Chloroplast Biogenesis, Its Inhibition and Modification by New Herbicide Compounds

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Z. Naturforsch. **39 c**, 492–499 (1984); received November 29, 1983

Chloroplast Biogenesis, Herbicide Action, Mevinolin, Prochloroplast, Sethoxydim

Biogenesis and development of chloroplasts are summarized in the light of new findings; the interactions of different groups of photosynthetic herbicides with chloroplast development are indicated. The effect of two new active ingredients, mevinolin * and sethoxydim **, on chloroplast modification and biogenesis are described. Mevinolin blocks the cytoplasmic HMG-CoA reductase and sterol accumulation and modifies chloroplast differentiation *via* the formation of a sun-type chloroplast. Sethoxydim inhibits leaf growth in grasses and gramineous crop plants and strongly affects chloroplast biogenesis and differentiation at all stages of development from proplastids *via* prochloroplasts to young and mature chloroplasts. This applies to thylakoid formation and multiplication, grana formation as well as chloroplast division and chlorophyll and carotenoid accumulation. The chlorophyll fluorescence induction kinetics (Kautsky effect) indicate that photosynthesis is not affected. At higher doses sethoxydim stops all growth responses of maize plants and induces the accumulation of anthocyanins.

Introduction

Herbicides can have a direct influence on photosynthesis *e.g.* by blocking the photosynthetic electron transport (DCMU-type herbicides). They also may have an indirect effect on the photosynthetic function by modifying morphology and architecture of the photosynthetic apparatus (formation of sun-type or shade-type chloroplasts) [1–3]. Interference with carotenoid formation and chlorophyll accumulation (chlorosis inducing herbicides) [4–8] and with chloroplast biogenesis and differentiation are further possibilities for indirectly affecting photosynthesis.

Since chloroplast development is dependent on a functional cell metabolism and also on the activity of nuclear genes, the point of action of a herbicide or active ingredient which interferes with chloroplast formation can also be in the cytoplasm.

In this paper the interaction of the different known herbicide groups with chloroplast development is summarized in brief. The effects of two new active ingredients a) mevinolin (a highly specific inhibitor of cytoplasmic mevalonate formation) and b) sethoxydim (a very efficient herbicide in the control of grasses) on chloroplast modification and biogenesis are described.

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A, LHPC's, light-harvesting chlorophyll *a*/*b*-lutein-neoxanthin proteins.

* (1,2,6,7,8,8a-hexahydro-β-δ-dihydroxy-2,6-dimethyl-8-[2-methyl-l-oxobutoxy]-1-naphthalene heptanoic acid).

** (2-[l-ethoxyiminobutyl)-5-(2-ethylthiopropyl)-3-hydroxy-2-cyclohexanone).

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0341-0382/84/0500-0492 $ 01.30/0

Material and Methods

Barley (*Hordeum vulgare* L. cv. "Breuns Villa") and maize seedlings (*Zea mays* L. cv. "Protador") were germinated for 3 days in the dark and then grown in a 14 to 10 h day-night rhythm at 22 °C, 65% rel. humidity, at a medium light intensity of about 20 $W \cdot m^{-2}$ and approximately 6 klux. Fixation and preparation of leaf material for electron microscopy and the biometrical analysis of the electron micrographs were performed as described [2]. The method of fluorescence measurements and extraction of leaf pigments was applied as reported [1, 9]. For the quantitative determination of chlorophylls and total carotenoids we used the newly established equations of Lichtenthaler and Wellburn [10]. The anthocyanins of 10 secondary leaf blades were extracted in 1 *n* HCl and the absorbance determined at 525 nm before and after oxidation with a 30% H_2O_2 solution (0.1 ml per 10 ml extract) and

referred to a cyanidin standard. Sethoxydim (NP 55, BAS 9052OH), was obtained from the BASF agricultural research station, Limburgerhof as a herbicide formulation (Poast) with 18 to 20% active ingredients. Dilution was performed only before spraying. Mevinolin was a gift from Merck, Sharp and Dohme, NJ, USA.

