The Effect of the Herbicide Glufosinate (BASTA) on Astaxanthin Accumulation in the Green Alga *Haematococcus pluvialis*

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The addition of 2.5 mM glufosinate ammonium (BASTA), a well known plant killer, to *Haematococcus pluvialis* culture efficiently inhibits cell growth, blocks the activity of glutamine synthetase (GS) and induces astaxanthin accumulation. Conversely, methionine-S-sulfoximine (MSX), a well known GS inhibitor, had no effect on neither these parameters. When GS activity was tested *in vitro*, MSX inhibited the activity at high concentrations (mM), while glufosinate was effective in the μM range. We have found that in the presence of glufosinate, ammonia is excreted from the cells. Therefore, we suggest that this process enables *Haematococcus* cells to escape the potentially harmful effect of glufosinate. As a consequence of the inability to assimilate nitrogen, astaxanthin is accumulated. This situation resembles the response of *Haematococcus* cells to nitrogen starvation.

Introduction

Herbicides are agrochemicals used to control weeds during crop growth . Essentially, they are enzyme inhibitors affecting basic metabolic reactions essential for plant life (Böger and Sandmann, 1998). A prominent group include compounds which block ammonia assimilation via their inhibition of glutamine synthetase (GS), the key enzyme for ammonia assimilation (Miflin and Lea, 1977). An example of such inhibitor is glufosinate also called phosphinothricin, or BASTA in its commercial form (Bayer *et al.*, 1972). This glutamate analog acting competitively *vs.* the natural substrate as a transient state inhibitor finally binds covalently to the enzyme at a 1:1 ratio. In bacteria and plants, inhibition of glutamine synthetase leads to the intracellular accumulation of ammonia originating from either exogenous nitrate reduction or from endogenous catabolic sources. This accumulation results in toxic effects which may eventually cause cell death (Bartsch and Tebbe, 1989; Lea and Ridley, 1989; Leason *et al.*, 1982). The herbicidal action of glufosinate depends on an efficient inhibition of ammonia assimilation. The compound was reported to be less efficient in some algae compared with plants (Altenburger *et al.*, 1995). In these cases, the induction of a special transporter was suggested to be required to translocate the herbicide inside the cell in order to inactivate GS. With *Chlamydomonas* , the remarkable resistance to glufosinate was attributed to its poor permeability, relative to methionine-S-sulfoximine (MSX), a GS inhibitor highly effective in this alga (Franco *et al.*, 1996a).

In this communication we report that in the unicellular green alga *Haematococcus pluvialis* glufosinate efficiently inhibits cell growth, induces astaxanthin accumulation and blocks the activity of GS in cultures and in cell-free extracts. We provide evidence that in the presence of glufosinate, ammonia may be extruded from the algae. We suggest that the latter process enables *Haematococcus* cells to escape the detrimental effect of glufosinate, a well known plant killer (Tachibana and Kaneko, 1986; Tachibana *et al.*, 1986).
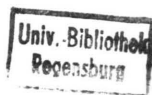
Materials and Methods

Materials

Haematococcus pluvialis Flotow (Chlorophyceae, order Volvocales) was obtained from the

Scandinavia Culture Center for Algae and Protozoa (SCCAP) at the University of Copenhagen, Denmark.

The source of glufosinate [D, L- 2-amino-4 (hydroxymethylphosphinyl) butanoic acid] in this study was a sample of BASTA (ammonium salt), at a nominal concentration of 500 mg/ml (calculated as 2.5 M) kindly provided by AgrEvo, Germany.

Growth conditions

The algae were cultivated in a modified BG_{11} medium (m-BG_{11}, containing nitrate 18 meq/l as the sole nitrogen source) as described previously (Lu *et al.*, 1998) in 500 ml sterilized columns placed in a transparent Plexiglas circulating water bath. Water temperature was maintained at 28 °C. Light was supplied at a photon flux density of 85 μmol·m^{-2}·s^{-1}. Continuous aeration was provided by bubbling air containing 1.5% CO_2. Under these conditions the pH was maintained between 6.5 to 7.0.

To test the effect of glufosinate or MSX on *Haematococcus*, the herbicide was added to a final concentration of 0.5 mg/ml to logarithmic growing cultures (2–2.5x10^5 cells/ml). Nitrogen starved cultures were obtained by resuspending logarithmic growing cells into fresh medium lacking any source of inorganic nitrogen.

Measurements of growth parameters

Cell number was determined with a Thomas blood cell counter. Chlorophyll and astaxanthin were extracted with dimethylsulfoxide (DMSO) as described previously (Boussiba and Vonshak, 1991). The absorbance of chlorophyll was determined at 666 nm (Seely *et al.*, 1972). The absorbance of astaxanthin was determined at 486 nm, after destroying chlorophyll, and calculated according to Davies (1976).

