Mode of Action of Herbicides Affecting Acetyl-CoA Carboxylase and Fatty Acid Biosynthesis

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The mode of action of cyclohexane-1,3-dione-type (cycloxydim, clethodim, sethoxydim, tralkoxydim) and aryloxyphenoxypropanoate-type herbicides (diclofop, fenoxaprop, haloxyfop, fluazifop) is summarized in this review. Both herbicide classes, though structurally completely different, specifically block the same target enzyme *i.e.* the plastid acetyl-CoA carboxylase (ACC) (EC 6.4.1.2). Most members of the Poaceae are sensitive towards both herbicide groups, whereas other monocotyledonous plants as well as the dicotyledonous plants appear to be resistant. This resistance, which can be found on the level of whole plants, in isolated chloroplasts and also on the level of ACC-enzyme preparations, is apparently due to a modification of the target enzyme ACC. Within the sensitive grass family some members (*Festuca* and *Poa* species) are partially tolerant against both graminicide groups. In the case of cyclohexanedione herbicides the tolerance seems to be due to a reduced sensitivity of the target enzyme. In the case of aryloxyphenoxypropionic acid herbicides the tolerance is apparently based on a combined action of cytoplasmic factors (metabolization?) and a slightly reduced sensitivity of the target enzyme. From differences in the sensitivity of certain grasses against the two herbicide classes it is concluded that both graminicide groups bind to the same binding domaine of the ACC enzyme but possess different subsites. The consequences of the block of *de novo* fatty acid biosynthesis in the plastids of sensitive plants is the lack of glycerolipid and biomembrane formation which finally causes cell death in the meristematic tissues.

Introduction

Several cyclohexane-1,3-dione derivatives (alloxydim, cycloxydim, clethodim, sethoxydim, tralkoxydim) have been developed in the past 10 years as commercial post-emergence herbicides which selectively control annual and perennial grass weeds (Poaceae) in cotton, flax, peanuts, soybeans, sugar beets, tomatoes and other broadleaf crops in doses of 0.2 to 0.5 kg a.i. ha^{-1} [1–6]. The usual time for complete control is 7 to 21 days following treatments. The first symptoms of the herbicides' phytotoxicity can be detected in whole grass plants after ca. 24 h, *i.e.* growth retardance and inhibition followed by wilting and necrosis of the meristematic regions of the grass seedlings.

A second class of commercial herbicides, which cause similar phytotoxic symptoms in grass weeds, are the aryloxyphenoxypropionic acid derivatives (some of them are diphenoxypropanoates) such as diclofop, fenoxaprop, fluazifop, haloxyfop, propaquizafop and quizalofop (Fig. 2) [7–16]. Though structurally completely different from the cyclohexanediones, the aryloxyphenoxypropanoates also control selectively many grass weeds in a variety of dicotyledonous crop plants.

Both classes of graminicides exhibit in the sensitive members of the Poaceae the same enzymic target *i.e.* the plastidic acetyl-CoA carboxylase (ACC) as was shown in 1987 by independent research using different methodical approaches in four laboratories [17–21]. This review summarizes the present knowledge of the mode of action of the two graminicide classes. It also provides information on the tolerance of certain Poaceae and on the apparent resistance of dicotyledonous plants and monocotyledonous plants outside the Poaceae.

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Results and Discussion

Initial observations

Cyclohexane-1,3-dione derivatives. To this group belong alloxydim [2], sethoxydim [1, 3] and the newer compounds cycloxydim [20–21], clethodim [16] and tralkoxydim [17] (Fig. 1). Sethoxydim inhibited the formation of the photosynthetic pigments (chlorophylls and carotenoids) and of thyla-

