Effects of Glufosinate on Anion Uptake in *Lemna gibba* G 1

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The duckweed *Lemna gibba* G 1 was used as a model to study inhibitory sites with the herbicide and glutamate analogue glufosinate (PPT). Growth and chlorophyll formation were partly inhibited by 25 μM, completely suppressed by 250 μM PPT. Photosynthesis showed partial inhibition within few hours, dark respiration (O_2 consumption) increased already within one hour. In the presence of 1 mM PPT in the light, the ammonium pool of *Lemna* increased to 600% within few hours, later to 1000%. The overall amino acid pool exhibited a slower increase to 300%, the nitrate pool only a slight increase, while total phosphate remained almost unchanged. In the dark all these effects were less pronounced than in the light. Nitrate, nitrite and phosphate uptake were partially inhibited by PPT, especially after 19 h PPT pretreatment. Nitrate reductase activity *in vitro*, after PPT treatment *in vivo*, showed an inhibition similar to that of nitrate uptake. Ammonium was not taken up but released under the same conditions.

The data are explained by a combined effect of PPT, by inhibition of glutamine synthetase leading to accumulation of ammonium from photorespiration and proteolysis, by membrane depolarization and inhibition of anion/proton cotransport, by secondary uncoupling of phosphorylation, and by secondary inhibition of nitrate reductase activity.

Introduction

Herbicides usually have a wide range of phytotoxic activities and varying specificity that should be known for adequate use. Glufosinate (PPT) has been reported so far to be relatively specific for the inhibition of glutamine synthetase. Thereby it causes strong ammonium accumulation which, in its part, will uncouple photophosphorylation and thus lead to metabolic breakdown of the plants [1]. PPT effects concerning ammonium accumulation, photosynthesis and isolated glutamine synthetase have been carefully studied with several cultivated plants [1−3].

In a preceding study [4] we reported on PPT uptake and on membrane effects linked to its uptake in *Lemna*. PPT was assumed to initially use the same amino acid carrier system as glutamate for influx, but it showed a secondary and irreversible interference with the electrical and mainly the transport proper-

ties of the plasmalemma [4]. These observations indicated another field of herbicidal action and encouraged us to the present study, in which effects on pool sizes and uptake of ammonium, nitrate, nitrite, and phosphate were investigated in comparison with those on photosynthesis, dark respiration and nitrate reductase activity.

Materials and Methods

Plants

Lemna gibba L., strain G 1 from the *Lemna* collection of Prof. R. Kandeler, Vienna, was cultured axenically in a medium containing 3.96 mM KNO_3, 5.47 mM $CaCl_2$, 1.22 mM $MgSO_4$, 1.47 mM KH_2PO_4, 18 μM Fe-EDTA, 8.1 μM H_3BO_3, 1.5 μM $MnCl_2$, 0.5 μM Na_2MoO_4, pH 4.8, and 29 mM sucrose as a carbon source. Prior to the experiments, the plants were kept for 10 days in a sucrose-free medium under the CO_2 of the air and at pH 6.5. The photoperiod was 8 h light to 16 h dark at 31 and 24 °C, respectively. Prior to nitrate or nitrite uptake experiments, the plants were N-starved for 5 days and finally "induced" by addition of 40 μM NO_3^- 16 h before the experiment started. Prior to phosphate uptake experiments the plants were kept on a phosphate-free medium for 12 days.

Experimental solutions

The basic experimental solution contained 4.65 mM KCl, 1.2 mM CaSO$_4$ and 10 mM MES and was buffered to pH 6.5 with KOH. For uptake experiments the respective anions were added as potassium salts; 0.1 mM NH$_4$Cl was added in ammonium experiments. For following the pool sizes of nitrogen compounds and phosphate, the basic solution was supplied with 0.1 mM KNO$_3$ + 0.05 mM KH$_2$PO$_4$. PPT concentrations are indicated at the figures.

Methods

Uptake rates were determined from concentration differences in the medium related to zero time, pool sizes in extracts obtained from 1 g of *Lemna* plants homogenized with 5 ml H$_2$O. The final volume of 10 ml extract was boiled for 7 min, centrifuged at 40,000 × g for 15 min.

