorbate peroxidase (APX).

MATERIAL AND METHODS

Organisms

Two strains of *Chlamydomonas reinhardtii* (Volvocales, Chlorophyceae) were used: the wild type $4A^+$ and the pleiotropic mutant *cur1* obtained by insertional mutagenesis with plasmid pSP124S (zeozin resistance). They were kindly provided by Dr K. Niyogi and Dr R. Dent (University of California at Berkeley). The mutant was selected as a copper-tolerant phenotype on agar plates of copper-enriched tris-acetate-phosphate (TAP) medium. Strain stocks are maintained at 15 μmol m^{-2} s^{-1} on TAP agar plates.

Chemicals

A previous screening for sublethal copper concentrations was carried out on agar plates in TAP media with the addition of 6.5 μM (control), 100 μM, 200 μM, 300 μM, and 400 μM copper chloride. Analytical grade copper chloride ($CuCl_2 \cdot 2H_2O$) was employed to obtain a 560 μM stock solution in deionised water. Solutions of varying concentrations were prepared by dilution of the stock with medium. In every case, appropriate volumes of distilled water were added to prevent changes in concentration of the other nutrients in the media.

At the onset of suspension cultures, the final concentration of free divalent copper cation (Cu^{2+}) in the culture solution was estimated in each case using MINEQL+ 4.0 (Table 1).

Growth conditions

Plates were inoculated with 3×10^4 cells and serial 1:10 and 1:100 cell dilutions of exponentially growing suspension cultures and incubated under continuous illumination (50 μmol m^{-2} s^{-1}), with growth scores being obtained periodically.

Suspension cultures were carried out photoheterotrophically in TAP media in a New Brunswick Innova 4340 culture chamber maintained at 23°C, with continuous agitation (200 rpm) and illumination by cool-white fluorescent lights (122 μmol m^{-2} s^{-1}). Copper-enriched TAP was prepared by the addition of a copper solution in order to reach the final desired nominal copper concentration. In every case, appropriate volumes of distilled water were added to prevent changes in concentration of the other nutrients in the media. Growth inhibitory concentrations of 50% (IC_{50}) at 72 and 96 h were calculated using the U.S. EPA Probit analysis program (v1.4). Bioassays with 120 μM copper chloride (TAP 120) were carried out in two series by triplicate, one harvested at 72 h and the other at 96 h. Initial cell density in all experiments was 5×10^3 cells ml^{-1}.

Cell density (number of cells per millilitre) was determined by direct counting in an improved Neubauer chamber, with an error less than 10%. Values were expressed as average cell count ± standard deviation. Specific growth rates were calculated from the semilog plot (Wood *et al.* 2005). At the same time, the rate of monadoid to palmelloid stages was determined.

Chlorophyll content

Cells from 1.5-ml cell suspension aliquots were harvested by centrifugation at 800 × g and thoroughly extracted in 80% acetone by vigorous vortexing. Acetone extracts absorbance at 664, 647, and 470 nm was measured in a UV/Vis Shimadzu spectrophotometer. Chlorophyll *a* and *b* and carotene contents were calculated according to the equations of Welburn (1994). Results were expressed as carotene:total chlorophyll ratio. In every case, caution was taken that the cellular debris was essentially colourless after solvent extraction.

Lipid peroxidation

Lipid peroxides were determined as thiobarbituric acid–reactive substances (TBARS) in 10-ml culture aliquots (Vavilin *et al.* 1998). After addition of butylated hydroxytoluene [final concentration, 0.01% (w/v)] to terminate lipid peroxidation chain reactions, cells were resuspended in freshly prepared trichloroacetic acid (with and without

Table 1. Estimation of divalent free copper cation (Cu^{2+}) and main inorganic and organic ligands in culture media TAP with different nominal copper chloride concentrations.[1]

