Phenolic Herbicides: Correlation between Lipophilicity and Increased Inhibitory Sensitivity of Thylakoids from Higher Plant Mutants

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Phenolic herbicides, being quite hydrophilic as compared to classical photosystem-II inhibitors do not exhibit a correlation between inhibitory activity and partition coefficient. Sensitivity against phenolic herbicides, however, as observed with atrazine-resistant thylakoids from higher plants mutants *(Brassica napus)* is increased by higher partition coefficients.

Introduction

Phenolic compounds, being inhibitors of photosystem II exhibit some peculiarities as compared with inhibitors like ureas or symmetric triazines [1, 2]. Inhibition needs a typical lag phase which can be alleviated by short pre-incubation in the light (or for a longer period in the dark), or by gentle trypsin treatment [3, 4]. The same holds for the binding of phenolic herbicides to thylakoids [5]. It has been claimed, although not convincingly, that at least some inhibitors of this type bind to 44- and 51-kD thylakoid peptides [6, 7], with an effect on the oxidizing side of photosystem II [8, 9]. Contrary to ureas or triazinones lipophilicity apparently does not influence their inhibitory activity [10]. Furthermore, atrazine-resistant thylakoids obtained from higher plants do not show resistance against phenol herbicides but an increased sensitivity is observed ([1, 11, 12], for cross resistance see [13]).

This communication will add additional results by comparing lipophilicity, electron transport inhibition and (increased) sensitivity toward phenolic herbicides using isolated thylakoids from atrazine-resistant *Brassica napus*.

Chemical names of herbicides used (either purchased or provided by gratuity from the companies listed): Atrazine, 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine (Ciba-Geigy AG, Switzerland); Metribuzin, 4-amino-6-*tert*-butyl-4,5-dihydro-3-methyl-thio-1,2,4-triazine-5-one (Bayer AG, Germany); Diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Riedel de Haen, Germany); DINP, 2,6-diiodo-4-nitrophenol (Riedel de Haen); DNOC, 2-methyl-4,6-dinitrophenol (Riedel de Haen); Dinoseb, 2-(1-methylpropyl)-4,6-dinitrophenol (Riedel de Haen); Dinoterb, 2-*tert*-butyl-4,6-dinitrophenol (Ehrenstorfer, Germany); Medinoterb, 6-*tert*-butyl-2,4-dinitrotoluol (Ehrenstorfer); Chloroxynil, 4-hydroxy-3,5-dichlorobenzonitrile (May & Baker, UK); Bromoxynil, 4-hydroxy-3,5-dibromobenzonitrile (May & Baker); Ioxynil, 4-hydroxy-3,5-diiodobenzonitrile (May & Baker); CL-9673, 6-chloro-3-phenyl-4-hydroxy pyridazine (Chemie Linz, Austria). This is the active metabolite (= free phenolic form) of pyridate (6-chloro-3-phenylpyridazin-4-yl-5-octylthio-carbonate.

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Materials and Methods

Cultivation

Brassica napus was grown in the greenhouse. The wild-type rape was *Brassica napus,* ssp. *sylvestris,* strain "Petranova" (summer rape). The atrazine-resistant mutant is also a summer rape, *B. napus,* strain OAC 82-1, bred by Ontario Agricultural College, Guelph, Canada (containing progenies from triazine-resistant bird rape, *B. campestris*).

Preparation of the thylakoid material

6 to 8 weeks old leaves were homogenized and the thylakoids were uncoupled by washing with pyrophosphate as published [1]. Then the pellet was washed two times with 300 mM sucrose, including 2 mM Tris/Tricine [Tris-(hydroxymethyl)aminomethane/N-[Tris(hydroxymethyl)methyl]glycine], pH 8, thereafter suspended in a mixture of 50 mM NaCl, 5 mM $MgCl_2$ containing 25 mM Tricine/NaOH, pH 8.0. All steps were carried out at 4 °C.

I_{50}-values

were determined in a system $H_2O \rightarrow$ potassium ferricyanide using a Clark-type electrode for oxygen gas exchange measurement at saturating red light [2]. Thylakoid material was equivalent to 25 µg/ml

chlorophyll. The reaction medium of 2 ml contained: 50 mM NaCl, 5 mM $MgCl_2$ and 25 mM Tricine/NaOH, pH 8.0, and 1 mM potassium ferricyanide. In the assays, the solvent was kept below 1%. It should be noted that before measurement the thylakoids were incubated with the phenolic inhibitors for 3 min at 25 °C in the dark to overcome the lag phase of inhibition (comp. [4, 5]).

