Inhibition by Sethoxydim of Pigment Accumulation and Fatty Acid Biosynthesis in Chloroplasts of *Avena* Seedlings

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The cyclohexanedione derivatives sethoxydim and alloxydim block leaf growth and inhibit the accumulation of photosynthetic pigments (chlorophylls, carotenoids) in expanding leaves of oat seedlings, but do not affect the rate of photosynthesis in the primary or secondary leaves. In isolated intact oat chloroplasts the incorporation of [2-^{14}C]acetate into the total fatty acid fraction is strongly inhibited by the two herbicides sethoxydim and alloxydim. The I_{50}-values for the inhibition of [^{14}C]acetate incorporation into fatty acids are 5×10^{-7} M for sethoxydim and 2×10^{-6} M for alloxydim. The desethoxyimino derivative of sethoxydim, M1-S, a degradation product of sethoxydim, does not interfere with the fatty acid biosynthesis of isolated oat chloroplasts. In isolated chloroplasts of the dicotyledonous plants pea and spinach, which as whole plants are tolerant towards sethoxydim, no significant inhibition of the fatty acid biosynthesis by sethoxydim could be found. It is assumed that the primary mode of action of sethoxydim and alloxydim is in blocking the fatty acid biosynthesis in the sensitive gramineous plants.

Introduction

The cyclohexanedione derivative sethoxydim (Fig. 1) is a very effective herbicide for postemergence control of annual and perennial grass weeds in a wide range of dicotyledonous crop plants such as soybean, sugar beet, peanuts, cotton, tomatoes [1–4]. The structurally related compound alloxydim possesses similar effects against gramineous weeds and is also applied in broadleaved crops [5]. After foliar application the herbicides are rapidly absorbed and basipetally transported (phloem) to the meristematic zone of leaves, roots and the shoot apex [2], where they inhibit growth and induce necrosis.

In newly expanding leaves of barley and maize seedlings sethoxydim blocks the accumulation of chlorophylls and carotenoids and inhibits chloroplast biogenesis at all stages from proplastids to mature chloroplasts as well as chloroplast replication and cell multiplication [6–9]. Sethoxydim expresses its herbicidal activity both in the dark and in the light [8]. The structurally related compound M1-S is a non-phytotoxic decomposition product of sethoxydim [10].

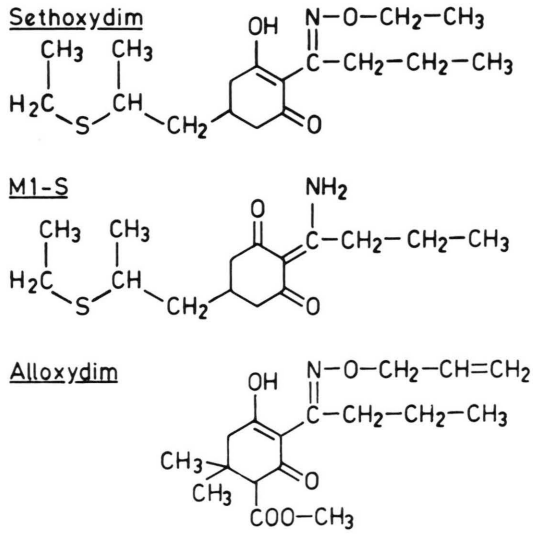

Fig. 1. Chemical structure of the herbicides sethoxydim and alloxydim and of the related cyclohexane-1,3-dione derivative M1-S.

Abbreviations: Alloxydim, 2-(1-allyloxyaminobutylidene)-5,5-(dimethyl-4-methoxycarbonyl)-cyclohexane-1,3-dione; sethoxydim,2-(1-ethoxyimino) butyl-5-(2-ethylthiopropyl)-3-hydroxy-2-cyclohexen-1-one; M1-S, 2-(1-aminobutylidene)-5-(2-ethylthiopropyl)-cyclohexane-1,3-dione.