Treatments	Total added copper (as chloride)	Free copper Cu^{2+}	$CuHPO_4$	Cu-EDTA	CuOH	$Cu(TRIS)_{tot}$[1]	Cu-$acetate_{tot}$[2]	pH	Dissolved copper (%)
Control	6.5	0.004	0.020	0.235	0.009	6.214	0.018	7.0	100
TAP 40	46.5	0.031	0.150	0.963	0.067	45.226	0.132	7.0	100
TAP 50	56.5	0.038	0.184	1.080	0.082	54.995	0.152	7.0	100
TAP 60	66.5	0.044	0.218	1.190	0.097	64.664	0.191	7.0	100
TAP 80	86.5	0.058	0.286	1.380	0.127	84.404	0.252	7.0	100
TAP 100	106.5	0.073	0.356	1.540	0.159	104.060	0.313	7.0	100
TAP 120	126.5	0.087	0.427	1.680	0.191	123.720	0.377	7.0	100
TAP 150	156.5	0.109	0.536	1.880	0.239	153.290	0.472	7.0	100
TAP 200	206.5	0.148	0.724	2.150	0.302	202.500	0.639	7.0	100

[1] Concentrations are expressed as micromoles per litre.
[2] $Cu(TRIS)_{tot}$ = CuTRIS + CuOH$(TRIS)_2$ + Cu$(TRIS)_2$ + Cu$(TRIS)_3$ + Cu$(TRIS)_4$.
[3] Cu-$Acetate_{tot}$ = Cu-Acetate + Cu-$Acetate_2$ + Cu-$Acetate_3$ + Cu-$Acetate_4$.

thiobarbituric acid). Following heating at 95°C for 30 min, TBARS were determined by absorbance at 532 nm with a correction for nonspecific absorbance at 440 and 600 nm (Hodges *et al.* 1999). A molar extinction coefficient for the thiobarbituric acid–malondialdehyde complex of 155 mM^{-1} cm^{-1} was used. Results were expressed as micromoles of TBARS per 10^6 cells.

Cellular homogenate preparation

Algal suspension (600 ml) was divided in two aliquots of 300 ml and cells were harvested by centrifugation at 1460 × *g*. Cells were resuspended as indicated: for homogenate A, in 3 ml of 200 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA and 1 mM mercaptoethanol; for homogenate B, in 2 ml of 50 mM potassium phosphate buffer pH 7.6 containing 1 mM EDTA and 5 mM ascorbate. Cell rupture was achieved by ultrasonication, employing fifteen 10-s successive cycles with a maximum frequency of 20 KHz with 50-s intervals. All procedures were performed at 4°C. The sonicated cells were centrifuged at 15,000 × *g* for 20 min to separate the supernatants for further analyses. Total protein content in homogenate was determined according to Lowry *et al.* (1951).

