Chlorophyll Proteins and Lipids in Paraquat Treated Sensitive and Resistant *Conyza Canadensis* Leaves

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Photosynthetic activity and molecular composition of thylakoid membranes of sensitive and atrazine/paraquat coresistant biotypes of *Conyza canadensis* (L.) Cronq was compared before and after 5×10^{-4} molar paraquat spraying. In contrast to the irreversibly damaged sensitive plants the Rfd values (vitality indices) calculated from parameters of fluorescence induction curves, and the *in vivo* CO_2 fixation in resistant plants showed – after a transitory inhibition – a recovery.

Resistant thylakoids contained higher amount of PS II and LHC II, (*i.e.* lower relative amount of CP I) which can be the result of an adaptation process. Fatty acid composition of total leaf extract was almost the same in both biotype even after spraying. The amount of the oligomeric form of LHCP II decreased in paraquat treated sensitive thylakoids, which was in correlation with the reduction of Δ^3-transhexadecanoic acid content. Decrease in oligomeric LHCP II and/or special lipids (PG) in sensitive thylakoids may change the association of LHCP II and photosystem II core as it is evidenced from lowering of the Mg^{2+} induced changes of the short wavelength fluorescence intensity and increase in the relative quantum requirement values. The results are discussed in connection with a possible effect of paraquat on PS II.

Introduction

Paraquat, active ingredient of nonselective contact herbicide preparations, is a well known photosynthesis inhibiting bipyridile compound. The first and characteristic effect of the bipyridylium herbicides is the disorganization of membranes. The membrane destructing effect is based on the ability of these compounds to accept electrons from photosystem I (PS I), to produce radicals and in complex series of reactions to generate reactive oxygen species as superoxide anion radical, hydroxyl radical, singlet oxygen and hydrogen peroxide. Superoxide and hydroxyl radical can directly damage membrane lipids. The amount of hydroxyl radicals in light exposed paraquat treated plants is extremely high and is quite sufficient to explain the rapid ceasing of top growth in treated plants [1].

The occurrence of paraquat resistance is well known among weeds. Up to now paraquat resistant populations of more than ten weed species has been detected [2]. The mechanism of resistance has been explained by several hypotheses. However, only two of them are supported by convincing experimental evidences. By the one the resistance is based on the enhanced activities or levels of protective antioxidant enzymes, as superoxide-dismutase, ascorbate-peroxidase, glutathione-reductase and dehydroascorbate-reductase, which enzymes in series all together are able to detoxify the various reactive oxygen forms generated by paraquat [3]. An other explanation may be that the resistance is due to the rapid sequestration of paraquat on inactive sites outside the chloroplast [4], or outside the leaf [5]. It is probable, however, that the high levels of the antioxidant enzymes protect resistant plants during the period necessary for the chemical or enzymatical degradation and/or sequestration of paraquat by some protective processes active after paraquat treatment only in the leaves of resistant biotype [6, 7].

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We have found a transitory sensitivity to paraquat followed by a rapid recovery of the photosynthetic activity in resistant plants [8]. Induced synthesis of paraquat inactivating enzyme(s) or binding protein(s) or other quicker repair mechanism can be supposed to occur, as increased activity of protective enzymes superoxide-dismutase, ascorbate-peroxidase, catalase and higher level of reduced glutathione in the total cell extracts could not be detected [9].

Although the membrane disorganizing effect of paraquat is well documented only very few detailed analysis on the membrane lipid content changes due to paraquat treatment has been reported [10]. Little is known about the effect of paraquat on the molecular organization of thylakoid membranes in sensitive and paraquat resistant plants (see *e.g.* Ref. [11]).

A comparative analysis of the photosynthetic capacities, molecular composition of chloroplast thylakoid membranes in sensitive and atrazine/paraquat coresistant biotypes of *Conyza canadensis* before and after paraquat (Gramoxone) treatment is given in the present paper in order to characterize differences accompanying resistance.