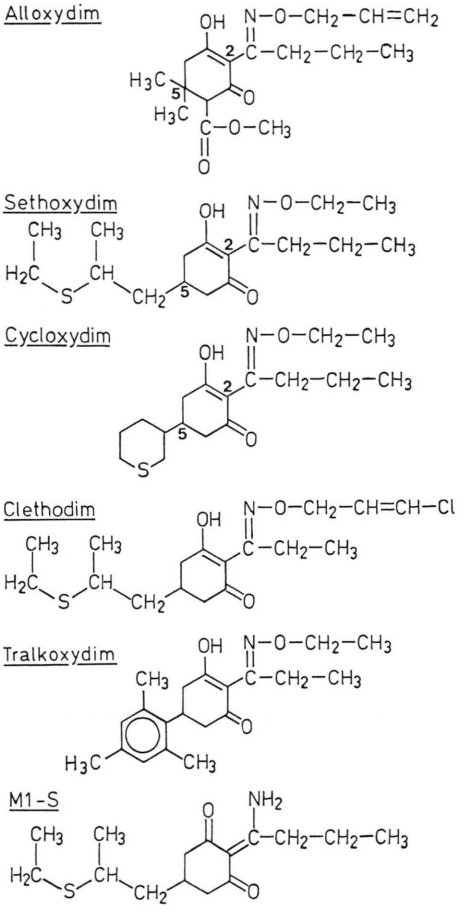

Fig. 1. Chemical structure of different cyclohexane-1,3-diones.

koids at all stages of chloroplast development [3, 22, 23]. It also blocked chloroplast replication and cell multiplication [3, 20, 22]. This resulted, at a medium dose of ca. 20 to 60 g sethoxydim per ha in the formation of white and pigment-free, chlorotic leaf zones in young still growing grass leaves. Despite of this, sethoxydim proved not to be a bleaching herbicide for two reasons: a) the biosynthesis of chlorophylls and carotenoids was not blocked, merely their accumulation [4, 24], and b) it was phytotoxic not only in the light but also in the dark [25].

The observation that in whole plants sethoxydim inhibits the accumulation of all cellular phospho- and glycolipids [26] and also their biosynthesis [4] pointed to an interaction with the plants' lipid metabolism. In fact, in isolated chloroplasts, the sole site of *de novo* fatty acid biosynthesis in higher plant [33], it could be shown that sethoxydim blocked *de novo* fatty acid biosynthesis from [^{14}C]acetate [4, 28]. That in sensitive grass plants the cyclohexane-1,3-dione herbicides specifically block the actyl-CoA carboxylase (EC 6.4.1.2) was then detected in independent research by four different research groups: for sethoxydim [17], for sethoxydim, clethodim and cycloxydim [18], for alloxydim, clethodim and sethoxydim [19] and for tralkoxydim [20].

Aryloxyphenoxypropanoates. Several esters of aryloxyphenoxypropionic acid derivatives such as diclofop, fenoxaprop, fluazifop, haloxyfop and quizalofop (Fig. 2) were found to cause similar damaging effects in most Poaceae [6–13] as those induced by cyclohexanedione-type herbicides mentioned above. The esters, which are regularly used in field application on the whole plants (two or three leaf stage), are, however, not the active in-

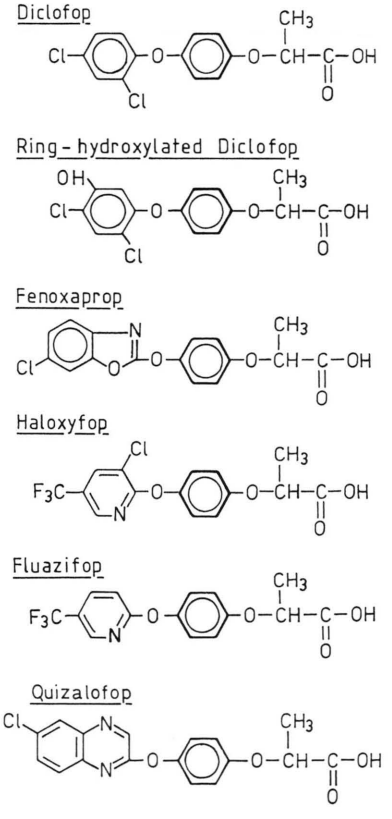

Fig. 2. Chemical structure of several aryloxyphenoxypropionic acid herbicides.

