Effect of abiotic stress on *Chlamydomonas acidophila* viability

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We isolated from the acidic mining water of Tinto river (Huelva, Spain) a *Chlamydomonas acidophila*, which can grow autotrophically, at pH 2.5, with nitrate and sulfate as unique N and S source, respectively. The algae has an unusually high intracellular activity of glutathione reductase (493.85 ± 5.2 mU·mg^{-1}) and ascorbate peroxidase (1,301.60 ± 20.5 mU·mg^{1}), which suppose a high protection against oxidative stress and thus against abiotic stresses.

Cd^{2+}, at 50 μM, in the culture medium results very toxic for the alga growth and inhibits nitrate uptake rate, even when the metal intracellular concentration was low (0.10 % of dry weight). The metal also induces the inactivation of the MVH- nitrite reductase (about 60 %), MVH-glutamate synthase (about 50 %), O-acetyl-serine-thiol-lyase (about 64 %), catalase (about 25 %), glutathione reductase (about 25 %), and ascorbate peroxidase (about 50 %) activities, after 24 h of treatment. The NADH-glutamate dehydrogenase activity remains unchanged.

However, *C. acidophila* can grow very well in the presence of Fe^{3+}, 1 mM, and it may resist even 5 mM of iron in the medium, when EDTA was simultaneously present. Under such condition the intracellular concentration of iron was 2.6 ± 0.06 % of dry weight.The presence of salt, 150 mM, in the culture medium inhibits the algae growth and nitrate uptake rate as well, however it does not afect the photosynthetic or respiratory activities. The algae may accumulate Na^{+} until 1.30 ± 0.01 % of dry weight.

Key words: abiotic stress, *Chlamydomonas acidophila*, oxidative stress, nitrate metabolism.

Introduction

Photosynthetic chain supplies metabolic energy and redox power for CO_2, nitrate and sulfate assimilation by microalgae, thus a healthy photosynthetic activity is critical for the alga adequate nutrition. Under normal growth conditions reactive oxygen species (ROS), like singlet oxygen, superoxide radical, peroxide and hydroxyl radical are formed at low rate in photosynthetic cells as by-products of aerobic metabolism, but many stresses can produce a dramatic increase in the ROS production rate, being the chloroplasts, the major ROS source in the leaf cells and algae [1]. An efficient removal of ROS from chloroplasts is critical since H_2O_2 concentrations, as low as 10 μM, can inhibit photosynthesis by 50 % [2]. The chloroplast antioxidant system involves enzymes like superoxide dismutase (EC 1.15.1.1), ascorbate peroxidase (EC 1.11.1.11) and glutathione reductase (EC 1.6.4.2). Catalase (EC 1.11.1.6) is able to scavenge large quantities of H_2O_2, but its location outside the chloroplasts limits its protective effect on photosynthesis [3].

Definition of "heavy metal" is that referring to all chemical elements with a density greater than 5 g·ml^{-1}, and there is about 40 elements in this category [4]. These metals are wide spread in the rocks, but the increase of industrial activity is raising the amounts of heavy metals in the biosphere and reaching easily the aquatic ecosystems [5; 6], and thus the human food chain.

Soil salinity is the main reason for limiting plant crop, and this affects to 1,000 millions Ha [7]. It is important to know how the photosynthetic organisms re-establish osmotic and ionic homeostasis after

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salt stress imposition and what biochemical mechanisms switch on to resists this adverse condition, in order to select the best adapted organisms. Besides, salt inhibits CO_2, nitrate and sulfate assimilation, thus increasing the interaction of O_2 with the photosynthetic electron flow, which rise the alga ROS production. The unicellular green alga *Dunaliella* is a dominant photosynthetic organism in many saline environments, because she responds to salt stress by massive accumulation of glycerol (an osmolite) and carotenoid (an antioxidant), elimination of Na^+ ions and increasing the photosynthetic CO_2 assimilation [8; 9]. In addition 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 antioxidant enzyme activities [10].

Extremophiles are organisms that live at extremes pH, temperature, pressure, salinity or high concentration of heavy metals or other recalcitrant substances [11]. They are valuable organisms to study the origin of life and also its direct application in biotechnological processes which may involve the organisms themselves or their biomolecules (e.g. enzymes). The Tinto river water is very acidic and highly contaminated with heavy metals and sulfur, derived from mine activities. We isolated a *Chlamydomonas* type microalgae and uses in this work in order to know how heavy metals and salt afect its viability when growing in axenic cultures.