Materials and Methods

Plant material

Seeds of susceptible and resistant horseweed (*Conyza canadensis* (L.) Cronq.) plants were collected from vineyards near Kecskemét. Plants were grown in soil under laboratory conditions (illumination 130 μE m^{-2} s PAR (photosynthetically active radiation), 16 h light/8 h dark period, 22–25 °C for 3–4 months. Then the plants in soil containers were transferred to field conditions. About 6-months-old plants – between the rosette and flowering stage – were used in the experiments. The resistant plants used in these experiments showed a high atrazine resistance, too.

Paraquat treatment

Intact *Conyza* plants were sprayed with commercial formulated paraquat (Gramoxone, 25% paraquat) solution. Paraquat concentration of the spraying solution was 5×10^{-4} M. The treatment was carried out on moderate light.

Fluorescence induction measurements

Leaf samples (discs of 10 mm in diameter) were taken at different times after spraying to follow kinetics of paraquat effect. Fluorescence induction curves were measured by a newly developed apparatus (Mikrolabor Ltd., Szeged, Hungary) as described earlier [8]. The quantum flux density on the surface of sample was 185 μE m^{-2} s^{-1}. Acquisition and storage of 20 points from 0 to 2 ms (100 μs sampling intervals) ensures a clear resolution of F_O. The characteristic fluorescence parameters derived from the original induction curves are means of 12 independent determinations.

CO_2 fixation

The rate of photosynthetic CO_2 fixation was determined according to [12] by placing the leaves in atmosphere containing $^{14}CO_2$ in a closed glass chamber. The leaves were illuminated with white light of 10 W m^{-2} intensity for 2 min. Discs 5 mm in diameter were cut from the leaves, dried and placed in scintillation vials. The radioactivity of the samples was determined by liquid scintillation technique (Beckmann LS 5000TD). 40–50 discs from different treated leaves were used for each experiment.

Chlorophyll-proteins (CP-s) and thylakoid proteins

Chloroplasts and thylakoid membranes were isolated according to [13]. Thylakoids were solubilized and their CP-s were separated as in [14] except that dodecyl-maltoside (DDM) was used for solubilization (DDM/Chl ratio was 10) and gels were pre-electrophoresed with 3 mA/tube for 20 min. The Chl *a*/*b* ratios and relative proportion of CP-s were estimated from densitograms measured with a Perkin-Elmer 554 spectrophotometer equipped with a gel scanner at 653 and 672 nm [15].

For fluorescence measurements chloroplasts were resuspended (5 μg Chl/ml) in 10 mM Tricine-NaOH buffer (pH 7.8) containing 10 mM NaCl and 50 mM sorbitol. Samples were incubated for 15 min at room temperature with or without 10 mM $MgCl_2$, then fluorescence spectra were measured at 77 K with a Perkin Elmer MPF 44B spectrofluorometer. Areas under short and long wavelength bands were measured and normalized for the long wavelength fluorescence. Mg^{2+} in-

duced increase was calculated in %, the normalized F 680 of samples without Mg^{2+} was considered as 100%.

Polypeptides were separated by the discontinuous polyacrylamide gel electrophoresis (PAGE) system by [16] using, however, 10–18% gradient gels. Membranes were solubilized for 0.5–1 h at room temperature. Gel slices containing CP-s were embedded in hot 0.5% agarose in stacking gel buffer. Gels were stained with Coomassie Brillant Blue according to [17].

Fatty acid analysis

Lipids were extracted from the leaf discs with chloroform/methanol 2:1 (v/v) by the procedure of Folch *et al.* [18]. Fatty acid methylesters were prepared from aliquots of total lipids (containing 17:0 as internal standard) by esterification in the presence of 5% HCl in methanol at 80 °C in ampoules sealed under CO_2 and quantitatively determined by gas-liquid chromatography with a JEOL JGS 1100 instrument. Lipid analysis were performed in a replicated experiment on three separate paraquat treatments.