Nitrate in the medium was determined after reduction with hydrazine sulfate [5]. Nitrate pool sizes in plant material were assayed with the salicylic acid method [6]. Phosphate in the medium was determined by formation of phosphomolybdate and reduction with ascorbic acid [7], in plant material total phosphate was assayed after acid digestion according to [8]. Ammonium was determined with the phenol blue method [9] in supernatant as well as in plant material. Evolution and uptake of O$_2$ in photosynthesis and respiration were measured manometrically using carbonate-bicarbonate buffers and carbonic anhydrase for constant CO$_2$ levels in photosynthesis.

Nitrate reductase activity was assayed in extracts: after treatment with or without PPT, 0.7 g of the plant material were frozen with liquid N$_2$ and homogenized in a microdismembrator (Braun, Melsungen, F.R.G.). The resulting powder was suspended in 5 ml extraction buffer [10] containing 0.25 M Tris-HCl, pH 8.5, 3 mM dithiothreitol, 1 mM EDTA, 1 μM Na$_2$MoO$_4$ and 5 μM FAD. Phenolic compounds were eliminated by immediately adding one tenth of the plant weight of polyvinyl pyrrolidone (Polyclar AT) and thoroughly stirring with an ultrasonic homogenizer. The resulting sap was centrifuged at 40,000 × g for 15 min. The supernatant served as crude enzyme preparation and was stable at 0 °C for several weeks.

The enzyme assay was performed in 2.1 ml reaction mixture containing: 60 mM phosphate buffer, pH 7.4, 28 mM KNO$_3$, 0.4 mM NADH, 13 mM MgSO$_4$ and 0.4 ml of the enzyme preparation. The reaction was started by adding the enzyme and was run at 30 °C for 15 min in a water bath. It was stopped by adding the reagent mixture for nitrite determination.

Amino acids were assayed using the overall reaction with ninhydrin [11], protein according to Lowry *et al.* [12], chlorophyll following extraction with hot 95% methanol [13].

All data shown in this paper are mean values of at least 3 experiments with 2 or 3 parallel samples each.

Results

Growth and chlorophyll content

Development and growth of new fronds of *Lemna gibba* started to become inhibited at about 25 μM PPT. They were completely suppressed by 250 μM or more. At 50 μM PPT or more the usual small colonies started to fall apart after 24 h, at high concentrations already after 5 h. Visible chlorosis developed after 48 h with 500 μM, after several days at low concentrations. This is also expressed in the chlorophyll content per fresh weight after 5 days in PPT as a % of the control samples (Table I).

Photosynthesis and respiration

Similarly to the observation by Wild *et al.* [1], photosynthetic O$_2$ evolution of *Lemna* was inhibited

Table I. Inhibition of growth and chlorophyll formation by glufosinate (PPT) in *L. gibba*. Values in % of control, or in days or hours. (−N) = N-free culture without PPT; nd = not determined. Experimental period: 5 days.

μM PPT	0	(−N)	10	25	50	100	250	500
Frond number increase (%)	100	50	110	95	80	10	0	0
Colony separation	−	−	−	−	±	+	+	+
Chlorosis visible after	−	−	5 d	5 d	5 d	65 h	52 h	48 h
Chlorophyll content after 5 days	100	nd	85	85	80	73	52	50

by PPT, at higher concentrations already within the first hour, but never completely (Fig. 1), in contrast to young *Sinapis* plants [1]. Respiratory O_2 consumption responded contrarily to photosynthesis. More rapidly than the latter, dark respiration increased after pretreatment with 1 mM PPT to about 200% but then remained constant for many hours (Fig. 1).