Phenolic compounds

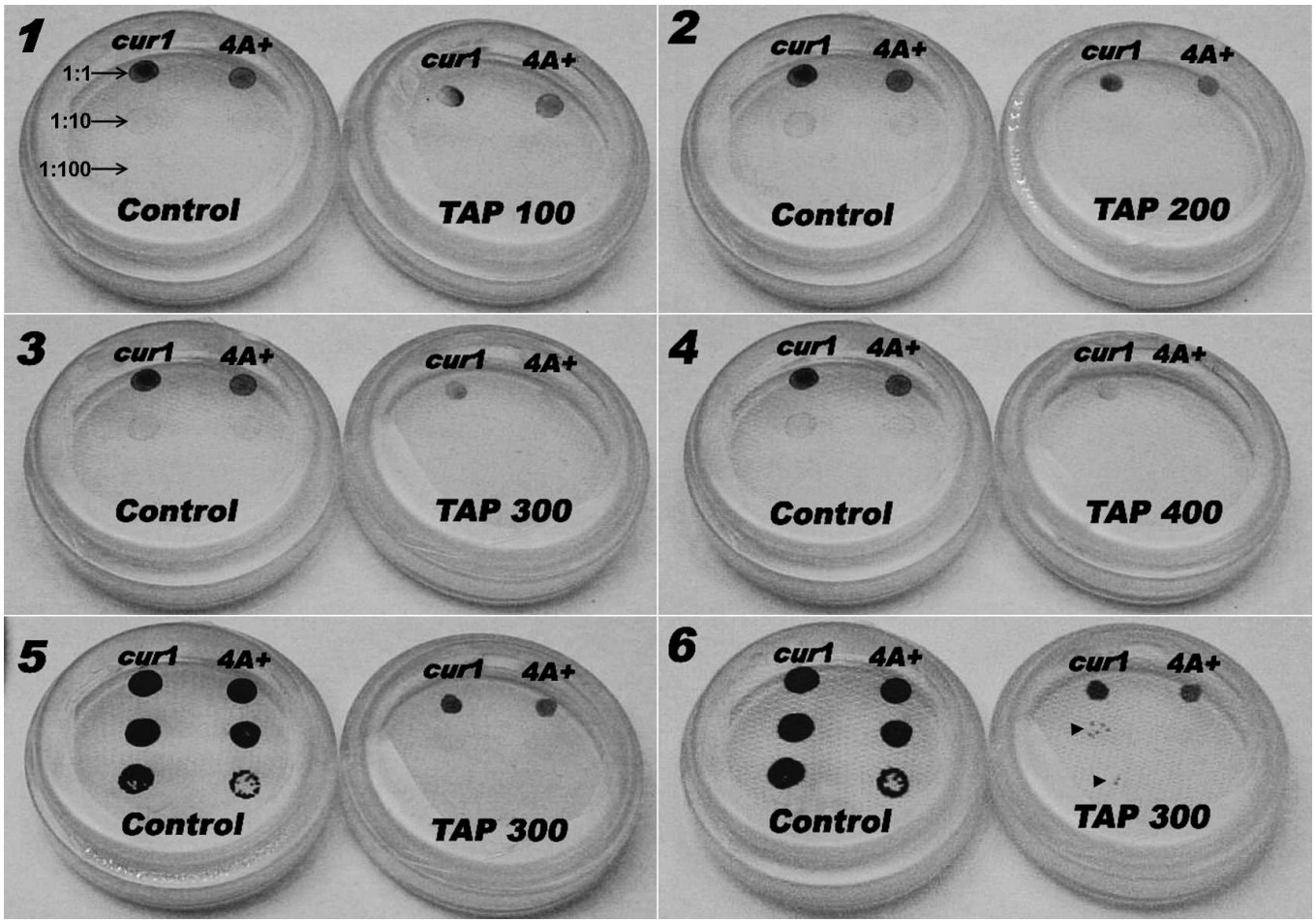
Phenolic compounds were determined in homogenate A (40–100 µl) according to Singleton *et al.* (1999), with the addition of 2.5 ml of 1:10 diluted Folin-Ciocalteau reagent followed by 2 ml of 0.5% Na_2CO_3 solution. After incubation at 45°C for 15 min, absorbance was read at 765 nm. Total phenolic compounds were expressed as microequivalents of gallic acid using a calibration curve prepared with 0 to 140 nmol of gallic acid dissolved in 95% ethanol.

Ascorbic and monodehydroascorbic acids

AA was determined after Okamura (1980) by adding 200 µl of homogenate A to a reaction mixture containing 2% trichloracetic acid, 8.8% orthophosphoric acid, 0.01% α,α′-dipyridyl and 10 mM ferric chloride (final volume = 1 ml). Absorbance was taken at 525 nm after incubation for 1 h at 37°C and referred to a calibration curve (0 to 160 nmol of AA). An identical procedure was applied for the homogenate previously reduced with 100 mM dithiotreitol (1 h at room temperature). Monodehydroascorbic acid (DHA) concentration was calculated as the difference between the absorbance of the reduced and nonreduced homogenates.

Starch

Starch cellular content was determined after exhaustive oxidation with perchloric acid. Namely, 100 and 200 µl of homogenate A were oxidized in ice bath during 20 min with the addition of two drops of 30% perchloric acid. The procedure was repeated twice to ensure total oxidation. After neutralization with 400 µl of a saturated $NaHCO_3$ solution, total carbohydrate content was determined by the phenol sulphuric method (Dubois *et al.* 1956). Glucose was used as standard and results were expressed as micrograms of carbohydrate per 10^6 cells.



Figs 1–6. Plaque bioassays in TAP medium (control) and TAP media with final nominal $CuCl_2 \cdot 2\ H_2O$ concentrations of 100, 200, 300, and 400 μM. Serial cell suspension dilutions in all assays is indicated by arrows in Fig. 1.
Figs 1–4. Growth score at day 3.
Fig. 5. Growth score at day 6.
Fig. 6. Growth score at day 12. Arrowhead indicates incipient colony development.