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The exact mode of action of sethoxydim and alloxydim is not yet fully worked out. Several observations point, however, to interaction with the lipid metabolism of plants. Sethoxydim blocks the accumulation and biosynthesis of all cellular glycolipids (bound to chloroplasts) and phospholipids (present in part in chloroplasts and also in the cytoplasm) [11, 12], but does not affect the biosynthesis of prenyllipids such as sterols or prenylpigments [13]. The main target of the herbicide action seems to be the fatty acid synthesis [12, 13], which in higher plants is exclusively located in chloroplasts and other plastid forms [14]. In a first experiment with isolated maize chloroplasts sethoxydim blocked the incorporation of [2-^{14}C]acetate into the total fatty acid fraction [12].

In order to further establish this possible main herbicide target we investigated whether the fatty acid biosynthesis of chloroplasts of another gramineous plant *(Avena sativa)* can also be blocked by sethoxydim and by the structurally related alloxydim. We also wanted to check whether the non-phytotoxic M1-S, a decomposition product of sethoxydim, which is nonphytotoxic to whole plants, is without effect in the isolated chloroplast test system. That the oat plants used in this investigation were sensitive to sethoxydim and alloxydim treatment was tested beforehand by studying the inhibition of pigment accumulation. We also investigated whether the fatty acid biosynthesis in isolated chloroplasts of dicotyledonous plants, which as whole plants are tolerant to the cyclohexane-1,3-dione-type herbicides, is unaffected by these active ingredients.

Materials and Methods

Oat seedlings (*Avena sativa* L. var. Flämingnova) were cultivated on peat in a 14/10 h day/night cycle at 25 °C at a light intensity of 1500 μE m^{-2} s^{-1} (light source: Osram HQI-E 400 W, 5 cm water filter). The seedlings were sprayed with an aqueous solution of sethoxydim or alloxydim sodium salt (62.5 g·ha^{-1}, 125 g·ha^{-1}) at the two-leaf stage. For the determination of photosynthetic pigments leaf-blades were taken before and 3, 5 and 7 days after sethoxydim treatment. The chlorophylls and total carotenoids were determined photometrically using the new absorption coefficients and equations of Lichtenthaler [15], which allow the simultaneous determination of chlorophylls and carotenoids in one extract solution. The measurements of net CO_2 assimilation were performed at light saturation (2500 μE m^{-2} s^{-1}) using a new CO_2/H_2O porometer according to Schulze *et al.* 1982 [16].

Oat chloroplasts were isolated from 7 d old seedlings using a medium described before [19]. The incubation with [2-^{14}C]acetate was carried out at room temperature in 1 ml chloroplast suspensions, which were shaken in a special apparatus, to guarantee that each of the 12 samples received the same light intensity of 1400 μE m^{-2} s^{-1}. The light was applied from below through a 4 cm filter of running water. The reaction mixture contained 300 mM sorbitol, 50 mM tricin, 50 mM phosphate buffer (pH 7.9), 30 mM $NaHCO_3$, 2.5 mM DTT, 2 mM ATP, 0.5 mM CoA, 0.5 mM $MgCl_2$, 0.2 mM NADH, 35 μM [2-^{14}C]acetate (1 μCi per ml) and chloroplasts with a chlorophyll content of ca. 100 μg per ml. The herbicides alloxydim, sethoxydim and M1-S were added in methanolic solution, the methanol concentration in all suspensions including controls was 0.2%. After an incubation time of 20 min the reaction was stopped by adding 1 ml 30% KOH, the lipids were hydrolyzed at 70–80 °C for 90 min. After acidification with ca. 500 μl 12 M sulfuric acid per sample and subsequent addition of 300 μl 30% trichloracetic acid the fatty acids and remaining non-acyllipids were extracted with light petrol (b.p. 50–70 °C) [17]. The fatty acids were separated by TLC (silicagel Merck Nr. 5626, solvent: light petrol/diethylether/acetic acid: 90/20/1, by volume) [18] and the radioactivity was measured with a liquid scintillation counter. We found in all labelling experiments that at least 97% of the total radioactivity taken up was incorporated into the total fatty acid fractions. The incorporation rate of the applied radioactivity amounted to 3 to 4% for oat and 7 to 8% for spinach and pea chloroplasts.