Effect on pool sizes

It is well known from previous reports that, by inhibition of glutamine synthetase, PPT causes a considerable accumulation of ammonium which, in its part, seems to be the main reason for the breakdown of photosynthesis [1, 3]. In *Lemna* ammonium showed a drastic increase by PPT in the light, far beyond the normal ammonium level, and reached 600% of the control within a few hours with 1 mM PPT. In the light-dark cycle after a relatively constant value for over 40 h another rise occurred to 1000% (Fig. 2 A). In the dark ammonium accumulation was similarly rapid, but in continuous dark the level scarcely exceeded 200% of the control values (Fig. 2 B). Although inhibition of glutamine synthetase would prevent ammonium metabolization and formation of amino groups, the total level of amino acids increased during PPT incubation, probably due to protein hydrolysis which could not be overcome by synthesis under those conditions. The nitrate pool responded only in the light and after
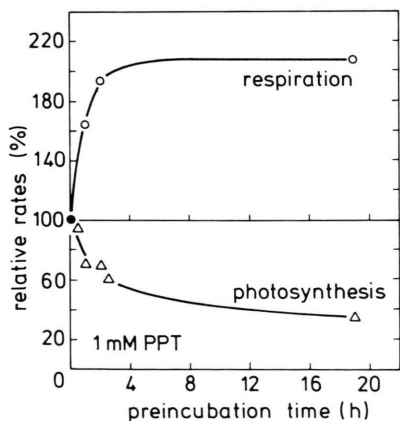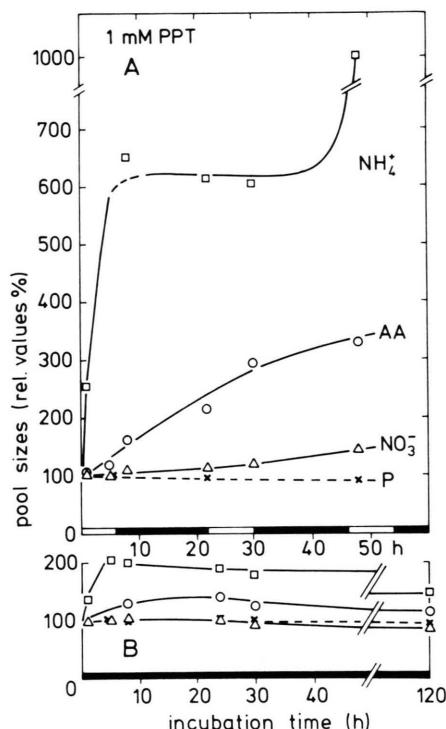

Fig. 2. Changes in pool sizes of NH_4^+, NO_3^-, total P and total amino acids by 1 mM PPT in the light (A) and in the dark (B). Values in % of control without PPT. Plants N-starved for 5 days, 0.1 mM KNO_3 and 0.05 mM $H_2PO_4^-$ in the experimental medium. Pool sizes of control plants (100%): 0.5 (±0.35) µmol NH_4^+ g^{-1} FW, 0.11 (±0.04) µmol NO_3^- g^{-1} FW, and ca. 45 µmol total P g^{-1} FW.

longer exposure to PPT, while total phosphate remained constant or was slightly reduced in comparison with untreated controls, probably due to inhibition of uptake and partial uncoupling of phosphorylation by ammonium.

Inhibition of ion uptake

Ion accumulation in PPT-treated *Lemna* can be due to blocking of metabolization as in the case of ammonium, or to changes in ion uptake rates. PPT had been found to induce remarkable changes in membrane properties [4], so it became important to study also fluxes of anions in the presence of the herbicide.

Transport of nitrate and nitrite was inhibited as dependent on PPT concentration and exposure time (Fig. 3 A and B). Compared with nitrate phosphate

Fig. 1. Relative rates of photosynthetic O_2 evolution and respiratory O_2 consumption after preincubation with 1 mM PPT. Control rates (100%): 150 µmol O_2 g^{-1} FW h^{-1} for photosynthesis and 8 µmol O_2 g^{-1} FW h^{-1} for respiration on average.
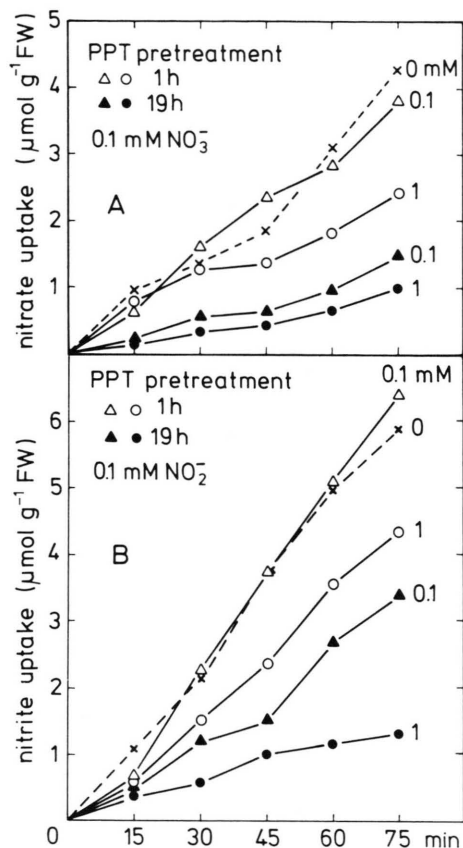
Fig. 3. Nitrate uptake (A) and nitrite uptake (B) after pretreatment with PPT in the light. PPT 0.1 or 1.0 mM, nitrate or nitrite 0.1 mM; plants N-starved for 5 days.

showed a more severe inhibition, after 19 h with 1 mM PPT even down to zero (Table II). In all cases an increase in PPT inhibition developed mainly during the light periods of the short-day rhythm.

