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Effect of Abiotic Stress on Photosynthesis, Respiration and Antioxidant System in *Chlamydomonas reinhardtii*

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K e y w o r d s : Abiotic stress, *Chlamydomonas reinhardtii*, photosynthesis, respiration, antioxidant, nitrate uptake, sulfate uptake.

S u m m a r y

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Our results indicate that 200 mM NaCl, 0.2 µM methyl viologen, 4 mM arsenite or 300 µM of cadmim in the culture medium are highly toxic for *C. reinhardtii* productivity and they inhibit photosynthesis, while respiration remains in all cases relatively high. Salt stress bloks immediately the photosynthetic activity of the alga which is partially recovered, and it increases catalase, ascorbate peroxidase (APX) and glutathione reductase (GR) activities. Catalase activity was increased almost 20-fold during salt stress, reaching a value of 338.0 µmoles O_2 formed/min/mg Chl, after 24 h treatment. In MV-treated *C. reinhardtii* cells catalase, APX and GR activities result increased, while CAT and GR are the most efficiently induced activities by arsenite-stressed cells. Cadmium induces CAT and APX, but not GR, activities. These data support that abiotic stress induces in *C. reinhardtii* an increase of active oxygen species (AOS), which parallel a significant inhibition of the photosynthetic electron flow.

On the other hand, nitrate and sulfate uptake rates by *C. reinhardtii* were significantly inhibited, under the indicated abiotic stresses. In addition, glutamate synthase (MVH-GOGAT), glutamate dehydrogenase (NADH-GDH) and O-acetylserine(thiol)lyase (OASTL) activities were differently affected by abiotic stress in *C. reinhardtii*. Salt enhances MVH-GOGAT and OASTL activities; methyl viologen produces a 30-fold increase of NADH-GDH activity, while MVH-GOGAT and OASTL activities are moderately enhanced. Cadmium produces an increase in NADH-GDH and OASTL activities. However, the three mentioned activities decreased with respect to control in arsenite-treated cells. These data indicate that an active synthesis of glutamate and cysteine is produced in *C. reinhardtii* cells treated with salt, methyl viologen or cadmium.

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Introduction

Under normal growth conditions active oxygen species (AOS) are formed at low rate in photosynthetic cells as by-products of aerobic metabolism, but many stresses cause a dramatic increase in the rate of AOS production, being the chloroplasts the major source of AOS in the leaf cells and algae (FOYER & NOCTOR 2000). An efficient removal of AOS from chloroplasts is critical since H_2O_2 concentrations as low as 10 μM can inhibit photosynthesis by 50 %. Antioxidant systems of plant chloroplasts include enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), ascorbate peroxidase (APX; EC 1.11.1.11) and glutathione reductase (GR; EC 1.6.4.2). Although catalase (CAT; EC 1.11.1.6), is capable of scavenging large quantities of H_2O_2, its location in peroxisomes and its relatively low Km limit its ability to keep H_2O_2 concentrations low enough to prevent chloroplast damage (APEL & HIRT 2004).

Information of literature indicates that salt tolerance have been achieved in plants overexpressing: a) the plasma membrane Na^+/H^+ antiporter SOS1 in *Arabidopsis* (WARD & al. 2003); b) ascorbate peroxidase activity in tomato (MITTOVA & al. 2002) and in tobacco chloroplasts (BADAWI & al. 2004); c) glutathione peroxidase (YOSHIMURA & al. 2004) and glutathione transferase (YU & al. 2003) activities in tobacco. In eukaryotic algae, we have: a) halotolerant *Chlamydomonas* sp. strain W-80 which can grow in medium containing 2.5 M of NaCl, or even in the presence of 100 μM of MV, have particularly high the glutathione peroxidase and ascorbate peroxidase activities (MIYASACA & al. 2000); and b) salt-tolerant *Dunaliella* which responds to high salinity by enhancement of photosynthetic CO_2 assimilation (LISKA & al. 2004).

