# Interference of herbicides with $CF_0$ of spinach chloroplast $H^+$ -ATPase

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## ABSTRACT

Acetochlor and propachlor from acetanilide group, glufosinate from amino acid synthesis inhibitors group, and cycloate from thiocarbamate group have been found to act as an inhibitors of photophosphorylation in spinach chloroplast. ATP synthesis and phosphorylating electron flows as well as light-activated membrane-bound $Mg^{2+}$ -ATPase were inhibited by all compounds without affecting basal and uncoupled electron transports and heat-activated $Ca^{2+}$ -dependent ATPase activity of isolated coupling factor protein. Thylakoids partially stripped of coupling factor by EDTA were unable to accumulate protons in the light. However, increasing concentrations of acetochlor, propachlor, glufosinate, and cycloate restored this ability. It is concluded that the four compounds effects result from blocking proton transport through the $CF_0$ channel. These results suggest that the herbicides, access inhibition sites in $CF_0$.

## INTRODUCTION

ATP synthesis from ADP and phosphate in chloroplasts is catalyzed by the $H^+$ -ATPase associated with the thylakoid membranes. Its stucture is bipartite with a membrane embedded, proton-conducting portion, $F_0$, and a peripheral portion, $F_1$, which protrudes from the membrane toward the stroma. One of the main experimental approaches to study the mechanism of photophosphorylation is the use of specific inhibitors that affect the light-dependent ATP synthesis in chloroplasts by blocking electron transport coupled to ATP synthesis or by directly inhibiting photophosphorylation reactions. This latter inhibition (energy transfer inhibition) may be localized at $CF_0$ (proton channel of ATP synthase) or at $CF_1$ (chloroplast coupling factor 1). For example, phlorizin, DIO-9, and N-ethylmaleimide, appear to inhibit $CF_1$ (Izawa *et al.* 1966; McCarty *et al.* 1965; McCarty and Racker 1968). However, dicyclohexylcarbodiimide (DCCD), triphenyltin chloride, and 5-0-β-D-galactopyranosyl-7-methoxy-3', 4' –dihydroxy-4-phenylcoumarin seem to interact with $CF_0$ (McCarty *et al.* 1965; Gould, 1976; Calera *et al.* 1995).

Moreover, in the chloroplast $H^+$ -ATPase comlex, the membrane sector is composed of four subunits I-IV. A major constituent of $CF_0$ is a hydrophobic protein of about 8 Kda, component III, commonly referred to as a proteolipid, which is the binding site of DCCD (McCarty and Racker 1968).

There are many examples of herbicidal interaction with the structure and function characteristics of plant chloroplasts (Yanase *et al.* 1990 ; Trebst and Draber 1992). Nevertheless, the effects of herbicides on plant cell appear to be complex and their mode of action is not completely established. For this purpose, the aim of the present study was to evaluate the effect of herbicides on several photosynthetic activities including ATP synthesis, electron flow (basal, phosphorylating, and uncoupled), chloroplast $Mg^+$ -ATPase, chloroplast coupling factor 1 ($CF_1$), and proton channel of ATP synthase ($CF_0$) in isolated spinach chloroplasts.

## MATERIALS AND METHODS

### Tested Herbicides

Pure (99.9%) Acetochlor and propachlor (Acetanilide group), glufosinate (Amino acid synthesis inhibitors group), and cycloate (Thiocarbamate group) were purchased from Zeneca Agrochemicals Co., UK. For all assays one millimolar stock solutions were made for all compounds. Solution of glufosinate was prepared in distilled water. Solutions of acetechlor, propachlor, and cycloate were made in pure ethanol and the final concentration of ethanol did not exceed 1%.

### Chloroplast Isolation, Chlorophyll Determination

Chloroplasts were isolated from market spinach leaves (*Spinacea oleracea* L.) as previously described by Strain *et al.* 1971 and suspended in 400 mM sucrose, 5 mM $MgCl_2$, 10 mM KCl, and 0.03 M $K^+$ -tricine (pH 8.0). The chlorophyll concentration was measured, as previously reported by Achnine *et al.* 1999

### Measurement of ATP synthesis

ATP synthesis was measured as the pH rise between 8.0 and 8.1 as described by Macias *et al.*1999. The pH changes were registered using a Gilson recorder. The ATP synthesis reaction medium contained 100 mM sucrose, 5 mM $Mg^+Cl_2.6H_20$, 10 mM KCl, and 1 mM and $K^+$ -tricine (pH 8.0) in the presence of 1 mM ADP and 3 mM $KH_2P0_4$ (Mills *et al.* 1980). In the assay media given above, 100 µM methylviologen (MV) was used as electron acceptor.