According to the observed accumulation ammonium responded differently, it was in excess within the tissue after short incubation with PPT, and was released to the medium instead of being taken up, especially at high concentrations of and after longer exposure to PPT (Fig. 4).

Inhibition of nitrate reductase

From the above described results one should expect a decrease in the nitrate pool rather than the observed slight increase. Hence nitrate reduction might be equally or more inhibited than nitrate uptake. Actually the inhibition of nitrate reductase activity was similar to that of nitrate uptake (Table II), but nevertheless, inhibition of transport could not be explained by that of the enzyme, since the inhibition concerned also nitrite uptake and since in all cases the uptake was inhibited from the very beginning, whereas inhibition through the subsequent metabo-

Table II. Inhibition of phosphate and nitrate uptake and of nitrate reductase activity (NRA) by PPT in the light. Between 2 and 18 h dark period. Plants either N-starved for 5 days or P-starved for 10 days. Control rates (100%): 3.5 μmol NO_3^- g^{-1} FW, and 0.7 μmol $H_2PO_4^-$ g^{-1} FW on average.

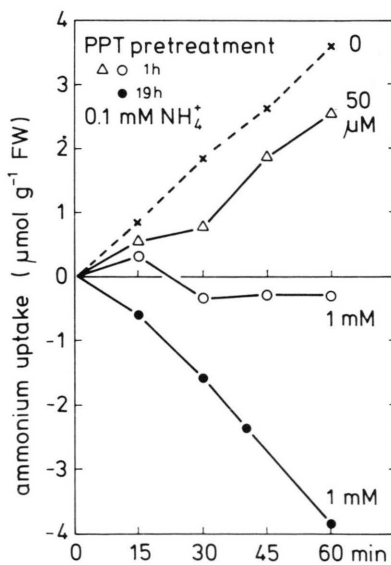| | mM PPT | Hours of preincubation | | | |
		1	2	19	24
Nitrate uptake	0.1	86	81	52	35
	1.0	53	54	34	18
Phosphate uptake	0.1	65	65	40	32
	1.0	35	33	9	0
NRA	1.0	65	61	38	23

Fig. 4. Ammonium uptake/release after pretreatment with PPT in the light. PPT 0.1 or 1.0 mM, 0.1 mM NH₄Cl; plants N-starved for 5 days.

lism would be reflected in uptake curves by changes in slopes after short periods of unaffected influx.

Discussion

The inhibitory and stimulatory effects of glufosinate reported here raise the question how far they can be explained by one single site of action, the inhibition of glutamine synthetase. According to the recent own measurements [4] at least two additional effects are involved. The results of this study seem to indicate even more sites of action.

Consequences of glutamine synthetase inhibition

Together with a poor ammonium assimilation rate *via* glutamate dehydrogenase, an enzyme that is not affected by PPT [2], inhibition of glutamine synthetase must necessarily cause a rapid and strong accumulation of ammonium. Its strong stimulation by light suggests a high contribution from photorespiration under the experimental conditions in air (0.03% CO_2), similar to accumulation and release of ammonium and glycolate in unicellular algae in the presence of methionine sulfoximine [14]. As indicated by low nitrate uptake rates and only minor changes in its pool size, the contribution from nitrate reduction is completely insufficient to explain the extent of ammonium accumulation shown in Fig. 2. In the dark a large proportion of the ammonium seems to originate from protein decomposition, as also indicated by the increase of amino acid pools in the presence of PPT in light and dark.