Plants and eukaryotic algae mainly use soil and water-dissolved nitrate as main nitrogen source, while sulfate and atmospheric SO_2 and SH_2 are used as sulfur source (HAWKESFORD & WRAY 2000). Nitrate and sulfate assimilation are specifically required for algae productivity, but also for protection mechanism against abiotic stress. Photosynthetic chain supply metabolic energy and redox power for nitrate and sulfate assimilation, which proceeds through out two independent metabolic pathways only connected at the biosynthesis of cysteine inside of the chloroplast (MOSULÉN & al. 2003). In *C. reinhardtii*, primary biosynthesis of glutamate proceeds through the glutamine synthetase (GS; EC 6.3.2.1)-glutamate synthase (Fd-GOGAT; EC 1.4.7.1 and NADH-GOGAT; EC 1.4.1.14) pathway, while NADH-glutamate dehydrogenase (GDH; EC 1.4.1.2) is an enzyme which may be involved in additional synthesis of glutamate under specific metabolic circumstances (DOMÍNGUEZ & al. 2003). Biosynthesis of cysteine is catalyzed, in *C. reinhardtii* by the cysteine synthase complex, which includes two enzymatic activities, serine acetyltransferase (SAT; EC 2.3.1.30) and O-acetylserine(thiol)lyase (OASTL; EC 4.2.99.8) (RAVINA & al. 2002).

Cadmium (HU & al. 2001) and arsenite (SCHMÖGER & al. 2000) induce very efficiently in *C. reinhardtii* and plants, the biosynthesis of phytochelatins, which are peptides consisting of repeating units of γ-glutamylcysteine followed by

a C-terminal glycine. This means a high requirement for glutamate and cysteine to display defense mechanism.

Thus, we consider interesting to analyse photosynthesis, respiration and representative antioxidant system enzymes in *C. reinhardtii* under different abiotic stress, and also study the nitrate and sulfate assimilation capacity in the alga cells during different adverse circumstances.

Material and Methods

Culture conditions and biomass production

In this work we use the eukaryotic algae *Chlamydomonas reinhardtii*, strain 21 gr, which was grown in climatized rooms, at 25 ºC using 1l bottles containing 500 ml of Sueoka liquid medium with 10 mM nitrate and 0.3 mM sulfate. The culture was bubbled with air supplemented with 5 % (v/v) CO_2 and continuously illuminated with white light from fluorescent tubes, 250 $\mu E/m^2$, at the surface of the tube. Under such conditions the generation time for *C. reinhardtii* was 8 h. The growth rate was determined by measuring the chlorophyll content in 1 ml of culture at different times. Biomass production was usually determined by the dry weight contained in 300 ml of alga culture, at the end of the experiment (More details in MOSULÉN & al. 2003).-

Cells permeabilization and crude extract preparation

Cells were permeabilized immediately before used by adding 100 µl toluene to a 2 ml cells suspension containing approximately 20 µg Chl/ml, and shaking vigorously during 20 s. Crude extract was prepared by freezing the recently harvested cells with liquid nitrogen and thawed with 50 mM Tris-HCl, pH 7.5, containing 0.5 mM EDTA and 14 mM β-mercaptoethanol (5 ml buffer per g of fresh weight cells). After 15 min, the suspension was centrifuged at 15,000 rpm during 15 min and the supernatant constituted the crude extract where enzymatic activities were studied.

Measurements of photosynthesis and respiration in *C. reinhardtii*

Photosynthetic light response curves were determined including in the electrode chamber 2 ml of the *C. reinhardtii* culture (about 20-25 µg Chl/ml) treated as indicated in each case. Respiration was determined in the same sample by darkening the chamber containing cells. The activity of photosystem I (PSI) was determined in permeabilized cells resuspended in 2 ml of 15 mM phosphate buffer, pH 7.5, containing ascorbate, 5 mM, 2,6-dichlorophenol-indo-phenol (DPIP), 50 µM, azide, 0.5 mM, methyl viologen, 0.1 mM and [3-(3',4'-dichlorophenyl)-1,1-dimethylurea)] (DCMU), 10 µM. Changes in oxygen concentration were monitored at 25 ºC with a Clark-type O_2 electrode. The reaction chamber was illuminated with 1,500 $\mu E/m^2$ of actinic light provided by a tungsten lamp.

Nitrate and sulfate uptake rates

They were determined by measuring periodically the remaining concentration of nitrate and sulfate in the *C. reinhardtii* culture medium. More details as in MOSULÉN & al. 2003.