### Measurement of non-cyclic electron transport rate

Photosynthetic non-cyclic electron flow from water to methylviologen was determined with a yellow Spring Instrument Model 5300 oxygen monitor and a Clark-type electrode. The basal electron transport reaction medium was the same as in the ATP synthesis assay, except for the tricine concentration (15 mM) and the absence of ADP and $KH_2P0_4$. For the uncoupled electron transport measurement, 6 mM $NH_4Cl$ was added to the

basal electron transport medium. For the phosphorylating electron transport medium, 1 mM ADP and 3 mM $KH_2P0_4$ were added to the basal electron transport medium. The mixture reactions were illuminated for 1 min. as reported by Macias *et al.*1999.

### $Mg^{2+}$ - and $Ca^{2+}$ -ATPase and EDTA-treated Chloroplast Assays

$Mg^{2+}$ -ATPase activity bound to thylakoid membranes was measured according to the technique reported by Mills *et al.* 1980 .To obtain $CF_1$-depleted chloroplasts and solubilized $CF_1$, an aliquot of fresh chloroplasts was diluted with 0.75 mM ethylenediaminetetraacetic acid (EDTA) pH 7.6 and incubated for 10 min at 20 °C. $CF_1$ –depleted membranes were then removed by centrifugation. Of this EDTA extract (containing $CF_1$ complex), 0.5 ml were added to 0.5 ml of 20 mM tricine (pH 8.0), o.2 mM EDTA, 10 mM dithiothreitol (DTT), and 40 mM ATP, and heated at 60 °C for 4 min. Of this activated mixture, 0.1 ml was incubated for 20 min at 37 °C with 0.9 ml of a medium containing 50 mM Tris, pH 8.4, 5 mM Ca $Cl_2$, and 5 mM ATP according to Achnine *et al.* 1999). Reaction was stopped with 2% trichloroacetic acid (TCA) and inorganic phosphate was determined as previously described by Taussky and Shorr 1953. Protein was detemined by the method of Lowry *et al.* 1951, using bovine serum albumin (BSA) as a standard.

### The Washing Procedure of EDTA-Treated Thylakoids

The washing procedure of $CF_1$-depleted chloroplasts was repeated four times as described by Gould 1976. A small aliquot (200 µg chlorophyll) was assayed for proton uptake measured as the pH rise between 8.0 and 8.1 according to Dilley 1972. The reaction medium contained 100 mM sucrose, 2.5 mM NaCl, 2 mM $MgCl_2$, 100 µM methylviologen, and 0.5 mM Hepes-KOH (pH 7.5). The reaction was measured as the restoration of $H^+$ -uptake in the presence of the herbicides. All biochemicals were purchased from Sigma Chemical Co. St. Louis, Missouri. The results are presented as mean values (±SD) of measurements made with chloroplast preparations from four independent isolates.

## RESULTS AND DISCUSSION

### Effects of acetochlor, propachlor, glufosinate, and cycloate on ATP synthesis and electron transport

Photosynthetic photophosphorylation coupled to electron transfer from water to methylviologen in freshly lysed spinach chloroplasts was inhibited by the herbicides acetochlor, propachlor, glufosinate, and cycloate (92, 86, 82, and 84%, respectively, at 200 µM) (Table 1).

**Table 1: ATP synthesis as a function of concentrations of acetochlor, propachlor, glufosinate, and cycloate.**

| Activity (%) | | | | | |
|---|---|---|---|---|---|
| Compound Conc. (µM) | | | | | |
| Tested Compounds | 0 | 50 | 100 | 150 | 200 |
| Acetochlor | 100±2 | 64±2 | 28±3 | 8±2 | 8±2 |
| Propachlor | 100±3 | 72±3 | 38±2 | 14±2 | 14±2 |
| Glufosinate | 100±3 | 78±2 | 40±4 | 18±3 | 18±2 |
| Cycloate | 100±2 | 68±2 | 36±3 | 16±2 | 16±2 |

Details of the experiments are given in Materials and Methods. The control rate value for ATP synthesis was 950 µmol. $h^{-1}$. mg $Chl.^{-1}$. Each point represents the mean of four determination, ±SE.

The light-dependent formation of ATP can be inhibited by either blockage of the electron transport, direct inhibition of the $H^+$ -ATPase complex, or uncoupling of ATP synthesis process from the electron transport (Good *et al.* 1981). To know the effect of acetochlor, propachlor, glufosinate, and cycloate on ATP synthesis, its mechanism of action on various electron transport activities was determined. The results show that phosphorylating electron flow was inhibited by all compounds (Table 2). However, basal and uncoupled electron flows (5 mM ammonium chloride) from water to methylviologen were unaffected by these compounds (Tables 3, and 4).