Enzymatic activity assays

ICL (EC 4.1.3.1) activity was measured using threo-D_sL_s-isocitric acid as substrate. Glyoxylate accumulation was estimated by the formation of the hydrazone in the presence of phenylhydrazine-HCl (Cooper & Beevers 1969), reading absorbance at 324 nm (extinction coefficient $14.63 \times 10^3\ M^{-1}\ cm^{-2}$). The reaction mixture (1 ml) contained 87 mM potassium phosphate buffer (pH 6.9), 4.6 mM mercaptoethanol, 87 mM $MgCl_2$, 30–40 μl of cellular homogenate A, and 10 mM phenylhydrazine. At the onset, 13 mmol of D_sL_s-isocitric acid were added and the reaction was monitored for 5 min. One ICL unit was defined as the enzyme amount that catalyzes the formation of 1 mmol of glyoxylate phenylhydrazone per minute at pH 6.9 and 30°C. Enzyme activity was expressed as ICL units per mg protein.

CAT (EC 1.11.1.6) activity was measured with H_2O_2 as the substrate and calculated by using an extinction coeffcient of $40\ M^{-1}cm^{-2}$ (Aebi 1984). The decay of peroxide was monitored during 50 s at 240 nm in a reaction mixture containing 40 mM potassium phosphate (pH 7.5) and 30–40 μl of homogenate A and 10 mM H_2O_2 added at the onset of the reaction. One CAT unit was defined as the enzyme amount that transforms 1 mmol of H_2O_2 per min. Enzyme activity was expressed as CAT units per milligram of protein.

APX (EC 1.11.1.11) activity was determined according to Nakano & Asada (1987) in a reaction mixture (1 ml) containing 50 mM phosphate buffer pH 7.0, 10–40 μl of homogenate B. The reaction was started with the addition of 50 nmol of L-ascorbic acid and 10 μl of 100 mM H_2O_2. Substrate consumption was monitored by the decrease in

Table 2. Growth parameters of *Chlamydomonas reinhardtii* $4A^+$ and *cur1* in control and copper-enriched cultures in TAP media.

Strain	Culture media	r (day^{-1})[1]	Final yield (cell ml^{-1})
4A+	control	1.82 ± 0.207	$9.51 \times 10^6 \pm 0.48 \times 10^6$
	TAP 120	1.34 ± 0.319*	$10.91 \times 10^6 \pm 0.71 \times 10^6$
cur1	control	2.18 ± 0.100	$10.97 \times 10^6 \pm 0.62 \times 10^6$
	TAP 120	1.12 ± 0.468*	$12.58 \times 10^6 \pm 0.83 \times 10^6$

[1] r = specific growth rate $[\ln (N_f/N_i)/\Delta t]$ where N_i and N_f are the initial and final number of cells per millilitre, respectively.
* Significant differences between control and respective treatments: $P < 0.05$, $n = 3$.