gredients. The esters are hydrolyzed in the cell to the free acids which represent the real phytotoxic inhibitors. In the case of diclofop and fenoxaprop it was first demonstrated in isolated maize chloroplasts by Hoppe [9] that both herbicides inhibit *de novo* fatty acid biosynthesis from [^{14}C]acetate, whereas their esters were not effective [10]. In 1987, the plastidic acetyl-CoA carboxylase was detected as the actual target also of the aryloxyphenoxypropanoate herbicides as shown in an independent work for haloxyfop [14, 17], for diclofop, fenoxaprop, fluazifop and haloxyfop [18] as well as for fluazifop [13].

The acetyl-CoA carboxylase as target

The acetyl-CoA carboxylase of higher plants (EC 6.4.1.2) is a biotin-dependent enzyme which catalyzes the following reaction in two steps:

acetyl-CoA + ATP + CO_2 → malonyl-CoA + ADP + Pi.

In a first step the biotinyl group is carboxylated and the carboxyl group is then transferred in a second step to acetyl-CoA to form malonyl-CoA [29]. The proposed mechanisms of the enzyme-catalyzed ATP-dependent carboxylation of biotin involves an adduct which decomposes to yield N_1-carboxybiotin and inorganic phosphate [30]. In contrast to bacteria, where the biotin carboxylase and the transcarboxylase are two separate peptides which cooperate with a biotin carboxyl carrier protein (BCCP), the acetyl-CoA carboxylase of higher plants consists of one major polypeptide of *ca.* 220 kDa containing all 3 subunits and is thought to be of the eucaryotic type [31].

The detection of the acetyl-CoA carboxylase as target enzyme for the cyclohexanedione and aryloxyphenoxypropanoate herbicides was based upon the application of ^{14}C-labelled precursor compounds such as acetate, acetyl-CoA, malonate and malonyl-CoA [15, 18]. It was shown a) in the isolated chloroplast test system as well as b) in an enzyme preparation containing ACP, both of which were able to synthesize fatty acids *de novo* from [^{14}C]acetate, that the incorporation of [^{14}C]acetate and [^{14}C]acetyl-CoA into the total fatty acid fraction was inhibited in a dose-dependent manner by both herbicide classes [15, 18, 32] as shown in Fig. 3. In contrast, the incorporation of

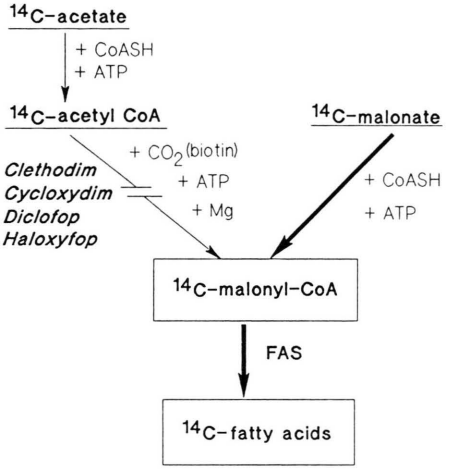

Fig. 3. Scheme of biosynthesis and labelling of fatty acids in chloroplasts from different ^{14}C-labelled substrates with indication of the inhibitors site by cyclohexanedione and aryloxyphenoxypropanoate herbicides. FAS = fatty acid synthetase.

^{14}C-label from malonate and malonyl-CoA was not inhibited by either of the graminide classes.