Results

The oat seedlings are sensitive to the treatment with sethoxydim in the two-leaf stage as can be seen from the inhibition of the accumulation of photosynthetic pigments (chlorophylls and carotenoids) in the tertiary, still expanding leaf (Fig. 2 and 3). The pigment accumulation of this leaf is affected to a larger degree than the development and length of its leafblade (Fig. 3). This results in the formation of longer chlorotic white zones in the tertiary leaves in the sethoxydim-treated plants. The inhibition of the

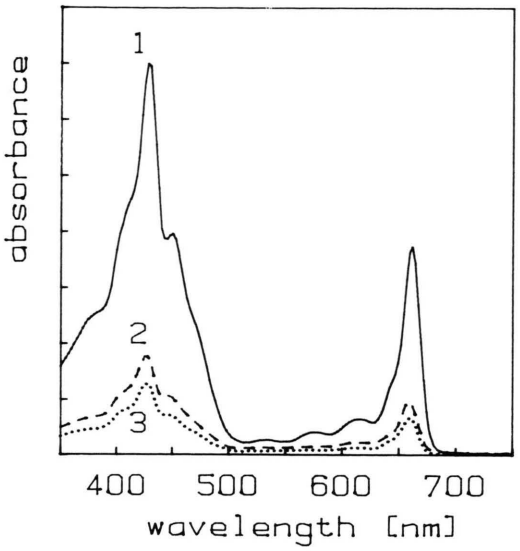

Fig. 2. Absorption spectrum of a leaf-pigment extract (chlorophylls + carotenoids) of the tertiary oat leaf-blades of control plants and equally old plants sprayed with the herbicide sethoxydim (at two different concentrations 62.5 and 125 g·ha^{-1}) 7d before extraction.

pigment accumulation proceeds for chlorophyll *a* and *b* as well as for carotenoids to the same degree. This can be seen from the pigment ratios, chlorophyll *a*/*b* and chlorophyll/carotenoids *(a + b/x + c)*, which do not change after sethoxydim treatment (Table I). In the primary and the secondary leaves of the oat seedlings, which had almost fully been developed before the spray application of sethoxydim, no or little effect of the herbicide could be detected. These results show that the *Avena* seedlings are sensitive to sethoxydim and that the induction of chlorotic zones and the inhibition of pigment accumulation in expanding leaves proceed in the same manner as described before for barley and maize seedlings [7, 8, 23]. In a further experiment, in which oat seedlings in the two-leaf stage were sprayed with the cyclohexanedione derivative alloxydim, we were able to demonstrate that alloxydim had similar inhibitory effects on growth and pigment accumulation, though it was slightly less effective than sethoxydim.

Sethoxydim and alloxydim did not affect photosynthetic function of the green primary or secondary leaves of *Avena* seedlings which had almost fully

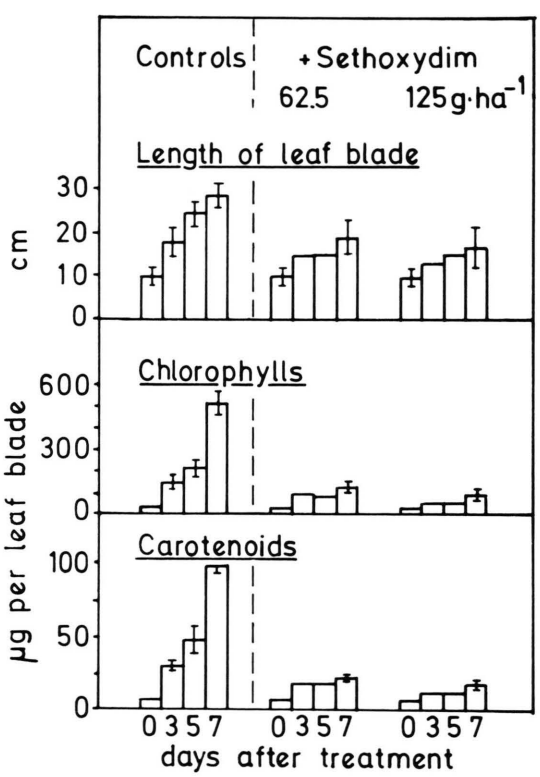

Table I. Values for the pigment ratios *a*/*b** and *a* + *b*/*x* + *c*** in tertiary leaves of *Avena* seedlings without (control) and with sethoxydim treatment. Mean of 4 experiments (deviation < 6%) with 2 to 4 leaves per extract depending on the leaf size.