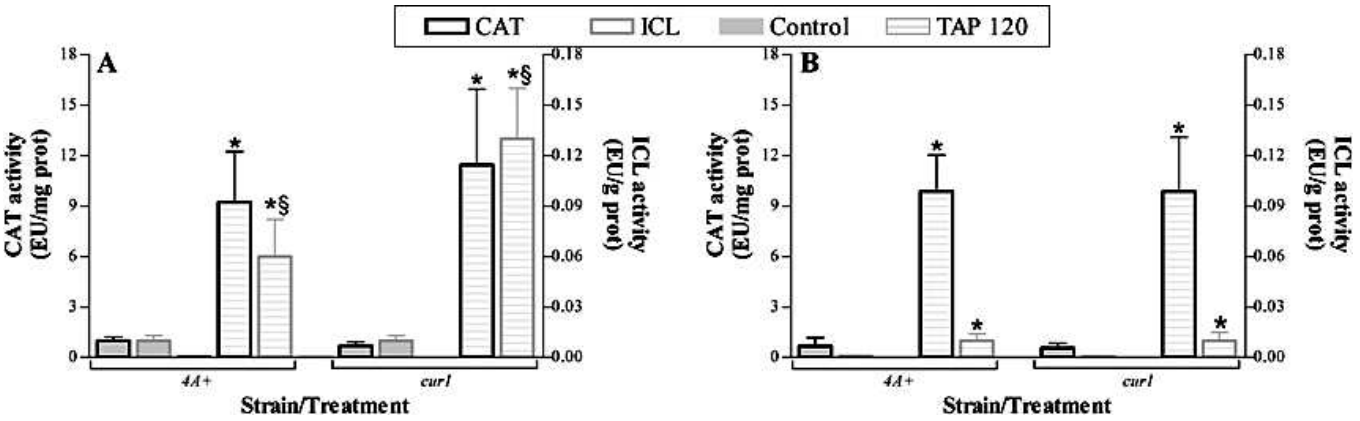


Fig. 7. Catalase (CAT) and isocitrate lyase (ICL) activities (EU mg^{-1} protein) in *4 A+* and *cur1* cultured in TAP medium containing 120 μM copper chloride. Controls: TAP medium. A. Cells harvested 72 h after culture onset. B. Cells harvested 96 h after culture onset. Significant differences between control and treatments: $*P < 0.05$. Significant differences between strains: $§P < 0.05$.

absorbance at 290 nm for 2 min and the activity was calculated using the extinction coefficient of ascorbate ($\varepsilon = 2.8\ mM^{-1}\ cm^{-2}$). A blank without homogenate was run to determine the unspecific spontaneous oxidation of ascorbate. Enzyme activity was expressed as APX units per milligram of protein.

Statistical analysis

The results of three replicates from different treatments were compared statistically by a two factor analysis of variance (ANOVA). The suppositions of normality and homogeneity of variances were tested with Lillieford and Bartlett tests, respectively (Steel & Torrie 1985), with the aid of STATISTICA software (StatSoft 1999). The following variables were considered as independent fixed factors: strain (*4A+*, *cur1*) and treatment (control, TAP 120). Differences for the IC_{50} values were considered statistically significant when the higher IC_{50}/lower IC_{50} ratio exceeded the critical value (95% confidence interval) established by the American Public Health Association (West 1980).

RESULTS

Plaque bioassays

Growth was always slower for both strains in media with 100 μM, 200 μM, and 300 μM $CuCl_2$ (Figs 1–6). Assays with 400 μM copper proved lethal (Fig. 4). After 72 h *cur1* colonies were the first to show up, even with the highest sublethal copper concentration employed. Colonies in copper-enriched media showed bleaching with respect to the controls. With prolonged incubation *cur1* developed colonies even in the 1:10 dilution in TAP 300 (Fig. 6). Besides, this strain exhibited denser growth in the control 1:100 dilution (Figs 5, 6).

Preliminary suspension cultures: estimation of free divalent cation concentration and sampling time for bioassays

In liquid TAP media, a linear relation of divalent free cation vs total micromoles of added $CuCl_2 \cdot 2\ H_2O$ was sustained for the range 0–200 μM ($r^2 = 0.9593$), according to the estimation of free divalent copper cation with MINEQL software (Table 1).

In order to determine the exposure time and copper concentration for batch bioassays, suspension cultures were carried out in TAP with 40, 60, 80, 100, 120, and 200 μM $CuCl_2$ added. Incubation in 200 μM $CuCl_2$ was lethal in suspension cultures, suggesting that lower diffusion and adsorption of the cation in agarized media led to an overestimation of sublethal concentration in plaque bioassays. A prolonged lag phase was observed in 120 μM $CuCl_2$ (TAP 120). In fact, considering two different exposure times (72 and 96 h) the $IC_{50,72h}$ were 92.3 (87.8–96.7) μM and 88.6 (83.8–93.0) μM for *4A+* and *cur1*, respectively. The $IC_{50,96h}$ for the same strains resulted in 109.8 (105.4–114.0) and 105.0 (99.6–109.4) μM. No significant differences in the IC_{50} values were found between strains ($P < 0.05$).

Batch bioassay

TAP 120 was selected for suspension bioassays. This copper concentration diminished specific growth rate 1.4- to 1.9-fold for *4A+* and *cur1*, respectively (Table 2). No significant differences ($P > 0.05$) were obtained in final cell yield but this was reached at 120 h for control cultures and at 144 h for copper-exposed ones. Neither specific growth rate nor final cell yield showed significant differences between strains ($P > 0.05$) (Table 2).

Both at 72 and 96 h, ICL activity showed a significant increase ($P < 0.05$), with respect to the controls, in copper stressed cultures (Fig. 7). Besides being higher, ICL activity at 72 h differed significantly between strains ($P < 0.05$), being more pronounced for *cur1*.