In a direct ACC enzyme assay the acetyl-CoA carboxylase as the specific target was confirmed by studying the incorporation of [^{14}C]HCO_3 into malonyl-CoA [14, 16, 17, 32–34]. In extensive kinetic studies with enriched ACC enzyme preparations it could be shown that both graminicide classes specifically interfere with the transcarboxylase activity of the acetyl-CoA carboxylase [33]. The inhibition is of the non-competitive type [33], which is also concluded from studies with isolated etioplasts [35]. The inhibition by the ACC inhibitors is reversible and can be reversed by removing the herbicide [35].

Structure and function

Cyclohexane-1,3-diones. Besides the cyclohexane-1,3-dione ring, the essential structure of this class of herbicides needed for an efficient phytotoxic activity is the ethoxyimino group (or ethoxyamino group at the C-atom bound to the cyclohexanedione skeleton in position 2), which is found in sethoxydim, cycloxydim and tralkoxydim (Fig. 1). In contrast, the substance M 1-S, which lacks the ethoxyamino group, is a well known *non-phytotoxic* degradation product of sethoxydim [36]. It has no inhibitory effect on *de novo* fatty acid bio-

synthesis, neither on the whole plant level, nor in the chloroplasts' test system [23] nor in the direct acetyl-CoA carboxylase enzyme assay. An allyloxyamino group (alloxydim) or a chloro-allyloxyamino group (clethodim) also appear to be good substituents for a phytotoxic response (Fig. 1).

The second substituent of the C-atom bound to the cyclohexanedione structure in position 2 may be an ethyl or propyl group. It appears that the substitutions in position 5 of the cyclohexanedione structure can vary considerably without large differences in the herbicidal activity. Of the compounds tested in our chloroplast test system, cycloxydim and clethodim proved to be the most efficient herbicides, followed by sethoxydim and alloxydim, as is documented in the I_{50}-values of the inhibition of *de novo* fatty acid biosynthesis (Table I). A more detailed study of the herbicidal efficacy of a variety of structurally different cyclohexane-1,3-diones is found in [37].

Aryloxyphenoxypropanoates. The common feature of all phytotoxic compounds in this group of herbicides is the presence of a diphenoxy- or an aryloxyphenoxypropionic acid structure (Fig. 2). The phenoxypropionic acid part of the structure is unaltered in all compounds shown and seems to be essential for the herbicidal activity; substituents in this near phenoxy-ring structure (next to the propionic acid) are not favourable to inhibitory activity. In contrast the second or distant phenoxy-ring structure can, however, vary considerably. It may be an aryloxy-type ring or quite different double-ring structures such as found in fenoxyprop, fluazifop and quizalofop. From the given structures (Fig. 2) it is evident that the halogenated derivatives containing chlorene or trifluoromethyl groups are essential for the herbicidal activity. In the case of diclofop, ring hydroxylation (Fig. 2) decreases the phytotoxic activity, as is seen from the increasing I_{50}-value (Table I). Ring hydroxylation and subsequent glucosylation of diclofop is a detoxification mechanism which exists in wheat seedlings [7].

All aryloxyphenoxypropanoates possess a chirality center in the C-atom of the propionic acid as marked in Fig. 2. The *R*(+)-enantiomers or D(+)-forms represent the real phytotoxic structures, whereas the *S*(−)-enantiomers or L(−)-forms exhibit only little or no effect. This has been shown on the level of the whole plant for quizalofop and also for diclofop [10]. It is also demonstrated for diclofop with the newly developed etioplast test system (Table II). Even a high concentration of 3.8 μM L(−)-diclofop did not increase or decrease the percentage inhibition of *de novo* fatty acid biosynthesis which was set by 0.2 μM D(+)-diclofop. This also show that the inactive L(−)-form does not expel the active D(+)-form from the binding site of the acetyl-CoA carboxylase.

Table I. I_{50}-values for the inhibition of *de novo* fatty acid biosynthesis in isolated oat chloroplasts by different cyclohexanedione and aryloxyphenoxypropanoate herbicides, which block the plastidic acetyl-CoA carboxylase. Measured was the incorporation of [^{14}C]acetate into the total fatty acid fraction. The chloroplasts of the resistant plant spinach are either not (cyclohexanediones) or very little affected (aryloxyphenoxypropanoates).