Analytical determinations

Chlorophyll was extracted with acetone (80%, v/v) and quantified using the absorbance at 652 nm ($\varepsilon = 34.5\ mg^{-1}.ml.cm^{-1}$). Protein was determined by the method of BRADFORD 1976 using bovine serum albumin as standard. Nitrate in the culture medium was determined spectrophotometrically by mixing 0.75 ml of sample, containing 0 - 100 nmoles of nitrate, with 0.05 ml of a solution of 10 % (w/v) sulfamic acid. After mixing vigorously during 2 min, 0.2 ml of a 20 % (v/v) solution of perchloric acid was added and the absorbance at 210 nm was determined ($\varepsilon = 7\ mM^{-1}.cm^{-1}$) (MOSULÉN & al. 2003). Sulfate in the culture medium was determined as sulfur in the ICP spectrophotometer, mod. 3410, Fisons Instruments (USA) using a wavelenght of 180.715 nm.

For Na^+, arsenic and cadmium determinations, the dried algae samples were digested with 8 ml nitric acid (65 %) plus 2 ml H_2O_2 (30 %) in a microwave oven (Mod. ETHOS 900, Milestone S.r.l. Italy), according MLS software report 229 for green algae, between 250-650 watt. Sodium and cadmium ions was determined in the dissolved samples using an inductive coupling plasma spectrophotometer (ICP), mod Fisons 3410, at wavelength of 588.99 and 226.502 nm, respectively. In the case of arsenic the samples were derived to its hydride form and the wavelength used was 190.76.

Enzyme assays

Catalase activity was determined *in vivo,* at 25 °C, by following the H_2O_2-dependent oxygen production, using the Clark O_2-electrode, which included in the cell chamber 2 ml of the *C. reinhardtii* culture, 4-fold diluted with water, to which 20 µl of a 2.5 M H_2O_2 solution was added to start the reaction. One unit (U) of activity represents the amount of cells required to produce 1 µmol of O_2 per min. The ascorbate peroxidase activity (APX) was determined spectrophotometrically by measuring, at 290 nm (absorption coefficient 2.8 $mM^{-1}.cm^{-1}$) and 25 °C, the rate of ascorbate peroxidation in 1 ml reaction mixture containing 1 mM EDTA; 50 mM K-P (pH 7.5), 0.2 mM ascorbate, 0.5 mM H_2O_2 and 100 µl of soluble crude extract. Glutathione reductase (GR) was determined following the absorbance change, at 340 nm, in 1-ml reaction mixture containing 25 mM Tris-HCl buffer, pH 7.5, 0.5 mM EDTA, 1 mM oxidized glutathione; 100 µl of crude extract and 0.25 mM NADPH, according to LAMOTTE & al. 2000. One unit of activity represents the amount of enzyme required to oxidize 1 µmol of NADPH per min.

OASTL activity was determined, at 40° C, as indicated previously (RAVINA & al. 2002). Total GOGAT activity (includes Fd-GOGAT plus NADH-GOGAT) was determined, at 30° C, with dithionite-reduced MV as electron donor, as indicated by MOSULÉN & al. 2003. NADH-glutamate dehydrogenase activity was measured at 30 °C in 1 ml reaction mixture containing: 50 mM Tris-HCl (pH 9.0), 100 mM NH_4Cl; 17 mM 2-oxoglutarate, 0.7 mM NADH and the appropriate amount of crude extract (about 200 µl). The oxidation of NADH was followed at 340 nm in aliquots of reaction mixture 5 fold diluted with water (DOMÍNGUEZ & al. 2003). One unit of activity represents either the transformation of 1 µmol of substrate or the formation of 1 µmol of product per min.