Copper induced the formation of palmelloid stages ($P < 0.05$) together with starch accumulation at 72 h (Table 3) in both strains. Starch accumulation was concomitant with a significant increase in the carotene:chlorophyll ratio for both strains at 72 h ($P < 0.05$) (Table 3). Such difference was no longer significant at 96 h ($P > 0.05$).

At 72 h, copper exposure also resulted in an approximate 70% increment in phenolic compounds with respect to the controls ($P < 0.05$) (Table 3). Phenolic compounds and TBARs dosage showed significant differences between

Table 3. Comparative results of measured variables at two end-points (72 and 96 h) in batch bioassays for strains *4A+* and *cur1* of *Chlamydomonas reinhardtii* in TAP (control) and TAP 120 μM $CuCl_2$.

Exposure time	72 h				96 h			
Strain	*4A+*		*cur1*		*4A+*		*cur1*	
Treatment	Control	TAP 120	Control	TAP 120	Control	TAP 120	Control	TAP 120
Palmelloid stages (%)	0.00 ± 0.00	69.9 ± 9.5*	0.00 ± 0.00	42.9 ± 25.3*	0.00 ± 0.00	49.4 ± 16.3*	0.00 ± 0.00	61.9 ± 11.4*
Carotenoids/ chlorophyll	$2.09 \times 10^{-5} \pm 1.00 \times 10^{-6}$	$6.32 \times 10^{-2} \pm 4.00 \times 10^{-2*}$	$2.00 \times 10^{-5} \pm 1.00 \times 10^{-6}$	$1.28 \times 10^{-1} \pm 0.95 \times 10^{-2*}$	$1.00 \times 10^{-1} \pm 1.00 \times 10^{-2}$	$8.65 \times 10^{-2} \pm 1.06 \times 10^{-1}$	$1.18 \times 10^{-1} \pm 5.00 \times 10^{-3}$	$4.94 \times 10^{-2} \pm 6.20 \times 10^{-2}$
Phenolic compounds (μmol 10^6 $cells^{-1}$)	6.01 ± 0.81	11.52 ± 2.45*†	6.07 ± 0.73	8.89 ± 1.52*†	3.41 ± 0.44	3.24 ± 0.34†	2.81 ± 0.95	2.47 ± 0.28†
Starch (μg 10^6 $cells^{-1}$)	0.65 ± 0.13	0.90 ± 0.15*	0.59 ± 0.17	0.99 ± 0.41*	0.74 ± 0.16	0.53 ± 0.10	0.49 ± 0.14	0.40 ± 0.34
Ascorbic acid (μg 10^6 $cells^{-1}$)	1.08 ± 0.52	0.14 ± 0.17*	0.86 ± 0.17	0.12 ± 0.07*	0.58 ± 0.04	0.64 ± 0.09	0.58 ± 0.24	0.52 ± 0.10
TBARs (nmol 10^6 $cells^{-1}$)	0.22 ± 0.04	1.51 ± 0.30*†	0.15 ± 0.03	1.06 ± 0.21*†	0.09 ± 0.01	0.45 ± 0.19*	0.07 ± 0.01	0.62 ± 0.19*

* Significant difference from the respective control ($P < 0.05$).
† Significant difference between strains ($P < 0.05$).

strains ($P < 0.05$.). Copper-enhanced lipid peroxidation (measured as TBARs) at both exposure times ($P < 0.05$), though the values were approximately 60% higher during acute exposition (72 h). The increase in TBARs was accompanied at both exposure times by the significant rise in CAT activity ($P < 0.05$) (Fig. 7). On the other hand, AA:DHA ratio was inversely related to APX activity (Fig. 8). Activation of this detoxifying enzyme was significantly induced for both strains in copper-stressed cultures at both exposure times ($P < 0.05$). But at 96 h, the difference with respect to the controls was smaller.

DISCUSSION

This work allowed us to distinguish two stages in copper-stressed cultures: an acute and a restoration phase, with signs of acclimation after 96 h (the IC_{50} increased at 96 h for both strains). Under continuous moderate illumination, copper-exposed cultures showed enhanced ICL activity. In contrast, control cultures expressed only basal constitutive ICL activity.