Results and Discussion

Chloroplast biogenesis

Differentiation and formation of photosynthetically active chloroplasts starts from proplastids, which not only represent the smallest plastid form, but also the one with the strongest reduction in morphology. On electronmicrographs of meristematic plant tissues, proplastids appear as round, often spherical organelles of 0.5 to 1 μm diameter surrounded by a double membrane with some osmiophilic plastoglobuli in the mostly dense stroma [11]. In recent years it has been shown that there exists for different plants a single basic pathway of chloroplast development from proplastids [12, 13], which includes at least two new transitional plastid stages. When during normal leaf growth the proplastid develops, it rapidly accumulates starch and turns into an *amyloplast*. In the further development starch disappears. In some plants an amoeboid configuration (amoeboplast) may follow the amyloplast stage [12]. In any case carbohydrate reserves are built up and stored before major biosynthetic and structural changes occur [12, 14]. The next step is the appearance of vesicles and tubules in the plastid stroma, which form an irregular structure, the *protubular body* [12, 13]. This plastid stage, which shows up in the dark and in the light, is termed here *prochloroplast* (Fig. 1).

In continuous darkness prochloroplasts are transformed into etioplasts which contain a highly ordered, crystallinic prolamellar body and many plastoglobuli. The etioplasts are not in the direct line of chloroplast development, they represent an artefact, which arises since normal light-dependent chlorophyll and thylakoid formation are blocked. Upon illumination of etiolated tissue the prolamellar body breaks down, the plastoglobuli disappear during thylakoid formation and the etioplast is gradually transformed into a young chloroplast [15]. In light-grown plants, however, the young chloroplast directly derives from the prochloroplast stage (Fig. 1).

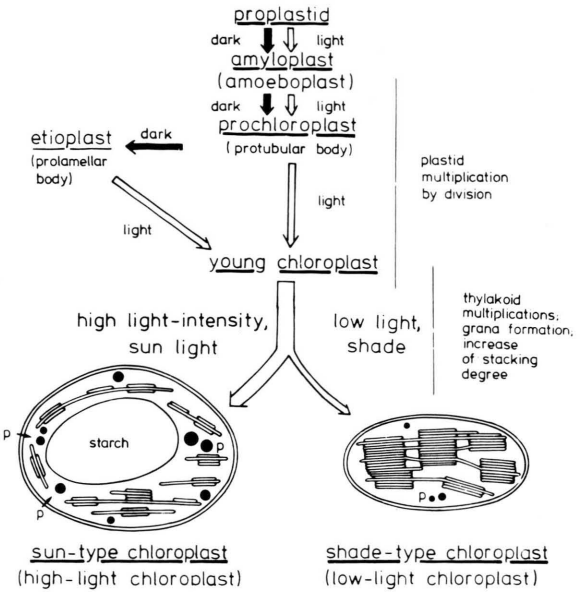


Fig. 1. Biogenesis and development of chloroplasts from proplastids in the dark (➡) and in the light (⇒). Modified scheme [2] including the results of [11, 12, 13, 17].

In the further stages of chloroplast differentiation there occurs rapid thylakoid multiplication and grana formation, small starch grains and also the plastoglobuli, which function as stores for excess lipids, reappear. During the differentiation from a young to a mature chloroplast it depends upon the environmental factors *e.g.* light intensity [1, 2] or cytokinin content [16] of the tissue, whether sun-type (high light) or shade-type (low light) chloroplasts are formed [17, 18] (Fig. 1). Sun-type chloroplasts, which at light saturation are very efficient in photosynthetic quantum conversion, are characterized by a lower frequency of thylakoids per chloroplast section, by fewer thylakoids per granum stack, by lower amounts of the light-harvesting chlorophyll *a*/*b*-proteins (LHCP's) per total chlorophyll and by a higher proportion of exposed thylakoid membranes; they also contain large starch grains and more plastoglobuli than the shade-type chloroplasts [2, 17–19]. The larger size of the photosynthetic unit (larger pigment antenna) of the shade-type chloroplasts is based on a larger width and height of the grana stacks, on more LHCP's and a higher stacking degree of thylakoids [17, 19].

During chloroplast biogenesis in the light the transition from amyloplasts *via* prochloroplasts to young chloroplasts may proceed so fast that one can see within one chloroplast the typical structural elements of all three plastid forms *e.g.* some remaining starch grains, vesicles and/or protubular structures and some thylakoids.

Another aspect of chloroplast biogenesis is the division and multiplication of chloroplasts, which proceeds in parallel to chloroplast differentiation and leaf growth. Mature green leaf cells exhibit much higher plastid (chloroplasts) numbers than meristematic or growing leaf cells [20]. Chloroplast division mainly occurs on the developmental stage of prochloroplasts and young chloroplasts (Fig. 1).