Measurement of partition coefficients

$P = H_c/H_w$ [P, partition coefficient; H_c, herbicide concentration in the organic phase; H_w, herbicide concentration in the aqueous (buffer) phase] of phenolic herbicides was determined by the partition method between water and an organic phase [14, 15]. For the organic phase cyclohexane (purity grade for spectroscopy, "Uvasol", Riedel de Haen, Germany), was used, and the water phase buffered with 25 mM phosphate of pH 8.0 (organic buffers are partially transferred into the organic phase). For CL-9673, since not soluble in cyclohexane, octanol (for spectroscopy) was used as organic phase. Shaking was done in test tubes on a Vortex mixer 3 times for 15 sec, allowing for phase separation between the shaking intervals. Cyclohexane or octanol (containing 10 μM and 100 μM of the herbicide) was 1 ml, the water phase 10 ml. Unlabelled derivates were determined spectroscopically (Shimadzu UV-300) by measuring the difference of the herbicide concentration in the clear cyclohexane phase before and after shaking. With ^{14}C-labelled ioxynil, bromoxynil and CL-9673 the spectroscopic determination was checked by scintillation counting of the organic phase (Rack-Beta II LSC, from LKB-Wallace, Turku, Finland) and identical partition coefficients were obtained.

Each experiment, including the preparation of thylakoids, was repeated 3 times. Unless mentioned otherwise, chemicals, analytical grade, were purchased from Merck, Darmstadt, Germany.

Results and Discussion

The Table shows the experimentally determined partition coefficients *(P)* for the phenolic herbicides assayed in this study. Furthermore the I_{50}-values to inhibit photosynthetic electron transport are listed as well as the "resistance factors" (*R/S*-ratio) using an atrazine-resistant strain of *Brassica napus*. As checked for dinoterb, CL-9673 and bromoxynil the same trend of I_{50}-values *vs.* P was found using thylakoids isolated from *Chenopodium album*.

Table. Partition coefficients of phenolic inhibitors pI_{50}-values, and resistance factors of *Brassica napus*, wild-type *vs.* mutant thylakoids.

Herbicides	P (partition coefficient)	pI_{50} wild-type	pI_{50} mutant	*R/S* ratio*
Dinoterb	4.87	5.42	6.56	0.071
Dinoseb	1.45	5.30	6.22	0.12
Medinoterb	0.89	5.79	6.48	0.20
CL-9673	0.72	4.11	4.93	0.15
DNOC	0.245	4.57	5.23	0.22
DINP	0.168	6.48	6.79	0.48
Ioxynil	0.0316	6.57	6.92	0.45
Bromoxynil	0.026	5.17	5.32	0.71
Chloroxynil	0.0155	4.17	4.24	0.85
Metribuzin	50**	7.25	5.00	181
Atrazine	400**	6.40	3.92	300
Diuron	480**	6.96	6.88	1.2

* = Ratio I_{50} mutant/I_{50}, wild-type ("resistance factor") determined by inhibition of photosynthetic electron transport of isolated thylakoids.
** Ref. [17].

Evidently the pI_{50}-values, both of wild-type and mutant thylakoids do not correlate with the partition coefficient. For example medinoterb with a $P = 0.89$ and dinoterb with $P = 4.9$, both exhibit about the same I_{50}-value for the wild-type chloroplasts. This finding corroborates a previous report on (wild-type)

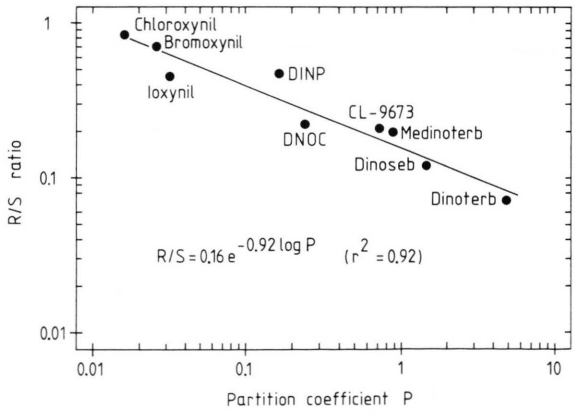

Fig. 1. Correlation between the *R/S*-ratio (resistance factor defined as I_{50}-value, mutant thylakoid per I_{50}-value, wild-type thylakoid) and the partition coefficient for various phenolic inhibitors. The double-logarithmic plot yielded a straight line as indicated by the equation.