Results and Discussion

Effect of abiotic-stress on the photosynthesis and respiration in *C. reinhardtii*

We have studied the effect of salt, methyl viologen, arsenite and cadmium on the photosynthetic and respiratory activities in *C. reinhardtii* (Table 1). Most algae and plants can be adapted to moderate salinities, but their growth is seriously limited above 200 mM. Salt toxicity is evident for *C. reinhardtii*, at this concentration and produces a rapid and complete inhibition of the photosynthetic activity, which is recovered about 33 % after 1 hour of salt treatment, value which is maintained during at least 24 h. Respiratory activity of the alga is less sensitive than photosynthetic activity is and its recovery is about 77 % of the original value in the absence of salt. In general salt stress produces inactivation of photosynthesis in plants (UEDA & al. 2003). *C. reinhardtii* cells can accumulate Na^+ until 0.66 ± 0.02 % of its dry weight, when growing with the non-toxic salt concentration of 100 mM, which suppose a 60-fold higher than the control cells. However the intracellular Na^+ content decreases until 0.36 ±0.02 %, at 200 mM NaCl, thus indicating the activation of a Na^+ extrusion mechanism in the alga.-The effect of 0.2 µM methyl viologen in the *C. reinhardtii* culture produces a total, but gradually inhibition of photosynthetic activity, while respiratory activity remains high during the 24 h of

the experiment (Table 1). PSI activity was not significantly affected by this MV concentration in *C. reinhardtii* (data not shown).

Table 1. Effect of abiotic stress on photosynthesis, respiration and antioxidant enzymes in *C. reinhardtii*.

Treatment	Photosynthesis	Respiration	CAT	APX	GR
Control	100	100	100	100	100
+ NaCl, 200 mM	33.1	77.3	1,878.0	198.1	130.0
+ MV, 0.2 μM	8.5	72.9	433.9	141.2	178.8
+ Arsenite, 4 mM	0.0	88.2	388.9	66.8	154.7
+ Cd^{2+}, 300 μM	39.0	80.2	222.8	143.0	44.8

Cells growing under standard conditions were added with the indicated compounds. After 24 h growing under continuous illumination, we measured in vivo the photosynthetic (100% = 407 ± 5.3 μmol O_2 evolved ·mg $Chl^{-1}·h^{-1}$) and respiratory (100 % = 52.4 ± 3.2 μmol O_2 consumed·mg $Chl^{-1}·h^{-1}$) rates, and also the CAT activity (100 % = 18.0 U.mg Chl^{-1}). In addition, APX (100 % = 99.0 ± 3.5 $mU·mg^{-1}$) and GR (100 % = 167.4 ± 3.3 $mU·mg^{-1}$) activities were determined in the corresponding crude extract. The data are representative of three different experiments.

In the presence of 4 mM arsenite, *C. reinhardtii* cells losses photosynthetic, but not respiratory, activity after 24 h treatment (Table 1). The amount of arsenic detected inside the *C. reinhardtii* cells was about 0.05 ± 0.002 % of its dry weight, which supposes less than 1% of the total arsenic in the culture medium. In addition 300 μM cadmium was toxic for *C. reinhardtii* and partially inhibits the photosynthetic activity of cells, while respiration remains high (Table 1). The alga may accumulate Cd^{2+} about 0.9 ± 0.02 % of its dry weight, which means about 30 % of the total cadmium in the medium, and this amount decreased when 1 mM Ca^{2+} was also present in the culture medium (MOSULÉN & al. 2003). The donor side of photosystem II is apparently impaired in a Cd^{2+}-tolerant mutant strain of *C. reinhardtii* (VOIGT & NAGEL 2002). Other metal, like zinc, partially inhibits photosynthetic activity in microalgae (MAGALHAES & al. 2004).

Effect of abiotic-stress on the *C. reinhardtii* antioxidant enzymes

We mostly observed that abiotic stress induces an increase of the antioxidant enzyme activities in *C. reinhardtii*. Particular attention deserves the induction of catalase activity, which reach values of about 20-fold higher than the control culture in salt-treated cells as compared with the 3-fold increase observed in cells treated with methyl viologen, arsenite or cadmium (Table 1). The salt promoted induction of CAT activity depends on NaCl concentration in the culture medium (Fig. 1) and it may be used as a specific indicator of salt stress in *C. reinhardtii*. Salt also induces in the alga a high expression of other antioxidant enzymes, such as ascorbate peroxidase and glutathione reductase (Table 1). These data suggest an important role of these enzymes as AOS scavengers in *C. reinhardtii* during salt stress, as indicated in other photosynthetic organisms (HASEGAWA & al. 2000). The toxic effect of salt is mediated in plants by the production of AOS (GOSSETT & al. 1996) and the level of antioxidant enzymes increases when plants are exposed to

biotic or abiotic stresses (LEVINE 1999). Consistent with this, a long-term salt stress induces superoxide dismutase activity in pea chloroplasts (GÓMEZ & al. 2004). Conversely, in higher plants catalase activity is not clearly induced by arsenite (CAO & al. 2004), or methyl viologen stresses (MANO & al. 2001), and even it is inhibited by cadmium stress (SKORZYNSKA-POLIT & al. 2003/4).