Ammonium is a cation. Wherever the product ammonia (NH_3) comes from, from nitrite reduction or protein hydrolysis, around or below its pK of 9.24, it will remove protons and cause an alkalinization. Moreover, in photorespiration anionic carboxyl groups are eliminated, and in *Lemna*, in contrast to unicellular algae, only a part of this alkalinizing metabolite is released to the medium (Fig. 2 and 4) [14, 15].
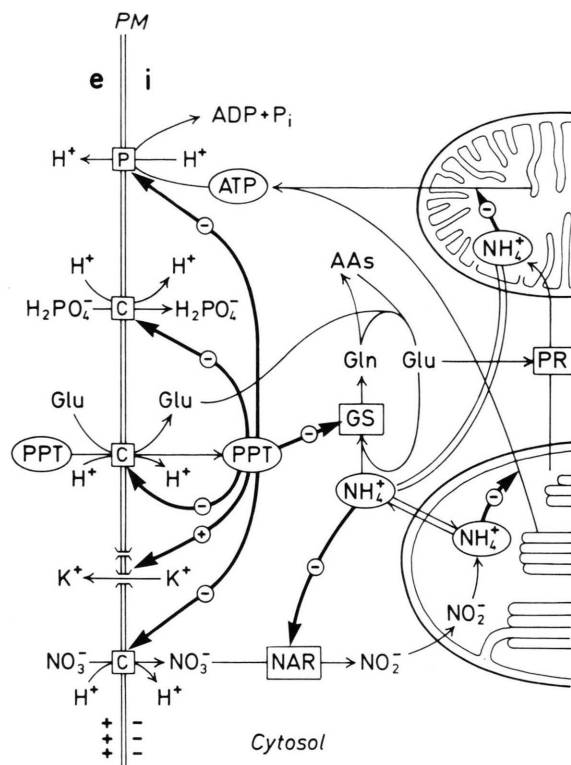
Alkalinization and uncontrolled proton consumption within the thylakoids are probably also the main reason why photosynthesis suffers inhibition from PPT. The mechanism is likely to be an indirect "uncoupling" [1, 3]. Mitochondrial respiration may be less sensitive to ammonium than photosynthesis, but much of the stimulated respiration rate may also be due to uncoupling, which in mitochondria would not easily imply breakdown of the electron flow.

Inhibition of ion transport and nitrate reductase

The uptake experiments with nitrate, nitrite and phosphate, and with ammonium show an increasing inhibition with increasing PPT concentration and exposure time. Direct inhibition of the transport is indicated by the instant effect, whereas a feedback inhibition from metabolism of the ions would result in a short initial time of unaffected transport followed by the inhibition when the cytosolic and/or vacuolar pools are saturated. In *Lemna* this saturation of nitrate and ammonium pools normally requires 15 to 20 min [16]. However, the mechanism of uptake inhibition is not revealed by such experiments. In the preceding study [4] we have shown that PPT after short exposure electrically depolarizes the plasmalemma of *Lemna* cells to an extent that it affects the K^+ equilibrium potential (E_K^+ of ca. -90 mV). This will induce K^+ efflux or even passive ion fluxes due to increased permeability [17, 18]. In addition it will essentially reduce the driving force for anion/proton cotransport as for the energy-dependent K^+/proton cotransport into the cells as supposed to be the main uptake mechanism for anions in *Lemna* [17, 19, 20] and for K^+ as shown recently in *Neurospora* [21]. Alkalinization and uncoupling by ammonium will also prevent the action of membrane ATPases and thus contribute to the decay of the electrical potential.

As shown previously [16] and in Fig. 3 and 4, reduction of E_m to E_K^+ [4] does not imply a complete inhibition of anion uptake. At E_K^+ K^+ fluxes become predominant and hide proton fluxes still driven by the ATPase. Thus H^+/anion cotransport may still proceed at a lower rate.

Simultaneous inhibition of anion transport and metabolization is indicated by the small changes in nitrate and phosphate pools in the presence of PPT when extracellular nitrate or phosphate are available (Fig. 2). Reduction of nitrate and nitrite could be independent of ATP formation, but the inhibition of nitrate reductase activity is about as strong as that of nitrate and nitrite uptake (Table II). Since extracted nitrate reductase does not show sensitivity to PPT (for *Lemna* R. Plasa, unpublished data, for *Sinapis* personal communication by A. Wild), its inhibition *in vivo* can be attributed to the high ammonium levels. Finally, a large proportion of the cellular nitrate pool is located in the vacuoles and thus only slowly accessible to metabolization. Our present

Fig. 5. Model of proposed interactions by PPT. Thick arrows indicate stimulation (+) or inhibition (−). Important substrates marked by circles, enzymes or enzyme systems by squares. K$^+$ efflux is attributed to channels. AAs = amino acid pool; c = carrier protein; e = extracellular space; GS = glutamine synthetase; i = intracellular space; NAR = nitrate reductase; P = proton ATPase; PM = plasmalemma; PR = photorespiratory chain.

knowledge about the action of PPT in *Lemna*, as taken from [4] and from the data presented here, is schematically summarized in Fig. 5.

Acknowledgement

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