Interaction of herbicides with chloroplast development

The classical DCMU-type herbicides such as bentazon and diuron not only inhibit photosynthetic electron flow and decrease the sugar concentration in the leaves but have additional physiological effects on plant and chloroplast morphogenesis. They induce the formation of shade-type chloroplasts [1–3], which are less efficient in photosynthetic quantum conversion, a response which contributes to their effectivity as herbicides [9] (Fig. 2 and Table I). The chlorosis-inducing herbicides ("bleaching" herbicides) interfere with the formation of cyclic carotenoids [5], the chlorophyll which yet accumulates at very low light intensities is photobleached at higher light fluence rates [6–8]. At non-lethal concentrations one obtains rudimentary sun-type chloroplasts and at higher doses pigment-free leucoplasts (Fig. 2 and Table I). Other active ingredients (*e.g.* cytokinins, triadimefon) through affecting plant metabolism outside the chloroplast, also modify the structure and composition of the photosynthetic apparatus [1, 16]. A new compound of the latter-type is mevinolin. To a fourth group

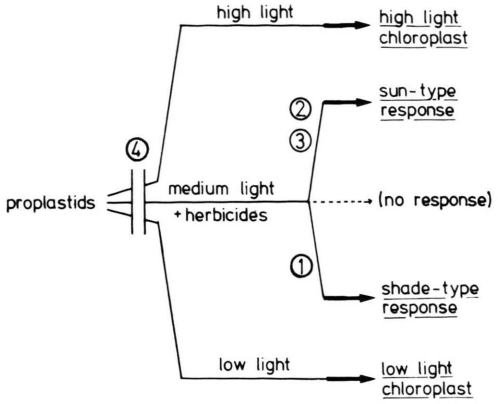


Fig. 2. Modification of the photosynthetic apparatus by light intensity, herbicides and other active ingredients. The sites of interaction of the different groups of herbicides and active ingredients (1, 2, 3 and 4) with chloroplast formation is indicated. Confer Table I for the herbicide groups 1 to 4.

Table I. List of different types of herbicides and active ingredients that interfere with chloroplast formation at an early or later stage of the development.

Herbicide types	Interference with	Result
1. *DCMU-type herbicides:*		
Diuron, bentazon, atrazin, some pyridazinones [1–3]	photosynthetic electron transport	shade-type chloroplasts
2. *"Bleaching" herbicides:*		
Amitrole, difunon, some pyridazinones (*e.g.* SAN 6706) [4–8]	carotenoid biosynthesis (bleaching of chlorophylls)	rudimentary sun-type chloroplasts + leucoplasts
3. *Special compounds:*		
Mevinolin [31]	cytoplasmic sterol formation	sun-type chloroplast
Tridimefon [1]	GA_3-synthesis	
Cytokinins [16]	change of phytohormone balance	
4. *Herbicides which block chloroplast differentiation at an early stage:*		
Sethoxydim Alloxydim	biogenesis and multiplication of chloroplasts	proplastids

belong the new herbicides sethoxydim and alloxydim (Table I and Fig. 2). All chemicals modify chloroplast development to some extent, the signal "no response" is never obtained.

Q_B and Q_B-protein

DCMU-type herbicides [21] bind to the Q_B-protein, which exhibits a rapid light-dependent turnover [22]. More recently it is suggested that Q_B and the DCMU-type inhibitors compete for the same binding site and can expel each other [23, 24]. The nature of Q_B is not known. It could be a plastoquinone [23, 24], but also phylloquinone, the endogenous naphthoquinone K_1, which is bound to the thylakoids [25]. Since halogenated naphthoquinones specifically block photosynthetic electron transport at the inhibition site of the DCMU-type herbicides [25, 26], it is concluded that this is the functional site of the endogenous naphthoquinone K_1 [25, 26]. Phylloquinone K_1 is certainly a good candidate for the unknown Q_B, and it would make sense if the electron (+ proton) flow into the large plastoquinone pool were regulated by a quinone which is not a plastoquinone. The turnover of the Q_B-protein increases with increasing light intensity [22] and it is supposed that it is destroyed under high light stress and photoinhibition conditions. The phylloquinone K_1 and β-carotene exhibit the highest labelling degree of all photosynthetic pigments and quinones. After 1 and 2 h of $^{14}CO_2$ photosynthesis in *Chlorella* the labeling degree of phylloquinone and of α- and β-carotenes amounts to 13 and 18% of the total carbon skeleton, whereas that of chlorophylls, lutein and plastoquinone is below 1 and 3%, respectively [27]. Whether the Q_B-protein contains phylloquinone and β-carotene has so far not been investigated, because of the high turnover of all three components this can, however, be anticipated.