It is important to take into account the transcriptional down-regulation of ICL exerted by light (Petridou *et al.* 1997) and the post-transcriptional reversible inactivation by glutathionylation (Bedhomme *et al.* 2009). Undetected ICL activity in the control cultures under moderate light intensity is in agreement with down-regulation at the transcriptional level of the ICL gene. Similar results were reported by Paulson *et al.* (2010).

On the other hand, low irradiance and darkness promote ICL activity (Lalibertè & Noüie 1993; Martínez-Rivas & Vega 1993; Petridou *et al.* 1997; Paulson *et al.* 2010; Sabatini *et al.* 2011). Assuming ICL transcription is up-regulated under low irradiance, its activity might also be promoted at the post-transcriptional level. In the absence of oxidative stress, the cellular glutathione and ascorbate pools are presumably maintained in a reduced state. Under such conditions, ICL glutathionylation would not be expected, thus the enzyme is activated (Foyer & Noctor 2005; Dalle-Donne *et al.* 2008; Sabatini *et al.* 2011).

Under moderate irradiance, copper-induced oxidative stress enhanced ICL activity, especially in the acute phase. Copper-enhanced reactive oxygen species (ROS) production poses a different scenario, altering the cellular redox state. In the chloroplast, the Calvin cycle is the main consumer of reducing power. If the redox state changes, glutathionylation down-regulates the Calvin cycle (Michelet *et al.* 2005). As a consequence, more reductive species will be available for ROS detoxification and also for reduction of glutaredoxins, which mediate protein deglutathionylation (Dalle-Donne *et al.* 2008). In the case of ICL, this modification activates the enzyme (Bedhomme *et al.* 2009), as we observed in copper-exposed cultures. Additionally, some authors have recently reported ICL transcriptional up-regulation under abiotic oxidative stress conditions (Gillet *et al.* 2006; Dittami *et al.* 2009). Note that the mutant strain exhibited at least twofold more enzyme activity than the wild type in the acute phase. Within this context, a higher ICL activity can give an advantage to the

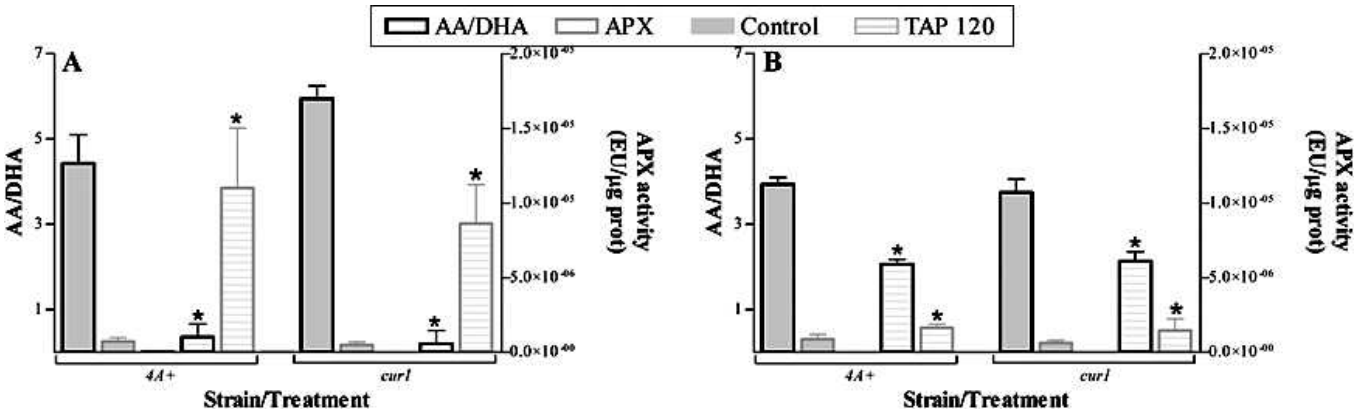


Fig. 8. AA:DHA ratio and APX activity (EU mg $^{-1}$ protein) in *4 A*$^{+}$ and *cur1* cultured in TAP medium containing 120 µM copper chloride. Controls: TAP medium. A. Cells harvested 72 h after culture onset. B. Cells harvested 96 h after culture onset. Significant differences between control and treatments: $*P < 0.05$.

mutant strain since it promotes mixotrophic organic carbon assimilation. Once restitution has begun (96 h) and the normal cellular redox state is presumably restored, ICL activity decays considerably for both strains.