spinach chloroplast [10]. Although the three benzonitriles tested in this study may implicate a correlation with lipophilicity no convincing relation could be found neither in our experiments nor in a recent study using various nitrile analogs [16].

With the compounds listed partition coefficients between 0.016 and 4.9 were determined, all yielding resistance factors below 1, that means increased sensitivity of mutant thylakoids against these phenolic compounds. Interestingly, the *R*/*S*-ratio, that is I_{50}-value of mutant thylakoids divided by that of the wild-type thylakoids, is positively correlated with the partition coefficient. The relationship is linear in a double-logarithmic plot (Fig. 1), and the corresponding equation as given in the figure was obtained by a curve-fitting program including all experimental values (comp. ref. [17]).

Using chloroplasts from *Amaranthus retroflexus* it has been noted previously that increasing lipophilicity counteracts resistance against triazinone derivatives [12] or ureas [18]. As further shown by the latter study with *Brassica* chloroplasts, this effect is not due to an altered fatty-acid composition. As not documented, the number of binding sites per chlorophyll (about 1 to 500 chlorophyll molecules) remained the same applying either (labelled) ioxynil, bromoxynil, CL-9673 or classical inhibitors like diuron, atrazine or metribuzin to isolated thylakoids from either wild-type or mutant.

It should be mentioned that the pk_a of the phenolic compounds used here, is about 5. That implies that at pH 8, as used for the assays, more than 99.9% of the herbicide molecules are present in the ionic form. As demonstrated by Fig. 2 longer pre-incubation times (35 min) were needed to obtain full inhibition with ioxynil at pH 8. At pH 6.5 full inhibition was observed immediately. Using longer incubation times the I_{50}-value is about constant over the pH range measured. Conclusively, I_{50}-value depends on the charged ioxynil species since its concentration does not vary substantially with pH. The uncharged form, however, increases 32-fold at pH 6.5 *vs.* pH 8, and the partition coefficient at the lower pH is about 4-times higher than at pH 8. Apparently the uncharged form facilitates accessibility of the phenolic

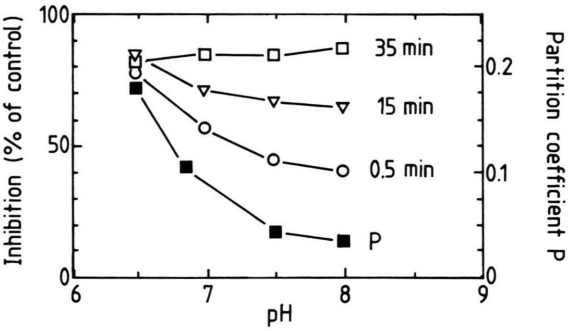

Fig. 2. Incubation time and pH-dependance of inhibition of photosynthetic electron flow using isolated *Brassica* thylakoids. 0.25 μM ioxynil was applied to obtain approximately a 50% inhibition at start of incubation.

compounds and thereby determines how fast the inhibition develops. Lack of pH-dependance of inhibition after long-term incubation was also found for ioxynil *binding* to the thylakoids of higher plants (*Spinacia; Chenopodium,* mutant and wild-type) under similar conditions as reported in [5].

In case of phenolic inhibitors we have the finding that in contrast to *e.g.* ureas or triazinones the binding constant itself (as concluded from the I_{50}-values) is essentially determined by other factors rather than lipophilicity (steric properties may be relevant but not yet proven [10]). On the other hand, in accordance with classical inhibitors, lipophilicity facilitates binding to the *mutant* thylakoids thereby giving rise to increased sensitivity *i.e.* yielding a higher pI_{50}-value *vs.* that of the wild-type. Increased sensitivity can be considered as counteraction of resistance.

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