Glutamine synthetase activity

Aliquots of 50–200 ml cultures were harvested in appropriate timing, resuspended in 5 ml of 20 mM tris[hydroxymethyl]-aminomethane hydrochloride pH 7.0 buffer and homogenized via a French Press cell (AMINCO, Silver Spring, Maryland) at a pressure of 18,000 psi. Under these con-

ditions more than 95% of the cells were broken. The resulting extract was used as a source of the GS enzyme and the activity was determined (in aliquots of 0.5 ml) by the transferase assay where the formation of γ-glutamyl hydroxamate is measured (Sampio *et al.*, 1979). Protein was determined after Lowry *et al.* (1951).

Ammonia and nitrate determination

Aliquots of the growth medium were analyzed for ammonia and nitrate by the Nessler method, as modified by Abeliovich and Azov (1979), and the Szechrome NAS method (Shilo and Rimon, 1982), respectively.

Results and Discussion

When added to logarithmic cultures at a nominal concentration of 2.5 mM the herbicide glufosinate drastically reduced cell division (Fig. 1A) to the level observed with no added nitrogen source. Microscopic observation and cell counting showed no sign of cell death or lysis even after 5 days in the presence of the inhibitor. Furthermore, while the biosynthesis of chlorophyll proceeded normally in the control culture, there was no sign of chlorophyll breakdown in the culture treated with glufosinate (data not shown). A lower concentration of glufosinate (1 mM) had no effect on the doubling time of the culture, nor on it's chlorophyll content (not shown). MSX, a classic GS inhibitor, had effect on neither growth nor chlorophyll parameters, when tested at the concentration which was found inhibitory for glufosinate (2.5 mM).

A most striking effect of this herbicide in *Haematococcus* was its effect on astaxanthin accumulation. In the presence of glufosinate, green *Haematococcus* cells transformed into red cysts and accumulated astaxanthin to the same extent as the ones lacking nitrogen in the growth medium (Fig. 1B). In accordance with the results for growth and chlorophyll content, a lower concentration (1 mM) of the herbicide did not promote astaxanthin accumulation, neither did MSX added at 2.5 mM.

We previously proposed that environmental stress (e.g. nutrient deprivation, salt, high light, etc.) impairing cell division promotes such transformation from green to red cells (Boussiba and
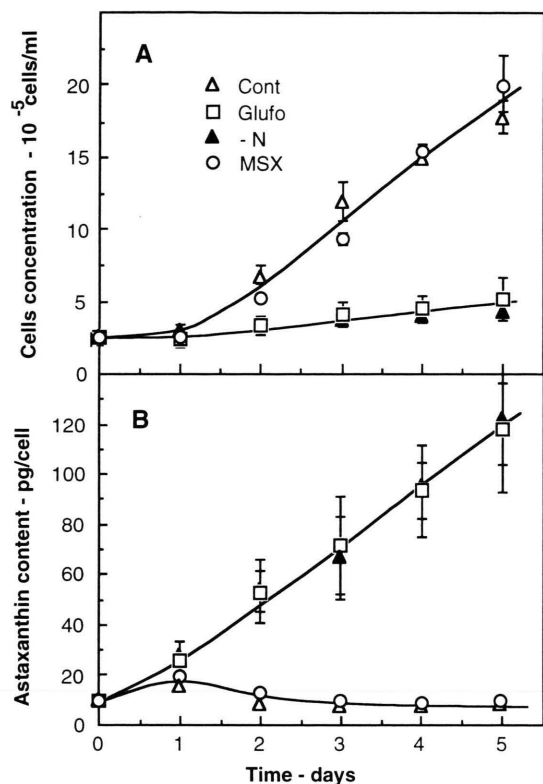
Fig. 1. Effect of glufosinate, methionine-S-sulfoximine (MSX) or nitrogen deprivation on cell growth and astaxanthin accumulation. Cells were grown in m-BG$_{11}$ medium (Cont) and analyzed for proliferation (A, upper panel) or astaxanthin content (B, lower panel) as described under Materials and Methods. Parallel cultures from the same inoculum were grown in the medium devoid of nitrogen (-N), in the presence of 2.5 mM glufosinate (Glufo), or the same concentration of MSX. The results are given as averages and standard errors of three experiments.

Vonshak, 1991; Boussiba *et al.*, 1992). In our view, inhibition of GS by glufosinate is metabolically equivalent to nitrogen deprivation, despite the abundance of nitrogen in the medium which cannot be utilized. Accordingly, this situation may eventually lead to astaxanthin accumulation. Our results demonstrate that in the green alga *Haematococcus,* the addition of glufosinate, while halting growth, did not cause cell death as was observed in higher plants (Böger and Sandmann, 1998), and even allowed active biosynthesis of astaxanthin.