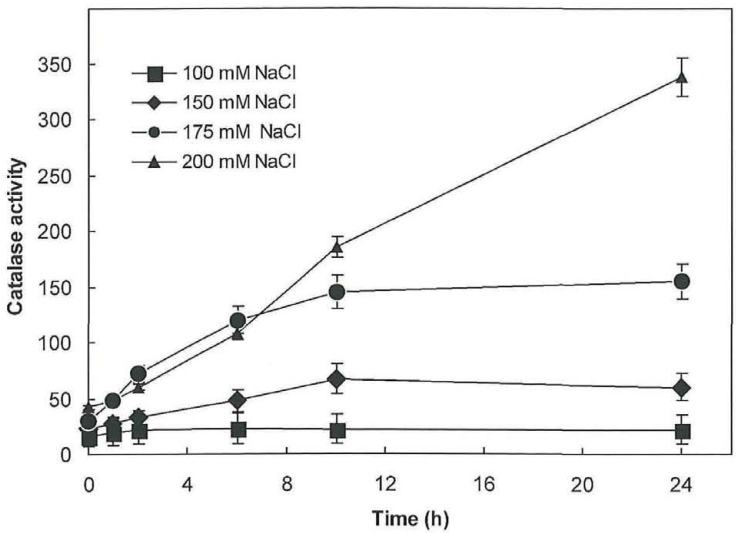

Fig. 1. Effect of salt concentration in the culture medium on the catalase activity induction in *C. reinhardtii.* Cells growing under standard conditions (about 20 µg/ml culture) were added with the indicated salt concentration. Periodically catalase activity (µmol $O_2 \cdot min^{-1} \cdot mg\ Chl^{-1}$) was determined *in vivo* all along the experiment. Given values are means ±SD from three independent experiments.

Methyl viologen has been reported to induce oxidative stress in photosynthetic organisms (DONAHUE & al. 1997) and chloroplastic ascorbate peroxidase seems to be the primary target in spinach leaves (MANO & al. 2001). In *C. reinhardtii* MV induces both the glutathione reductase and ascorbate peroxidase activities (Table 1) like it has been observed in plant leaves (DONAHUE & al. 1997, YU & al. 2003). In addition MV also induces increase in the SOD activity in plants (ALLEN 1995).

Glutathione reductase activity is induced in *C. reinhardtii* after 24 h treatment with 4 mM arsenite, but in this case we observed an inactivation of ascorbate peroxidase activity (Table 1). At low levels of arsenic exposure (20 mg kg^{-1}), enzymatic antioxidants are important for arsenic detoxification and accumulation in chinese brake fern, while non-enzymatic antioxidants, like glutathione, were more

important at high arsenic exposure (50-200 $mg.kg^{-1}$) (CAO & al. 2004). In addition arsenic triggers specific responses of antioxidant and detoxification related genes in tissue of maize (MYLONA & al. 1998). In the presence of 300 µM cadmium, the alga cells induce catalase and ascorbate peroxidase, while glutathione reductase activity is significantly inhibited (Table 1).

These data indicate that abiotic stress, such as high salinity, methyl viologen, arsenite, or cadmium induce in *C. reinhardtii* an oxidative stress, as part of its toxic effect, like it has been observed in other photosynthetic organisms, and the stimulation of antioxidant enzymes only prevent the alga from the toxic effect of the generated oxidative stress.-However this significant antioxidant effect can not prevent the high toxicity of these abiotic stresses on *C. reinhardtii*. According to this, ABA treatment enhanced 3-fold the gene expression of catalase and ascorbate peroxidase in *C. reinhardtii*, and partially released the growth suppression caused by salt stress (YOSHIDA & al. 2004). Some key proteins involved in photosynthesis and nitrate assimilation were down regulated, whereas some mitochondrial respiratory proteins were transiently up-regulated by oxidative stress in the green alga *Haematococcus pluvialis* (WANG & al. 2004). Copper also induces oxidative stress in the green alga *Chlorella vulgaris* (MALLICK 2004).