Results and Discussion

Floating of leaves on the herbicide solutions is a conventional method for investigation of herbicidal effects. However, spraying of the weeds with herbicide solution is the most usual practice in the agriculture. Under laboratory conditions the results achieved by Gramoxone (25% paraquat) treatment were markedly different depending on whether spraying or floating of the leaves was employed. In paraquat resistant *Conyza canadensis* treated with 5×10^{-4} M paraquat the transitory inhibition of photosynthesis measured as fluorescence induction kinetics was detected only in sprayed plants [8], but not in floated ones. Therefore spraying was used in the following experiments. 4 h after spraying with Gramoxone the sensitive plants were heavily damaged, strongly wiltered or dried. Similar symptoms could not be observed on resistant plants.

Changes of *in vivo* photosynthetic CO_2 fixation and Rfd values of sensitive and resistant horseweed leaves sprayed with Gramoxone are shown on Fig. 1. Rfd value (called vitality index) – *i.e.* the ratio of fluorescence decrease to the steady

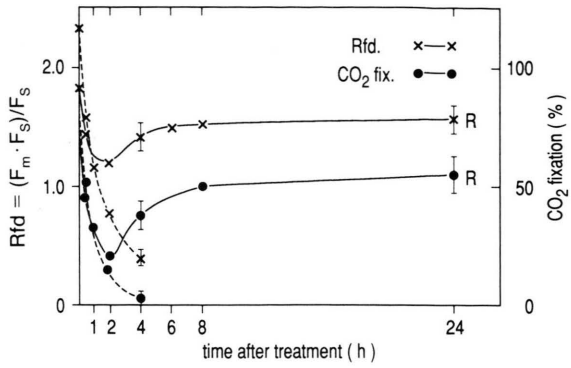

Fig. 1. Time dependence of photosynthetic activity of sensitive and resistant *Conyza canadensis* after spraying with Gramoxone (5×10^{-4} M paraquat) measured as *in vivo* CO_2 fixation and Rfd value (vitality index) calculated from parameters of fluorescence induction kinetics according to the equation: $(F_{max} - F_s)/F_s$ = Rfd.

state fluorescence $[(F_{max}-F_S)/F_S]$ – is considered to be an indicator for the potential photosynthetic activity of a leaf [19] and is extensively used in the ecophysiology [20, 21] and in other studies, using chlorophyll fluorescence induction for characterization of photosynthetic activity [22]. After a transitory inhibition the CO_2 fixation and Rfd values of resistant plants showed a recovery effect. Photosynthetic O_2 evolution measurements showed the same time course [23]. However, the functional activity of the sensitive plants was irreversibly damaged 4 h after the spraying.

Gramoxone treatment did not change the ultrastructure of chloroplasts of sensitive and resistant plants (not shown).

Lipids present in the chloroplasts contain a high percentage of polyunsaturated fatty acids and are very susceptible to peroxidation. The stimulatory effect of paraquat on lipid peroxidation is well known [24]. Floating the leaves on 5×10^{-4} M paraquat solution resulted in a decrease of linolenic acid content and an increase in the level of palmitic and linolic acid in total fatty acids of chloroplasts isolated from sensitive *Conyza canadensis* plants, while in resistant plants these effects were not observed [25]. There was no clear indication for fatty acid peroxidation in total lipid extract of sprayed sensitive or resistant plants (Table I). These results show that 4 h time period after spraying of sensitive plants is probably not long enough to cause detectable changes in the fatty acid composition of

Table I. Fatty acid content and composition of total lipids from untreated control and paraquat (5×10^{-4} M) treated (tr) sensitive (S) and resistant (R) *Conyza canadensis* leaves. Fatty acids denoted by number of carbon atoms/number of double bounds. The data are averages out of 3×2 determinations.