Materials and Methods

Culture conditions and biomass production

In this work we used *C. acidophila*, a microalga isolated from the Tinto river (Huelva, Spain), which pH ranges is 1.7-3.1 all along the year. The aquatic alga environment was highly contaminated by heavy metals, particularly magnesium (40-45 mM), iron and aluminum (both of them were about 10-15 mM), as consequence of the industrial activities in the area, and also contains a high concentration of sulfur (about 0.1 M). The alga was grown in climatized rooms, at 25 ºC using 1L bottles containing 500 ml of liquid medium with 22.6 mM nitrate and 22.6 mM sulfate, as N and S sources, respectively [12]. The cultures were bubbled with air supplemented with 5 % (v/v) CO_2 and continuously illuminated with white light from fluorescent tubes, 200 $\mu E \cdot m^{-2}$, at the surface of the tube. Under such conditions the generation time for *C. acidophila* was about 60 h. The growth rate was determined by measuring the chlorophyll content in 1 ml of culture at different times. Biomass production (productivity) was usually determined by the dry weight contained in 300 ml of alga culture, at the end of the experiment.

The growth of *C. reinhardtii* and experiments related are described in Mosulén *et al.* [13].

Measurements of photosynthesis and respiration in C. acidophila

Photosynthetic light response curves were determined including in the electrode chamber 2 ml of the *C. acidophila* culture (about 20-25 μg $Chl \cdot ml^{-1}$) treated as indicated in each case. The reaction chamber was illuminated with 1,500 $\mu E \cdot m^{-2}$ of actinic light provided by a tungsten lamp. Respiration was determined in the same sample by darkening the chamber containing cells. In both cases, changes in oxygen concentration were monitored at 25 ºC with a Clark-type O_2 electrode.

Crude extract preparation

For crude extract preparation the cells were broken in a vibration homogenator with glass beads of 0.25-0.30 mm diameter in the presence of 50 mM phosphate buffer, pH 7.5 (1 g cells/5 ml beads/1 ml buffer). The mixture was submited to high vibration during 3 periods of 1 min each and keeping the temperature to 0º C. The broken material was extracted with the buffer (5 ml/g of cells) and filtered through four cheese-cloth layers and the homogenate was centrifuged at 27,000 x g during 15 min. The supernatant was used as crude extract.

Enzyme assay

Catalase activity (CAT) was determined *in vivo,* at 25 ºC, 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. acidophila* 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 (APX) activity was determined spectrophotometrically by measuring, at 290 nm (ξ = 2.8 $mM^{-1} \cdot cm^{-1}$) and 25 ºC, 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 *et al.* [14].

O-acetylserine (thiol)lyase (OASTL, EC 2.5.1.47) activity was determined as indicated previously [15]. Total glutamate synthase activity (MVH-GOGAT), includes Fd-GOGAT (EC, 1.4.7.1) plus NADH-GOGAT (EC 1.4.1.14), was determined with dithionite-reduced MV as electron donor as indicated by Devriese *et al.* [16]. NADH-glutamate dehydrogenase (GDH, EC 1.4.1.2) activity was measured at 30 ºC 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 [17].

Analytical determinations

Chlorophyll was extracted with absolute methanol and quantified as peviously described [12]. Protein was determined by the method of Bradford [18] 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 (ε = 7 $mM^{-1}.cm^{-1}$) [16]. 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^+, Cd^{2+} and total iron determinations, the dryed 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 were determined in the dissolved samples using an inductive coupling plasma spectrophotometer (ICP), mod Fisons 3410, at wavelength of 588.99; 226.502 and 259.953 nm, respectively.

Statistics

The are representative of different experiments and standard deviations use to be lower than 3%.

Results and discussion

Effect of cadmium on the C. acidophila viability

Cadmium, at 50 µM, in the culture medium was toxic for *C. acidophila* and partially inhibits the enzymes of the cell antioxidant system, like catalase, glutathione reductase and ascorbate peroxidase, but the levels of these enzymes still remains high enough, as compared with those observed in *C. reinhardtii*, to protect the alga.