Herbicides	I_{50}-value [μM] Oat	Spinach
Cyclohexanediones		
Cycloxydim	0.15	n.i*
Clethodim	0.15	n.i*
Sethoxydim	0.15	n.i*
Alloxydim	2.0	n.i*
Aryloxyphenoxypropanoates		
Diclofop	0.1	100
Fenoxyprop	0.1	100
Haloxyfop	0.3	100
Hydroxydiclofop	2.0	100
Fluazifop	3.0	100

* No inhibition at a concentration of 200 μM.

Table II. Inhibition of the [1-^{14}C]acetate incorporation into the total fatty acid fraction of isolated oat etioplasts by active D(+)- and inactive L(−)-diclofop and mixtures of both forms (with standard deviation). * D(+)-diclofop had a contamination of 2% L(−)-diclofop and **L(−)-diclofop *ca.* 4.9% D(+)-diclofop. The [^{14}C]acetate incorporation is expressed as nmol acetate per mg carotenoids (x + c) and hour.

Additions	[^{14}C]Acetate incorporation	% Inhibition
Control	763 (± 41)	0
0.2 μM L(−)-diclofop*	750 (± 35)	0
0.2 μM D(+)-diclofop**	287 (± 7)	62
0.2 μM D(+)-diclofop + 0.2 μM L(−)-diclofop	292 (± 17)	62
0.2 μM D(+)-diclofop + 3.8 μM L(−)-diclofop	297 (± 15)	61

The aryloxyphenoxypropanoate herbicides are applied in the field as esters (diclofop-methyl, fenoxaprop-ethyl, fluazifop-butyl, haloxyfop-methyl, quizalofop-ethyl) in order to facilitate the uptake of the herbicides by the intact plants. The ester forms of the herbicides, though active on the whole plant level (after internal hydrolyzation), have no or very little inhibition effect on the level of isolated chloroplasts [18]. The I_{50}-values for the different ester forms of the aryloxyphenoxypropanoates are larger by a factor of *ca.* 100 [28] than those of the free acids given in Table I. In a direct enzyme assay the pure ester forms also did not exert any inhibition on the activity of the acetyl-CoA carboxylase.

Investigations on the level of whole plants, isolated chloroplasts and target enzyme preparations

Investigations on the sensitivity, relative tolerance or full resistance of plants against inhibitors of *de novo* fatty acid biosynthesis have to be performed on three levels a) the whole plants, b) the isolated intact chloroplasts [20], etioplasts [19] or leucoplasts (Table III) as a quasi *in vivo* test system and c) the isolated or enriched ACC target enzyme preparations. On the whole plant level visual effects, growth retardance and necrosis, are determined and in the case of excised shoots or leaf discs the rate and inhibition of [^{14}C]acetate incorporation into the total lipid fraction. In the whole chloroplasts, the incorporation of [^{14}C]acetate into the total fatty acids is measured, which includes the activity of the acetyl-CoA synthetase, acetyl-CoA carboxylase and fatty acid synthetase (FAS) (Fig. 4). In the direct enzyme assay, the incorporation of labelled $H^{14}CO_3$ into malonyl-CoA is determined.

Table III. Influence of cycloxydim and diclofop on the incorporation of [^{14}C]acetate into the total fatty acid fraction of leucoplasts from roots of sensitive maize plants. Incorporation time was 20 min. Mean of 4 determinations with standard deviation (SD).

Additions	Rate*	SD	% Inhibition
Control	562	66	–
Cycloxydim 1 µM	427	42	24
Cycloxydim 10 µM	197	8	65
Diclofop 1 µM	259	29	54
Diclofop 10 µM	141	6	75

* The rate is given as Bq per mg protein.