It is interesting to note that the herbicide sensitivity profile of *Haematococcus*, i.e. resistance to MSX and sensitivity to glufosinate, opposite to

that described for *Chlamydomonas* (Franco *et al.*, 1996a), is similar to that described in plants (Lea and Ridley, 1989), except that it doesn't kill the alga.

There are a few possible mechanisms which may confer resistance to GS inhibitors in auxotrophs: (i) the herbicide is poorly permeable (Altenburger *et al.*, 1995; Franco *et al.*, 1996b), (ii) the inhibition of GS occurs, but intracellular accumulation of ammonia to toxic levels is prevented. In our case, growth is inhibited without reaching cell death, ruling out the first possibility and supporting the second. We propose that *Haematococcus* cells could escape the toxic effect of glufosinate due to their ability to excrete excess endogenously generated ammonia.

In Fig. 2, the effect of glufosinate on GS activity in a crude protein extract from mid-logarithmic cultures of *Haematococcus* was tested in comparison with MSX, which had no effect on the algae in
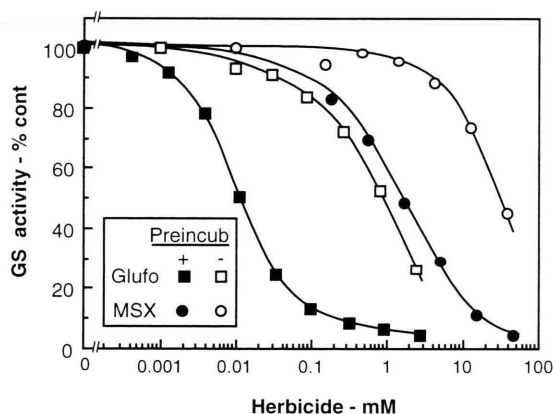

Fig. 2. Effect of glufosinate or methionine-S-sulfoximine (MSX) on glutamine synthetase (GS) activity in cells extract. Cell free extract was prepared from green cells (4 days old culture, 2.5 10^5 cell/ml) as described under Materials and Methods. GS activity in aliquots (1.1 mg prot) was assayed directly (open symbols) in reaction mixes (0.5 ml) containing the indicated concentrations of glufosinate (Glufo), or MSX for 15 min at room temperature in the presence of ammonium kept at a constant concentration (2.5 meq/l). The latter had no effect by itself on the initial GS activity (0.66 mM/min). In a second experiment, aliquots (1.1 mg prot) were preincubated with glufosinate or MSX under identical conditions in GS assay mixes devoid of glutamine. After 15 minutes the assay was started by the addition of glutamine (to reach 30 mM, the concentration in the standard assay) and stopped after additional 15 minutes (closed symbols). Preincubation with NH$_4$Cl alone did not affect GS activity in the control (0.44 mM/min).

culture. The results indicate a prominently higher efficiency (two orders of magnitude) in the inhibition of GS for glufosinate over MSX when the compounds were added to the extract in the presence of the substrates. However, after a short preincubation of the extract with the herbicides in the absence of glutamine, an additional dramatic decrease in the effective concentration for inhibition was observed. This change, reflecting the irreversible inhibition of GS by both the compounds, made the advantage of glufosinate over MSX more pronounced, yielding an effective concentration for glufosinate in the range of 10 μM. These results provide the molecular basis for the failure of MSX to provoke a physiological response in *Haematococcus* cells. To the best of our knowledge this is the first reported case for straightforward resistance of eukaryotic glutamine synthetase to MSX in cultures and in cell-free systems.

Ammonia has been shown to down-regulate GS in cyanobacteria (Boussiba, 1989; Merida *et al.*, 1990) and microalgae (Maurin and Le Gal, 1997). Since we used ammonium glufosinate in this study, it is possible that some of the observed effects could have been caused by ammonia itself. We therefore reassessed the effect of the herbicide in comparison to control cultures grown in the presence of ammonia. The results presented in Fig. 3 show that even in the presence of excess nitrate, ammonia is effectively assimilated in the control cells before nitrate, and with no significant effect on their growth as reported previously (Boussiba and Gibson, 1991). However in the presence of glufosinate, ammonia is first taken up from the medium, but this trend is subsequently reversed and its concentration increases back to its initial value within 3 days of the treatment, during which no cell proliferation is observed (Fig. 1A) and the green cells to red cysts transformation occurs (Fig. 1B).