Table 1. Effect of Cd^{2+} on the antioxidant system of *Chlamydomonas* sp. Cells were grown under standard conditions and at zero time $CdCl_2$ was added as indicated. The enzymatic activities were

measured after 24 h treatment using crude extracts obtained from aliquots of the corresponding culture. Productivity (mg of alga dry weigth·ml^{-1} culture) and the intracellular concentration of Cd^{2+} (% of alga dry weight) were determined at the end of the experiment. The data are representative of three different experiments. More details in materials and methods.

Biologic Parameter	*C. reinhardtii* Control	*C. reinhardtii* + Cd^{2+}, 300 μM	*C. acidophila* Control	*C. acidophila* + Cd^{2+}, 50 μM
Productivity	1.75	1.31	1.70	0.41
GR (mU·mg^{-1})	167.40	75.00	493.85	357.37
APX (mU·mg^{-1})	99.00	141.57	1301.60	689.80
CAT (U·mg^{-1} Chl)	18.00	40.10	32.80	24.70
Intracellular [Cd^{2+}]	0.00	0.90	0.00	0.10

The amount of Cd^{2+} accumulated by *C. acidophila* was very low, 0.1 ± 0.003, as compared with *C. reinhardtii*, 0.90 % ± 0.02 (Table 1) and the marine microalga *Tetraselmis suecica*, which may accumulate 60% of the external cadmium [19]. These data indicate that *C. acidophila* protects herself by avoiding the entrance of Cd^{2+} and keeping high levels of antioxidant activities, by contrast, it seems more efficient the protection system developed by *C. reinhardtii*, which can accumulated relatively high amounts of Cd^{2+}, previously complexed with phytochelatins, in her vacuole [20]. In this case Cd^{2+} reached toxic efectivity when was present in the medium at 300 μM. Cadmium ion has influence on the O_2-evolving photosystem II (PSII) and on the photosynthetic carboxylating enzymes ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoenolpyruvate carboxylase [21, 22], as well as on nitrate and sulfate assimilation [13].

On behalf of nitrate and sulfate metabolism Cd^{+2}, 50 μM, inhibits the activities MVH-GOGAT and OASTL, thus decreasing the ability of *C. acidophila* to produce glutamate and cysteine, which is a different situation from that occurred in *C. reinhardtii* (Table 2), where glutamate and cysteine are needed to produce the required phytochelatins [20].

Table 2. Effect of cadmium on nitrate metabolism in C*hlamydomonas sp*. Cells were grown under standard conditions and at zero time $CdCl_2$ was added as indicated. Periodically the nitrate remaining in the medium was determined all along the experiment, for nitrate uptake rate (μmol·h^{-1}·mg Chl^{-1}) measurement. After 24 h of growing under continuous illumination, the enzymatic activities (mU·mg^{-1} protein) were determined in the corresponding crude extract. The data are representative of three different experiments. More details in materials and methods.

Biologic Parameter	*C. reinhardtii* Control	*C. reinhardtii* + Cd^{2+}, 300 μM	*C. acidophila* Control	*C. acidophila* + Cd^{2+}, 50 μM
NO_3^- uptake	8.66	7.07	2.10	0.57
MVH-NiR	260.0	253.20	71.45	22.49
MVH-GOGAT	269.30	207.36	467.16	244.76
NADH-GOGAT	18.50	56.20	2.12	2.00
OASTL	29.30	60.36	13.89	5.02

These data supports the idea that *C. acidophila* lakcs of the effective phytochelatins complexing mechanism to detoxify the Cd^{2+} effect, and this is the reason of her high sensitivity to Cd^{2+}. Other eukaryotic algae protect themselves against Cd^{2+} by complexing the metal with phytochelatins and confining it inside the vacuoles, like *Chlorella fusca* [23], or inside the chloroplast, like *Euglena gracilis* [24]. Not many information concerning extremophiles are in the literature about Cd^{2+} resistant mechanism. However, an inhibitory level of 0.1 mM Cd^{2+} in *C. acidophila* was reverted by increasing iron concentration up to 90-180 mM, resulting in a recovery of the alga growth rates in artificial media as described Aguilera and Amils (2005) [25]

An important stress factor in rio Tinto water is the high concentration of iron. *C. acidophila* is particularly resitant to iron in the culture medium, because it may resists even 5 mM iron, in the presence of EDTA, and the alga may accumulate iron until 2.60 % ± 0.06 of dry weight (Table 3), thus indicating

why this alga is a dominant phytoplankton specie in acidic environments contaminated with heavy metals. In addition *C. acidophila* can be used in aquatic iron phytoremediation.