Effects of mevinolin

Mevinolin, a metabolite from the ascomycete *Aspergillus terreus*, is a highly specific inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) which catalyzes the biosynthesis of mevalonic acid and is the key enzyme for the regulation of plant isoprenoid synthesis. In radish seedlings mevinolin applied *via* the roots blocks the length-growth of roots and hypocotyls but has a much lower effect on leaf growth [28, 29]. Biosynthesis and accumulation of sterols (cytoplasm) are inhibited to a larger extent in roots than hypocotyls and cotyledons [30, 31]. Mevinolin, however, exerts little effect on the accumulation of chlorophylls and carotenoids in leaves [31]. This suggests that chloroplasts possess their own mevalonate synthesizing pathway and that mevinolin does not pass the chloroplast envelope.

At low concentrations, which already affect sterol and ubiquinone accumulation in the cotyledons, mevinolin exhibits even a stimulatory effect on the formation of chloroplast quinones such as plastoquinone-9, phylloquinone and α-tocopherol (Schindler/Bach/Lichtenthaler, in preparation). Mevinolin slightly shifts chloroplast development towards the formation of sun-type (high light-type) chloroplasts (Fig. 1, 2), which are known to possess more electron transport carrier [1, 16] as compared to the control plants grown under the same light condition. This sun-type response of chloroplasts can also be seen by a change of the chlorophyll fluorescence kinetics (Kautsky effect). In mevinolin-treated plants the values for the variable fluorescence ratios (rise and decrease signals), which are a measure of photosynthetic quantum conversion and capacity, are as expected higher than in control plants [31]. By blocking the cytoplasmic mevalonate and sterol formation as its primary target, mevinolin indirectly modifies the differentiation and composition of chloroplasts. With respect to chloroplast development its mode of action is similar to those of cytokinins [16] and of the fungicide triadimefon [1].

Effect of sethoxydim

The herbicide sethoxydim is applied for selective postemergence control of annual and perennial grass weeds in a wide range of broad leaf crops [32]. Sethoxydim is absorbed by the leaves and readily translocated (phloem) to the meristematic zones of the leaf, shoot and root, where growth is inhibited. A similar effect on grass weeds has also been reported for the structurally related alloxydim [33]. The exact mode of action of sethoxydim or alloxydim are, however, not known.

In our attempt to obtain more information on its way of action we studied the effect of sethoxydim on chloroplast development and pigment formation

in barley and maize seedlings. When 8 day old barley plants are sprayed with sethoxydim ($300\ g \cdot ha^{-1}$) the development of the leaf blade (length growth and growth in width) is blocked (Table II). This leaf development inhibition by sethoxydim works to a higher extent in the secondary leaf, which is still developing than in the primary leaf, which at the time of treatment (8 d old plants) was almost fully developed. From day 8 to day 11 a great part of the tertiary leaf is developed in the controls, no tertiary leaf is formed, however, in the herbicide treated plants. The total CO_2-assimilation area (the leaf area responsible for photosynthetic light absorption) thus amounts only to about one third of that of the controls (Table II).

Sethoxydim does not block photosynthesis, as was determined by measuring *in vivo* the chlorophyll *a* fluorescence induction kinetics (Kautsky effect) of the intact leaves. In predarkened leaves the chlorophyll fluorescence rises *via fo* to a maximum *fp* and then decreases with the onset of photosynthesis to the steady state fluorescence *fs* (Fig. 3). The kinetics are similar for plants treated with sethoxydim and do not provide an indication of a disturbance of the photosynthetic apparatus. Furthermore the fluorescence decrease ratio ($R = fd/fs$ see Fig. 3), which is a measure of the amount of photosynthetic light quanta conversion, is in the same range (values of 1.3 to 2.5) for treated and untreated plants.