In a similar experiment, aliquots of cells grown in the presence of ammonium glufosinate or NH_4Cl were withdrawn, and analyzed for GS specific activity in cell free extracts, as well as for ammonia concentration in the medium. The results presented in Fig. 4 indicate that a strong decrease in GS activity is observed after about two hours in both the control and treated cells before a significant uptake of ammonia is detectable. The GS in-
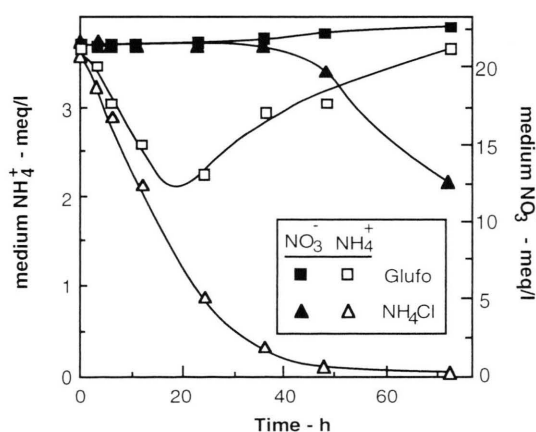

Fig. 3. Effect of glufosinate on ammonium and nitrate transport. Cells were grown in m-BG$_{11}$ medium supplemented with 20 mM Na-2-[N-morpholino]ethanesulfonate, pH 6.7, as described under Materials and Methods. The cultures contained in addition 0.5 mg/ml glufosinate (3.6 meq/l in NH_4^+, Glufo) or 3.6 mM NH_4Cl (NH_4^+). At the indicated time, aliquots were withdrawn, centrifuged to remove the cells, and analyzed for ammonium (open symbols) and nitrate (closed symbols) content in the medium.
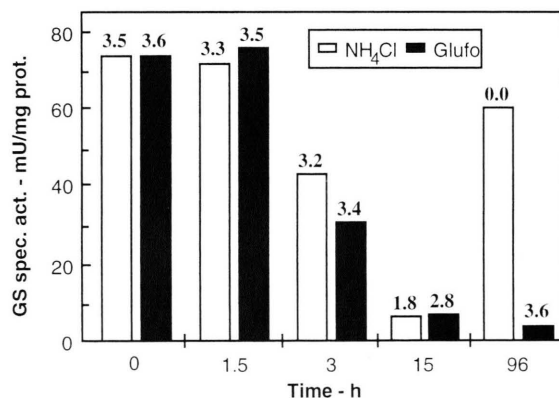

Fig. 4. Effect of glufosinate on cellular glutamine synthetase (GS) activity and extracellular ammonium. Cells were grown in m-BG$_{11}$ medium supplemented with 20 mM Na-2-[N-morpholino]ethanesulfonate, pH 6.7, in the presence of ammonium chloride or ammonium glufosinate, as described in Fig. 3. At the indicated time, aliquots containing about 50–75 10^6 cells were removed and centrifuged to separate the cells from the medium, in which the ammonium content was analyzed as described in Fig. 3. After cell disruption, crude extracts were assayed for GS activity and protein content as described in Fig. 2. The results for GS specific activity (1 mU = 1 nmol product/min) are presented as a histogram (open bars, NH_4Cl; closed bars, Glufo), and the corresponding ammonia concentrations in the medium (mM) are given as numbers above the bars.

hibition is complete after 15 hours, while more than 50% of the added ammonia is still present in the medium in both cases. However, while a full recovery of GS activity is observed in the green proliferating control cells after 4 days, it remains inhibited in the treated algae which underwent a green to red transformation. During that time the concentration of ammonia in the medium of the treated cells has been restored to its initial value. The occurrence of this process was reproducible in three separate experiments, as well in another one performed in the absence of nitrate (not shown). However, the kinetics and extent of the transient uptake of ammonia were variable, reflecting a delicate balance between the ability for ammonia uptake and assimilation, and the inhibitory effect of glufosinate. While the mechanism of the transient GS repression in the presence of ammonia alone remains to be resolved, one could safely conclude that the net effect of glufosinate is twofold: (i) the herbicide does inhibit rapidly and permanently GS activity *in vivo*, as well as *in vitro* and (ii) as a result of the consequent inhibition of nitrogen assimilation, ammonia is excreted back to the medium with relatively slower kinetics.

The excretion of ammonia following GS inactivation is a well known phenomenon in algae (Hipkin *et al.* 1982; Florencio and Vega, 1983; Boussiba *et al.*, 1984; Zimmerman and Boussiba, 1986). In our experiments, most of the excreted ammonia found in the external medium was equivalent to that added together with glufosinate. In some cases however, the final concentration of ammonia in the medium exceeded this value by up to 40%. Since nitrate was not utilized in the presence of glufosinate (Fig. 3), the extra ammonia could have been generated by protein breakdown or photorespiration as previously reported (Florencio and Vega, 1983; Boussiba *et al.*, 1984).

In conclusion, the main cellular target of glufosinate (BASTA) is the same in *Haematococcus* and in higher plants. The herbicide abolishes GS activity, resulting in ammonia accumulation. In *Haematococcus* the excretion of this ammonia relieves the cells from its toxic side effects, while in higher plants the accumulated ammonia causes cell deterioration and death.

Acknowledgements

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