Table 3. Effect of iron on C. acidophila viability. Fe3Cl, was added (zero time) as indicated to cells growing under standard conditions. Periodically the nitrate remaining in the medium was determined all along the experiment. Cell productivity and intracellular iron concentration were determined as indicated in Material and methods. The data are representative of three different experiments.

Treatment	Productivity (mg dry weight·ml^{-1})	NO_3^- uptake rate (μmol·h^{-1}·mg Chl^{-1})	Intracellular [Fe] (% alga dry weight)
Control (Fe^{2+}, 9 μM)	2.20	1.80	0.02
+ Fe^{3+}, 0.5 mM	2.76	2.28	0.51
+ Fe^{3+}, 1.0 mM	3.28	1.45	1.13
+ (Fe^{3+}- EDTA), 1 mM	2.55	1.77	0.66
+ (Fe^{3+}- EDTA), 2.5 mM	2.0	1.88	2.03
+ (Fe^{3+}- EDTA), 5.0 mM	1.30	1.30	2.60

Although relatively little is know about iron assimilation in green alga, Eckhardt and Buckhout [26] reported that *C. reinhardtii* cells reduce Fe(III)-chelates with a plasma membrane reductase to Fe(II), and then was transported through the membrane. The cells may accumulate a total of about 0.9 μmol Fe·mg^{-1} Chl [27], about 0.3-0.5 % on dry weight base. A *C. acidophila* isolated from acidic mining lakes in the Lusatia region of Germany has multiple adaptation mechanisms to increase tolerance to metals, like high accumulation of heat-shock proteins (Hsps) and/or decreased accumulation of Zn and Fe resulting from increased adsorption [28].

Most algae and plants can be adapted to moderate salinities, but their growth is seriously limited above 200 mM. The productivity of *C. acidophila* was significantly inhibited by 130 mM salt (Table 3). The photosynthetic and respiratory activities of *C. acidophila* were 5 times lower than those observed in *C. reinhardtii*, which may explain the high generation time of *C. acidophila*, 60 h, as compared with that of *C. reinhardtii*, about 8 h. Salinity stress leads in *C. reinhardtii* to a serie of changes in basic biosynthetic functions like photosynthesis and nitrate uptake rate, however in *C. acidophila* only nitrate uptake rate, but not photosynthetic activity, was significantly inhibited by salt (Table 4), even if this alga accumulated Na^+ at 1.30 ± 0.01 % of dry weight, as compared with the 0.36 ± 0.01 % of *C. reinhardtii*.

Table 4. Salt effect on *Chlamydomonas* sp. viability. When indicated, NaCl was added (zero time) to cultures growing at the logaritmic phase. Productivity: mg (alga dry weigth)·ml^{-1} culture; NO_3^- uptake rate: μmol·h^{-1}·mg^{-1} Chl; Photosynthetic and Respiratory activities: μmol O_2 evolved·h^{-1}·mg^{-1} Chl; and intracellular [Na^+]: % of alga dry weight were determined as above indicated. The data are representative of three different experiments.

Biologic Parameter	*C. reinhardtii* Control	*C. reinhardtii* + NaCl, 200 μM	*C. acidophila* Control	*C. acidophila* + NaCl, 130 μM
Productivity	1.83	1.02	1.70	0.60
NO_3^- uptake	8.33	3.92	2.47	0.61
Photosynthesis	407.03	134.70	58.22	64.35
Respiration	52.41	40.50	6.41	7.90
Intracellular [Na^+]	0.01	0.36	0.00	1.30

In general salt stress produces inactivation of photosynthesis in plants [29], and also induces apoptosis in *Anabaena* [30]. The mechanims of plant adaptation to saline medium are: a) the accumulation of compatible low molecular weight organic solvents, like sugars, aminoacids, or ammonium derived metabolites [31]; b) the activation of ionic chanels in order to reorganize the metabolite trafic throught the membrane [32]; c) overexpression of antioxidant enzymes, like SOD and APX [33]. Previous data indicate that saline toxicity in *C. reinhardtti* is mediated through oxidative etress [34].

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