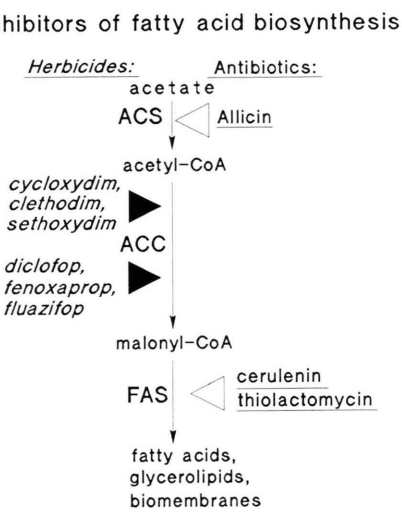

Fig. 4. Scheme of the *de novo* fatty acid biosynthesis in chloroplasts showing the points of inhibition by the two herbicide groups, the cyclohexanediones *e.g.* cycloxydim and the aryloxyphenoxypropanoates (*e.g.* diclofop). The specific site of inhibition by the naturally occurring antibiotics allicin, cerulenin and thiolactomycin is also indicated. ACS = acetyl-CoA synthetase; ACC = acetyl-CoA carboxylase; FAS = fatty acid synthetase. The scheme is based on results described in ref. [14–18, 37, 38, 40].

Isolated intact chloroplasts [15, 18, 39] or etioplasts [35] represent a very efficient test system for the determination of I_{50}-values, since the rate of *de novo* fatty acid biosynthesis is very close to the *in vivo* situation. The lowest I_{50}-values we always found in the chloroplast test system. In a direct assay of the ACC target enzyme, the I_{50}-values are about 5 to 10 times higher or even more, depending upon the plant and the isolation procedures applied. The results obtained in isolated intact chloroplasts (or etioplasts) thus fully reflect the binding and inhibition characteristics of the target enzyme acetyl-CoA carboxylase. This is of great importance for the screening of possible differences in sensitivity and tolerance towards both herbicide classes, which in our experience is best performed at the level of isolated chloroplasts.

Sensitivity, tolerance and resistance

In an attempt to screen the susceptibility and resistance of plants of different taxonomic groups

towards both herbicide classes we applied cycloxydim and diclofop in a comparative study. The sensitivity of plants to both herbicide classes seems to be restricted to members of the Poaceae. Dicotyledonous plants and monocotyledonous plants outside the Poaceae *(e.g. Chlorophytum comosum)* appear to be resistant on the whole-plant and the chloroplast level, as seen from their relatively high I_{50}-values towards cycloxydim and diclofop (Table IV). The resistance towards ACC inhibitors was confirmed in a direct ACC enzyme assay [32]. At the chloroplast level some resistant plants such as spinach proved to be slightly sensitive towards diclofop, as is documented by a clearly lower I_{50}-value (150 μM) than that of cycloxydim (>300 μM). Yet the very slight sensitivity of spinach chloroplasts towards diclofop is *ca.* 1500 times lower than that of sensitive oat plants.

Within the grass family (Poaceae) there exist some species which at the whole plant level are relatively tolerant to both graminicide classes, *e.g.*, two fescue species (*Festuca ruba* and *Festuca ovina*), whereas the tall fescue *(Festuca arundinacea)* is extremely sensitive. On the chloroplast level tolerant fescue species showed the expected high I_{50}-values towards cycloxydim (100 μM). Though the latter are lower than those of resistant dicotyledonous plants, they are *ca.* 1000 times higher than that of the sensitive tall fescue (Table IV). In the case of diclofop the I_{50}-values at the chloroplast level of the tolerant fescues are, however, much lower, and only 4 times higher than that of the sensitive fescue. Though a certain cross tolerance of cycloxydim and diclofop seems to exist on the chloroplast level, this is not sufficient to explain the tolerance of the two fescue species at the whole plant level. We conclude that the tolerance of the two *Festuca* species (*F. rubra* and *F. ovina*) towards diclofop is mainly determined by cytoplasmic properties (*e.g.* metabolization) and only partially by a lower sensitivity of the target enzyme [39].