Under low irradiance, reducing power from the photosystems might not be enough to reverse the oxidizing redox cellular state promoted by metal-enhanced oxidative stress. Under such conditions, ICL would be glutathionylated and lose activity. So even when transcription might not be down-regulated, a decrease in enzyme activity would be expected under oxidative stress conditions, as we reported in previous work (Sabatini *et al.* 2011). This transient inactivation of ICL favours the energy-yielding TCA cycle, in order to enable cellular recovery from stress (Fischer *et al.* 2006).

The increase of ICL activity is observed simultaneously with the enhancement of APX and CAT activities in copper-stressed cultures. Since *Chlamydomonas* ICL is very sensitive to irreversible inactivation by H_2O_2 (Bedhomme *et al.* 2009), increase in ICL activity denotes efficient H_2O_2 scavenging. At the same time, high APX activity ensures glutathione production contributing to maintain functional ICL.

Up-regulation of the transcription of the ICL gene has also been related to deflagellation (Chamberlain *et al.* 2008). Accordingly, we observed an increment in the percentage of cells in the palmelloid stage. This increment is in accordance with the delay in specific growth rate in copper-stressed cultures, often entitled as a good indicator in metal toxicity bioassays with microalgae (Miao *et al.* 2005). Yet, similar final cell densities in control and treatment cultures allow us to infer that stress responses are occurring within the homeostatic range.

Together with growth arrest, starch accumulation occurred during the first 72 h, as indicated by Libessart *et al.* (1995). Once restoration had begun, differences in starch content were dissipated. Different metal exposure times might explain contradictory reports of starch underproduction (Irmer *et al.* 1986; Ballan-Dufrançais *et al.* 1991; Morlon *et al.* 2005) or overproduction (Wong *et al.* 1994; Nishikawa *et al.* 2003; Sabatini *et al.* 2011).

Micromolar copper exposure evokes a state analogous to photoinhibition, enhancing the production of singlet oxygen radicals in the chloroplast (Küpper *et al.* 2002), that trigger the formation of lipid peroxides, as suggested by the increase in TBARS dosage. Simultaneously, an important increase in the carotene:chlorophyll ratio was detected at 72 h. Lipid-soluble antioxidants such as carotenes and tocopherols protect chloroplast membranes either by functioning as singlet oxygen quenchers or by trapping lipid peroxides (Asada 2006). The lower AA:DHA ratio can also be related to scavenging of lipid peroxides, since water-soluble antioxidants such as ascorbate and glutathione may function as direct reductive species or cofactors for peroxidases.

Phenolic compounds scavenge peroxyl radicals and protect membranes against lipid peroxidation (Mansouri *et al.* 2005). Stress exposure requires a minimum duration, probably to complete biosynthesis as indicated by Kovácik *et al.* (2010). These authors were unable to detect an increase in total phenols in *Scenedesmus quadricauda* after 1 h exposure to UV, while Duval *et al.* (2000) reported an increase in *Chlamydomonas nivalis* after 48 h of irradiation. On the other hand, chronically copper-exposed algal populations show no significant change in phenolic compounds (Ratkevicius *et al.* 2003; Contreras *et al.* 2005), suggesting that these metabolites are effective scavengers in the acute stage. Note that extending the exposure time up to 96 h also showed no significant variation in total phenolic compounds, even when lipid peroxidation and CAT activity still differed significantly with respect to control cultures.

CONCLUSIONS

Results from suspension batch bioassays in copper-enriched TAP media under moderate irradiance showed no significant difference in the performance of the copper-tolerant phenotype (*cur1*) and the wild type. The higher intrinsic ICL activity measured in *cur1* could explain faster development of the mutant strain in agar plates. Even when this higher intrinsic ICL activity persisted in copper-exposed suspension cultures, it was not translated into significant differences in copper stress tolerance among

strains. The higher ICL activity under copper stress can be interpreted as an adaptive strategy promoting mixotrophic organic carbon assimilation under circumstances of impaired photosynthetic carbon dioxide assimilation due to oxidative stress and results a useful parameter in bioassays with acetate-flagellates.

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