Sample	Total fatty acid [mg/mg Chl]	Fatty acid composition [mol %]					
		16:0	16:1	18:0	18:1	18:2	18:3
S	4.55 ± 0.40	15.9	0.2	3.3	5.0	18.4	57.2
S_{tr}	4.29 ± 0.37	15.2	0.5	2.9	5.9	19.5	56.0
R	3.99 ± 0.35	14.8	0.3	3.9	4.0	15.7	61.3
R_{tr}	4.58 ± 0.26	15.8	0.3	3.7	4.5	19.9	55.7

total leaf extract. In the leaves of the resistant biotype treated by paraquat the toxic effects did not develop even 24 h after spraying.

The investigation of chlorophyll proteins showed that the nature of CP bands – their chlorophyll *a*/*b* ratio and polypeptide pattern – was the same in sensitive and resistant plants and it did not change following the treatment (data not shown). Even their proportion was constant in untreated and treated plants (Table II).

Comparing sensitive and resistant plants, resistant thylakoids contained – on a chlorophyll basis – relative lower amount of PS I chlorophyll proteins (CP 1 and CP 1a), lower amount of the 70 kDa apoprotein (Fig. 2) and showed a less in-

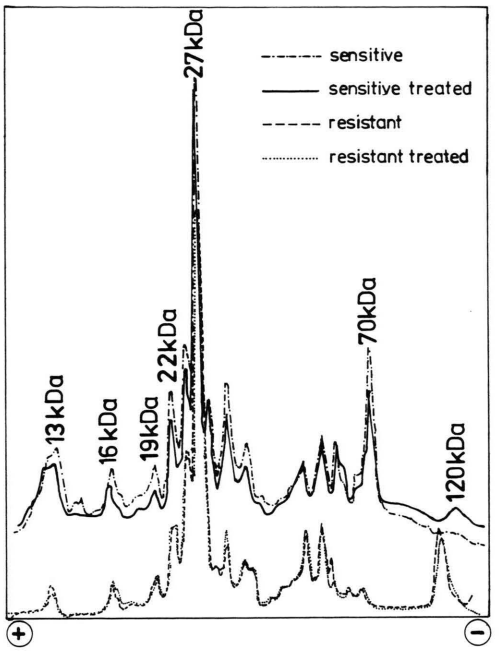

Fig. 2. Polypeptide composition of thylakoid membranes of chloroplasts isolated from untreated and Gramoxone (5×10^{-4} M paraquat) sprayed sensitive and resistant *Conyza canadensis* plants (2 h after treatment for S plants, 24 h for R plants).

Table II. Relative amount (in %) of chlorophyll proteins and indices of the amount of photosystem I in thylakoids isolated from leaves of untreated and paraquat (5×10^{-4} M) treated (tr) sensitive (S) and resistant (R) *Conyza canadensis* plants. Absolute values in μg Chl/cm² leaf surface are given in brackets. (S = 41 μg/cm², R = 48 μg/cm²). F730/F680 is the ratio of the integrated long and short wavelength fluorescences in spectra measured at 77 K. PS I (%) shows the relative amount of the 70 kDa protein among the thylakoid polypeptides.

Sample	CP 1 + CP 1a	CPa	LHCP 1–3	FP	F730/F680	PS I [%]
S	28.9 ± 5.5 (11.8)	14.7 ± 3.3 (6.0)	40.5 ± 3.3 (16.6)	13.5 ± 1.5 (5.5)	1.09 ± 0.07	11.4 ± 2.2
S_{tr}	27.9 ± 3.1 (11.5)	15.3 ± 2.8 (6.3)	40.0 ± 0.8 (16.4)	15.8 ± 3.3 (6.5)	1.04 ± 0.13	
S_{tr}/S (%)	96.5	104.1	98.8	117.0	95.4	
R	23.7 ± 5.6 (11.4)	14.5 ± 2.9 (7.0)	44.2 ± 2.9 (21.2)	15.2 ± 3.7 (7.3)	0.74 ± 0.07	8.0 ± 1.4
R_{tr}	21.5 ± 3.0 (10.3)	13.9 ± 6.0 (6.7)	42.4 ± 3.7 (20.4)	16.9 ± 2.1 (8.1)	0.80 ± 0.19	
R_{tr}/R (%)	90.7	95.8	95.9	111.2	108.1	
R/S (%)	82.0 (96.6)	98.6 (116.7)	109.1 (127.7)	112.6 (132.7)	67.9	70.2