Table IV. I_{50}-values for the inhibition of *de novo* fatty acid biosynthesis of chloroplast ([^{14}C]acetate incorporation) isolated from plants with different sensitivity towards cycloxydim and diclofop. Diclofop was a 50% mixture of the active D(+)- and the inactive L(−)-enantiomers.

Species	I_{50}-Value [μM] Cycloxydim	Diclofop
Chlorophytum comosum	>300	*ca.* 280
Nicotiana tabacum	>300	*ca.* 250
Pisum sativum	>300	*ca.* 250
Spinacia oleracea	>300	150
Festuca ovina	100	0.4
Festuca rubra	100	0.4
Poa annua	10	0.2
Poa pratensis	0.2	0.1
Avena sativa	0.15	0.1
Festuca arundinacea	0.1	0.1

We also determined the I_{50}-values of the two grasses *Poa annua* and *Poa pratensis,* of which the first is known to be tolerant towards cycloxydim and diclofop, whereas the second is sensitive. At the level of isolated chloroplasts the I_{50}-value towards cycloxydim was *ca.* 50 times higher in the tolerant than the sensitive *Poa* species. With respect to diclofop the I_{50}-value of *Poa annua* was very low (as compared to that of cycloxydim) and only 2 times higher than the diclofop I_{50}-value of the sensitive *Poa pratensis*. The tolerance of *Poa annua* on the whole plant level must therefore lie in cytoplasmic properties of the cells.

The relative tolerance of certain members of the Poaceae towards cycloxydim (and other cyclohexanediones) is due to a reduced sensitivity of the ACC target enzyme in the chloroplasts. In the case of diclofop the ACC target enzyme is almost as sensitive in the tolerant as in the sensitive *Festuca* and *Poa* species. Though a certain "cross tolerance" is seen on the whole plant level, this is, however, only weakly reflected on the level of the ACC target enzyme. Based on these results we conclude that cyclohexanediones and aryloxyphenoxypropanoates bind to the same binding niche at the target enzyme acetyl-CoA carboxylase but occupy different binding subsites. This view of different binding subsites is further supported by the observation that a mutant of Italian rye grass *(Lolium multiflorum)* is resistant to diclofop, however, still sensitive to sethoxydim [41], whereas the wild type is sensitive to both herbicides. Differences in subsites of binding may also exist within herbicides of the cyclohexanedione class and within the aryloxyphenoxypropanoate class. In the tolerant *Festuca rubra* we found a high sensitivity of the ACC enzyme in plastids towards diclofop (Tables II and IV), whereas only very weak sensitivity was found towards haloxyfop [42]. From the evidence available it appears that the different inhibitors of the

ACC enzyme share a common feature in binding. A true cross tolerance, however, does not exist.

Consequences for intact plants

As a result of the inhibition of *de novo* fatty acid biosynthesis, which proceeds solely within the plastid compartment [27], there is a shortage of fatty acids for glycerolipid biosynthesis in the cell. In fact, the biosynthesis of cellular glycolipids (MGDG, DGDG and SQDG) and phospholipids (PG, PC and PE) is blocked by the cyclohexanedione sethoxydim for a longer period after treatment with the herbicide, as was shown for excised maize shoots [4]. The lack of fatty acids and the block of glycerolipid formation and accumulation [26] results in an inhibition of the formation of all cellular biomembranes. Young developing leaves and the meristematic tissues, which are fully dependent on a functional *de novo* fatty acid biosynthesis, are firstly affected. The consequences are: block of plastid development, chloroplast biogenesis, thylakoid formation as well as plastid/chloroplast replication and cell division [3, 4], which causes the observed growth retardance. Finally necrosis and cell death develops in the meristematic parts of the plant.

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