**Table 2: Effects of acetochlor, propachlor, glufosinate, and cycloate on phosphorylating electron transport from water to methylviologen in freshly lysed spinach chloroplasts.**

| Activity (%) | | | | | |
|---|---|---|---|---|---|
| Compound Conc. (µM) | | | | | |
| Tested Compounds | 0 | 50 | 100 | 150 | 200 |
| Acetochlor | 100±3 | 66±4 | 42±3 | 27±2 | 27±3 |
| Propachlor | 100±3 | 68±2 | 45±4 | 32±2 | 32±5 |
| Glufosinate | 100±2 | 74±4 | 60±5 | 38±2 | 38±3 |
| Cycloate | 100±2 | 75±3 | 60±4 | 44±4 | 44±2 |

Details of the experiments are given in Materials and Methods. Control value was 500 µequiv. $e^-$ .$h^{-1}$ . mg of Chl. $^{-1}$. Each point represents the mean of four determination, ±SE.

**Table 3: Effects of acetochlor, propachlor, glufosinate, and cycloate on basal electron transport from water to methylviologen in freshly lysed spinach chloroplasts.**

| Activity (%) | | | | | |
|---|---|---|---|---|---|
| Compound Conc. (µM) | | | | | |
| Tested Compounds | 0 | 50 | 100 | 150 | 200 |
| Acetochlor | 100±2 | 100±4 | 100±2 | 100±3 | 100±2 |
| Propachlor | 100±4 | 100±3 | 99±2 | 100±4 | 99±2 |
| Glufosinate | 100±3 | 98±3 | 99±4 | 98±2 | 97±4 |
| Cycloate | 100±2 | 99±2 | 98±3 | 97±2 | 99±2 |

Details of the experiments are given in Materials and Methods. Control value was 400 µequiv. $e^-$ .$h^{-1}$ . mg of Chl. $^{-1}$. Each point represents the mean of four determination, ±SE.

**Table 4: Effects of acetochlor, propachlor, glufosinate, and cycloate on uncoupled electron transport from water to methylviologen in freshly lysed spinach chloroplasts.**

| Activity (%) | | | | | |
|---|---|---|---|---|---|
| Compound Conc. (µM) | | | | | |
| Tested Compounds | 0 | 50 | 100 | 150 | 200 |
| Acetochlor | 100±3 | 100±2 | 99±2 | 100±4 | 99±3 |
| Propachlor | 99±2 | 100±2 | 97±3 | 100±2 | 98±2 |
| Glufosinate | 98±2 | 99±4 | 99±2 | 99±3 | 100±3 |
| Cycloate | 99±2 | 99±3 | 99±2 | 99±3 | 99±3 |

Details of the experiments are given in Materials and Methods. Control value was 912 µequiv. $e^-$ .$h^{-1}$ . mg of Chl. $^{-1}$. Each point represents the mean of four determination, ±SE.

The light-dependent membrane bound $Mg^{2+}$ -ATPase was also inhibited in a concentration-dependent manner with acetochlor, propachlor, glufosinate, and cycloate (94, 92, 86, and 88%, respectively, at 200 µM) (Table 5). Therefore, these compounds act as an energy transfer inhibitors. This behavior is similar to that found for energy transfer inhibitors like DCCD (McCarty and Racker 1968); DIO-9 (McCarty *et al.* 1965); Phlorizin (Izawa *et al.* 1966) ; and triphenyltin (Gould 1976).

**Table 5: Effects at different concentrations of acetochlor, propachlor, glufosinate, and cycloate on the light-activated membrane-bound $Mg^{2+}$ -ATPase and the heat-activated $Ca^{2+}$ -ATPase activity of purified coupling factor 1 of chloroplasts**

| Compounds | $Mg^{2+}$ -ATPase (%) | $Ca^{2+}$ -ATPase (%) |
|---|---|---|
| Acetochlor (µM) | | |
| None | 100±2 | 100±4 |
| 50 | 24±2 | 98±3 |
| 100 | 10±0.12 | 97±2 |
| 150 | 8±0.12 | 95±2 |
| 200 | 6±0.11 | 96±3 |
| Propachlor (µM) | | |
| None | 100±3 | 100±5 |
| 50 | 32±2 | 96±2 |
| 100 | 18±0.14 | 97±4 |
| 150 | 9±0.12 | 98±3 |
| 200 | 8±0.14 | 96±2 |
| Glufosinate (µM) | | |
| None | 100±4 | 100±4 |
| 50 | 36±4 | 99±3 |
| 100 | 24±2 | 97±2 |
| 150 | 14±0.11 | 98±4 |
| 200 | 14±0.12 | 99±2 |
| Cycloate (µM) | | |
| None | 100±5 | 100±6 |
| 50 | 33±2 | 100±4 |
| 100 | 22±2 | 98±2 |
| 150 | 12±0.14 | 97±2 |
| 200 | 12±0.11 | 96±3 |

Details of the experiments are given in Materials and Methods. Control values for $Mg^{2+}$ - and $Ca^{2+}$ -dependent ATPases were 125 µmoles Pi released/mg Chl. h. and 186 µmoles Pi/mg protein., respectively. Each point represents the mean of four determination, ±SE.