Effect of abiotic stresses on the nitrate and sulfate assimilation in *C. reinhardtii*

Salt produces in *C. reinhardtii* a significant and immediate inhibition of nitrate and sulfate uptake rates by the cells and they remain low during the 24 h of the experiment. However, the MVH-GOGAT and OASTL activities are shown high 24 h after the cells treatment with 200 mM NaCl (Table 2). These data are consistent with a high glutamate and cysteine biosynthesis required for the alga defense mechanism against salt.

Methyl viologen 0.2 µM induces in *C. reinhardtii* an immediate and drastic inhibition of nitrate and sulfate uptake rates by cells. In this case it is particularly interesting the high stimulation of NADH-GDH activity which is 12-fold higher in MV-treated cells than in the control cells, while GOGAT activity is only moderately stimulated (Table 2). These data suggest two important ideas: a) NADH-GDH may be a specific indicator for MV stress in *C. reinhardtii* and b) a specific role of aminating NADH-GDH activity in the nitrogen circulation inside the MV-stressed cells, to produce the glutamate required for defense mechanisms. Similar conclusions have been suggested in cadmium treated *C. reinhardtii* (DOMÍNGUEZ & al. 2003) and plant cells (DUBOIS & al. 2003), or when the plant is carbon limited, or under various stress conditions (MIFLIN & HABASH 2002). Methyl viologen also stimulates the OASTL activity in *C. reinhardtii*, as consequence of a high cysteine demand of cells to overcome the oxidative stress. Transgenic tobacco plants overexpressing OASTL activity showed reduced damage following MV treatment (YOUSSEFIAN & al. 2001).

Table 2. Effect of abiotic stress on nitrate and sulfate assimilation pathways in *C. reinhardtii.*

Treatment	NO_3^- uptake	GOGAT	NADH-GDH	SO_4^{-2} uptake	OASTL
Control	100	100	100	100	100
+ NaCl, 200 mM	38.9	133.8	71.9	41.7	299.0
+ MV, 0.2 µM	0.0	138.9	1,303.8	0.00	678.5
+ Arsenite, 4 mM	40.0	24.2	60.0	25.0	95.6
+ Cd^{2+} 300 µM	73.7	77.0	305.4	100	206.5

Cells growing under standard conditions were added with the indicated compounds. Periodically nitrate and sulfate remaining in the medium were determined all along the experiment for nitrate (100 % = 9.5 ± 0.8 µmol·mg $Chl^{-1}·h^{-1}$) and sulfate (100 % = 0.12 ± 0.02 µmol·mg $Chl^{-1}·h^{-1}$) uptake rates. After 24 h of growing under continuous illumination, MVH-GOGAT (100 % = 269.3 ± 5.8 mU·mg^{-1}), NADH-GDH (100 % = 18.5 ± 0.3 mU·mg^{-1}) and OASTL (100 % = 29.3 ± 0.4 mU·mg^{-1}) activities were determined in the corresponding crude extract. The data are representative of three different experiments.

After 24 h of treatment of *C. reinhardtii* with either 4 mM arsenite or 0.3 mM cadmium, the alga cells show a descreased but significant capacity of nitrate and sulfate uptake rates (Table 2). The glutamate (MVH-GOGAT and NADH-GDH activities) and cysteine (OASTL activity) biosynthesis capacity are stimulated with respect to the control in cadmium-treated, but not in arsenite-treated cells (Table 2). This indicates that in *C. reinhardtii* cadmium is more effective than arsenite in the induction of phytochelatins biosynthesis. Cd also promotes a high increase of NADH-GDH activity in parallel with an increase of the intracellular ammonium concentration in bean (GOUIA & al. 2000), and in both NADH-GDH and OASTL activities in maize leaves (ASTOLFI & al. 2004).

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