	Days after treatment			
	0	3	5	7
Ratio *a*/*b*:				
control	3.12	3.09	3.57	3.10
+ 62 g a.i. · ha^{-1}	3.12	3.39	3.58	3.13
+ 125 g a.i. · ha^{-1}	3.12	3.17	2.92	3.22
Ratio *a* + *b*/*x* + *c*:				
control	4.71	5.28	4.72	5.23
+ 62 g a.i. · ha^{-1}	4.71	4.79	4.52	5.34
+ 125 g a.i. · ha^{-1}	4.71	4.65	4.81	5.26

* *a*/*b* = ratio chlorophyll *a* to *b*.
** *a* + *b*/*x* + *c* = ratio chlorophylls to carotenoids (x = xanthophylls; c = β-carotene).

Fig. 3. Development of the length of the leaf-blade and chlorophyll and carotenoid content of the tertiary oat leaf-blade in control plants and plants treated with the herbicide sethoxydim (62.5 and 125 g·ha^{-1}). The measurements were performed before (0) and 3, 5 and 7 days after spray application of the herbicide. Mean values of 4 determinations from 2 separate plant cultivations.

been developed at the time of herbicide treatment. The rate of photosynthetic CO_2 fixation in primary leaves (ca. 8 μmol CO_2 m^{-2} s^{-1}) and secondary leaves (ca. 12 μmol CO_2 m^{-2} s^{-1}) of the control plants varied to the same extent (+15%) as in the herbicide-treated plants.

In order to test whether sethoxydim and alloxydim affect the de novo biosynthesis of total fatty acids in the plastid fraction, we isolated intact chloroplasts from green untreated oat seedlings. By variation of cofactors, buffers and pH-value we ameliorated the initially low capacity of isolated Avena chloroplasts for de novo fatty acid biosynthesis up to an incorporation rate (of [2-^{14}C]acetate into the total fatty acids) of 4% of the applied radioactivity for a 20 min period. In isolated intact chloroplasts the fatty acid biosynthesis is only maintained for ca. 40 min, the incorporation studies have therefore to be performed immediately after isolation. The incorporation of [^{14}C]acetate proceeded in a linear manner to up to at least 20 min and was much higher in the illuminated samples (= 100%) than in the dark (10 to 15%). This is certainly due to photosynthesis (endogenous oxygen evolution rate of the intact isolated chloroplasts: 180 μmol O_2 per mg chlorophyll h), which also provides ATP and NADPH for fatty acid biosynthesis. Broken chloroplasts, which in the presence of Hill reagents may still have a high rate of oxygen evolution (Avena chloroplasts: ca. 200–220 μmol O_2 per mg chlorophyll h), have lost their capacity for fatty acid biosynthesis.

In the test system of isolated intact *Avena* chloroplasts we investigated the effect of sethoxydim and alloxydim on the [2-^{14}C]acetate in the total fatty acid fraction. This was inhibited by both herbicides in a dose-dependent manner (Fig. 4). As in intact plants sethoxydim was somewhat more effective than alloxydim (Table II). The desethoxyimino derivative M1-S, in turn, did not show a significant inhibition effect. The concentration for 50% inhibition of the fatty acid biosynthesis (I_{50}-values) was 5×10^{-7} M for sethoxydim and 2×10^{-6} M for alloxydim.

In isolated chloroplasts from the dicotyledonous plants pea and spinach, known to be tolerant towards treatment with cyclohexanedione-type herbicides, sethoxydim did not affect the de novo fatty acid biosynthesis from [^{14}C]acetate (Fig. 5). In spinach chloroplasts there appears to be a tendency for some inhibition only for the highest sethoxydim concentration of 100 μM. Alloxydim, tested in the isolated pea and spinach chloroplasts in a concentration of 10 μM, had no influence on fatty acid biosynthesis.