To localize the action site of the four compounds on the $H^+$ -ATPase complex, its effect on $CF_1$ isolated from EDTA-treated thylakoids were assayed. Table 5 shows that increasing concentrations of these compounds did not affect the $Ca^{2+}$ -ATPase activity. These results indicate that the four compounds do not interfere with $CF_1$ when acting as an energy transfer inhibitors.

To localize further the target of these compounds on $H^+$ -ATPase complex, its effect on the EDTA-treated thylakoids was studied. It is known that the removal of $CF_1$ from thylakoid membranes results in an enhancement of proton permeability (Neumann and Jagendrof 1964). Moreover, certain energy transfer inhibitors such as DCCD and triphenyltin interact with $CF_0$ and decrease the rate constant of proton efflux (McCarty and Racker 1967; Gould 1976). The proton uptake of thylakoids partially stripped of $CF_1$ can be restored by adding $CF_1$ (Klein-Hitpass and Berzborn 1984) or by adding DCCD (Gould 1976). Table 6 illustrates that increased concentrations of the four compounds restore the light dependent pH-rise to a suspension of EDTA-washed chloroplasts, as does triphenyltin, and DCCD. Therefore, acetochlor, propachlor, glufosinate, and cycloate inhibit photophosphorylation by blocking the $CF_0$ channel.

**Table 6: Restoration of light-driven proton uptake in EDTA-washed chloroplasts at different concentrations of acetochlor, propachlor, glufosinate, and cycloate.**

| % of Restoration | | | | | |
|---|---|---|---|---|---|
| Compound Conc. (µM) | | | | | |
| Tested Compounds | 0 | 50 | 100 | 150 | 200 |
| Acetochlor | 100±3 | 138±4 | 167±8 | 175±6 | 176±5 |
| Propachlor | 100±2 | 134±6 | 164±6 | 173±8 | 174±6 |
| Glufosinate | 100±2 | 128±5 | 160±5 | 170±6 | 170±8 |
| Cycloate | 100±2 | 122±8 | 160±4 | 168±7 | 168±6 |

Details of the experiments are given in Materials and Methods. Control value for proton uptake was 1.20 µequiv. $H^+$/ mg of Chl. Each point represents the mean of four determination, ±SE.

Therefore, it can be concluded from the present study that herbicides can act on the $CF_0$ target in plat chloroplasts. This interaction may constitute an essential mechanism of their mode of action in plant.

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## الملخص العربى

# تداخل مبيدات الحشائش مع الجزء الغير ذائب والناقل للبروتونات من انزيم الأدينوزين ثالث الفوسفاتيز فى كلوروبلاستيدات أوراق نبات السبانخ

شفيقة أحمد الكسباتى

المعمل المركزى للمبيدات-مركز البحوث الزراعية-الصبحية-الاسكندرية

وجد أن مبيدات الحشائش الأسيتوكلور والبروباكلور من مجموعة الأسيتانيليدات و مبيد الجليوفوسينات من مجموعة مثبطات الأحماض الأمينية و مبيد السيكلوات من مجموعة الثيوكاربامات تثبط عملية الفسفرة الضوئية فى أغشية كلوروبلاستيدات أوراق نبات السبانخ.

وقد أوضحت النتائج أن المركبات الأربعة تثبط تخليق مادة الأدينوزين ثالث الفوسفات وتدفق ألكترونات الفسفرة الضوئية فى النظام الضوئى و انزيم الأدينوزين ثالث الفوسفاتيز المعتمد فى نشاطة على أيونات الماغنسيوم ولكنها لا تؤثر على كل من أنسياب الألكترونات الأساسية و عملية سير الأنتقال الألكترونى بصورة منتظمة وانزيم الأدينوزين ثالث الفوسفاتيز المعتمد فى نشاطة على أيونات الكالسيوم. وكما وجد ان المركبات الأربعة تؤثر على الجزء الغير ذائب و الناقل للبروتونات من انزيم الأدينوزين ثالث الفوسفاتيز بصورة واضحة.

ومن هذا يتضح ان الجزء الغير ذائب من الأنزيم يعتبر هدف هام للمبيات السابقة فى النبات.