tensive 730 nm fluorescence compared with sensitive ones. This may be the cause of the lower photosynthetic activity and lower vitality index of untreated resistant plants (Fig. 1). The same initial sensitivity of resistant plants to paraquat (see the slope of curves on Fig. 1) excludes the possibility that the lowered amount of PS I would be the cause of resistance. Indeed, in absolute terms (Table II, numbers in brackets) resistant plants contained the same amount of PS I and an increased amount of PS II and its antenna (CPa + LHCP 1–3). Therefore resistant plants seems to adapt to atrazine and/or paraquat compensating their decreased photosynthetic efficiency by increasing the amount of PS II and its antenna. Paraquat alone may also affect the activity of PS II, the most sensitive part of the electron transport chain probably *via* the generated toxic oxygen forms. Toxic oxygen species seems to contribute to the degradation process of D 1 protein [26].

Oligomeric form of LHCP ($LHCP_o$) was lowered in sensitive but not in resistant plants following the paraquat treatment (Table III). Its decrease was in good correlation with the decrease in phosphatidyl glycerol (PG) and Δ^3-*trans*-hexadecanoic acid (16:1) content of leaves. This is in agreement with the current view that Δ^3-*trans*-hexadecanoic acid is a stabilizing factor of $LHCP_o$ [27–30] but a primary effect of paraquat treatment on protein conformation cannot be excluded. PG may play a role in the core complex building and/or function, too [31].

The showed changes in the state of light-harvesting complex of PS II or those of the lipid composition (Table III) in consequence of paraquat treatment may disturbe the light-gathering process in PS II and thus may lower the photosynthetic energy transformation processes. Indeed, the Mg^{2+}-induced increase in the short wavelength part of the fluorescence spectra – *i.e.* the increase of the PS II antenna size by a tighter association of LHCP II to the reaction core – is lowered due to the paraquat treatment of the sensitive plants (Table III). This well matches with an about 1.5–2 times increase of the relative quantum requirement values in sensitive chloroplasts after paraquat treatment calculated from the light intensity curves of DCPIP reduction according to ref. [32].

In conclusion, atrazine and/or paraquat resistance was concomitant with diminishing of changes in the amounts of PG, Δ^3-*trans*-hexadecanoic acid and $LHCP_o$ under paraquat treatment. Decrease in the overall photosynthetic activity and in the light-gathering process of PS II were also diminished. However, further work is necessary to decide which molecular organizational differences are the consequences of paraquat resistance alone and/or which ones reflect a more general pattern of herbicide resistance.

Table III. Changes in the phosphatidyl glycerol (PG), Δ^3-*trans*-hexadecanoic acid (16:1) and oligomeric LHCP ($LHCP_o$) content and in the Mg^{2+}-induced increase in the short wavelength part of 77 K fluorescence spectra in sensitive (S) and resistant (R) *Conyza canadensis* plants after paraquat treatment (4 h – S_{tr}, 24 h – R_{tr} plants). The data are averages out of 3–4 × 2 determinations.

Sample	PG [μg/mg Chl]	16:1	$LHCP_o$ [%]	Mg^{2+}-induced F 680 increase [%]
S	545	70.0	45.1*	145.4*
S_{tr}	375	34.5	30.8*	89.2*
S_{tr}/S (%)	68.8	49.3	68.3*	61.3*
R	394	48.0	39.1	154.2
R_{tr}	459	53.0	43.7	160.5
R_{tr}/R (%)	116.5	110.4	111.8	104.1
R/S (%)	72.3	68.5	86.7	106.1

* Determined 2 h after paraquat treatment.

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