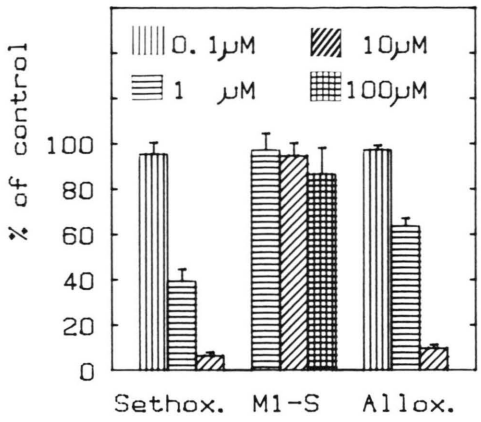

Fig. 4. Effect of different cyclohexane-1,3-dione derivatives on the incorporation of [2-^{14}C]acetate into the total fatty acid fraction of isolated chloroplasts from primary and secondary leaves of *Avena* seedlings. The incorporation rate of the [^{14}C]acetate in the untreated control chloroplasts amounted to 12 to 14 kBq per mg chlorophyll for a 20 min period. Mean of 4 different chloroplast isolations with 12 values per concentration with standard deviation.

Table II. Percentage inhibition of *de novo* fatty acid biosynthesis from [2-^{14}C]acetate in isolated *Avena* chloroplasts in the presence of different concentrations of the cyclohexane-1,3-dione derivatives alloxydim, sethoxydim and M1-S. Incorporation rates of control chloroplasts and number of measurements as in Fig. 4.

Concentration [M]	Alloxydim	Sethoxydim	M1-S
10^{-7}	3	5	–
10^{-6}	36	60	3
10^{-5}	90	93	5
10^{-4}	99	99	13
10^{-3}	–	–	6
I_{50} value ca.	2×10^{-6} M	5×10^{-7} M	–

Discussion

The results of this investigation show that the herbicides sethoxydim and alloxydim block the *de novo* fatty acid biosynthesis in a dose-dependent manner in isolated intact chloroplasts of *Avena* seedlings which are sensitive to these herbicides. After the first demonstration of inhibition by sethoxydim of fatty

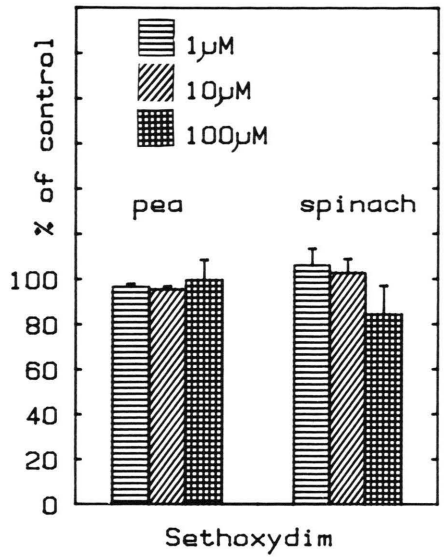

Fig. 5. Incorporation of [2-^{14}C]acetate in the total fatty acid fraction of isolated chloroplasts from herbicide-tolerant dicotyledonous plants pea and spinach with and without addition of sethoxydim. The incorporation rate of [^{14}C]acetate into the total fatty acid fraction amounted to 13 kBq (pea) and to 8 kBq (spinach). Mean of 3 determinations for each concentration with standard deviation.

acid biosynthesis in isolated maize chloroplasts [12 and cited in detail in 13], the results described here are further evidence for the hypothesis that the target for the sethoxydim action within the cell is the *de novo* fatty acid synthesis of the chloroplasts. The inhibition of [^{14}C]acetate incorporation into fatty acids by alloxydim supports this view. From the ineffectiveness of M1-S in the isolated chloroplast test system it appears that the ethoxyimino group is an essential component for the mode of action of sethoxydim and alloxydim. The *de novo* acid biosynthesis of isolated maize chloroplasts is also inhibited by compounds with a different chemical structure such as the diphenoxypropionic acid derivatives diclofop and fenoxaprop [20]. It is possible that the different chemical compounds affect different parts of the acetyl-CoA carboxylase, which is the target [21].

That the fatty acid biosynthesis of isolated chloroplasts of the tolerant dicotyledonous plants pea and spinach is also insensitive towards treatment with sethoxydim and alloxydim indicates that the tolerance of these plants is based on their chloroplasts. Whether the uptake of these herbicides into the chloroplasts is affected or whether the enzymes performing the fatty acid biosynthesis (from [^{14}C]acetate) in the tolerant species are modified as compared to sensitive species, so that the target is not accessible to these herbicides, is a matter of further research.

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