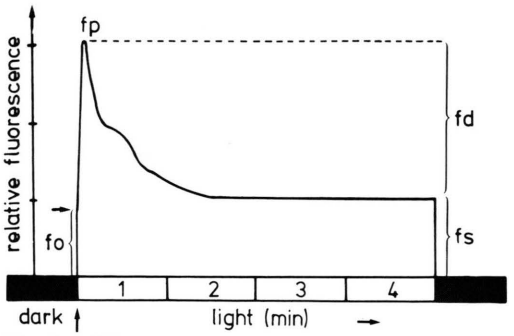


Fig. 3. Chlorophyll *a* fluorescence induction kinetic (Kautsky effect) in the secondary leaf of 11 day old barley seedlings. f_0 = ground fluorescence; f_p = maximum fluorescence; f_s = steady state fluorescence; f_d = fluorescence decrease.

Table III. Differences in the ultrastructure of chloroplasts from 11 day old barley seedlings, which were treated with sethoxydim ($300\ g \cdot ha^{-1}$) on the 8th day.

Secondary leaf, middle leaf region	Controls	+Sethoxydim
Thylakoid frequency [a]	194	124
Stacking degree of thylakoids in %	62	49
Ratio of stacked to exposed membranes	1.6	1.0
Average width of grana (μm)	0.61	0.46
Volume of chloroplasts (μm^3)	51	74

[a] μm thylakoid length per $10\ \mu m^2$ median chloroplast section.

Table II. Leaf parameters from 11d old barley seedlings grown without (controls) and with the herbicide sethoxydim ($300\ g \cdot ha^{-1}$). Mean of 20 leaf blades. The treatment was performed on the 8th day.

Parameters	Controls		+Sethoxydim	
Length of leaf blade (cm)				
primary leaf	14 ± 1		12.6 ± 0.5	
secondary leaf [a]	21 ± 2		6.2 ± 0.8	
tertiary leaf	8 + 1		not formed	
Width of leaf blade (mm)				
primary leaf	7.5 ± 1		7.1 ± 0.7	
secondary leaf [a]	9.0 ± 0.7		3.6 ± 0.5	
tertiary leaf	3.0 ± 0.6			
Area of leaf blade (cm^2)				
primary leaf [a]	10.1	**28.6**	7.6	**9.8**
secondary leaf [a]	16.3		2.2	
tertiary leaf	2.2			

[a] The differences between controls and herbicide treated leaves are significant ($P < 0.01$).

Sethoxydim has a strong inhibitory influence on biogenesis and differentiation of chloroplasts as is shown for barley seedlings. The chloroplasts of the upper an lower part of the secondary leaves from control plants exhibit the typical elongated chloroplast shape with a regular arrangement of grana and stroma thylakoids (Fig. 4a). In the upper, middle and lower parts of the 2nd leaf of sethoxydim-treated plants, the chloroplasts are nearly spherical and possess a larger volume (Fig. 4b and Table III). A lower thylakoid frequency, a lower stacking degree of thylakoids, lower values for the ratio of stacked to exposed photosynthetic membranes, and a lower average width of grana in the secondary leaves of sethoxydim-treated plants demonstrate that sethoxydim blocks the normal chloroplast differentiation and results in the formation of sun-type chloroplasts. Another sethoxydim effect is the much lower

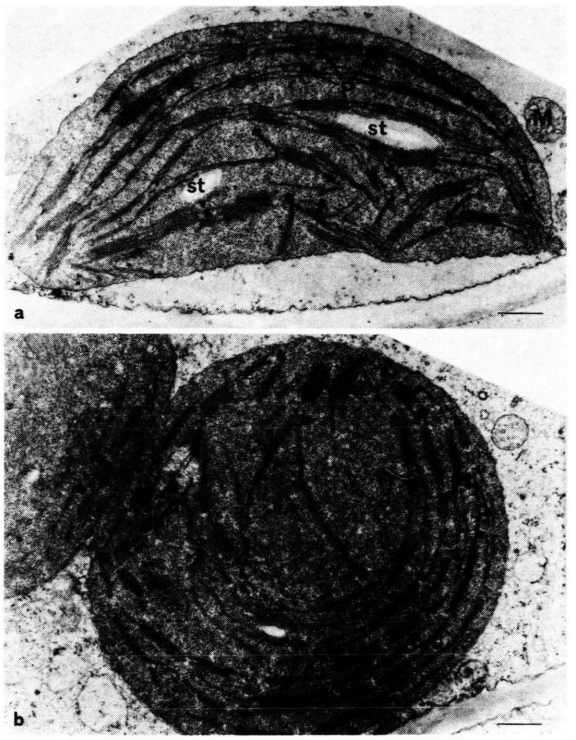
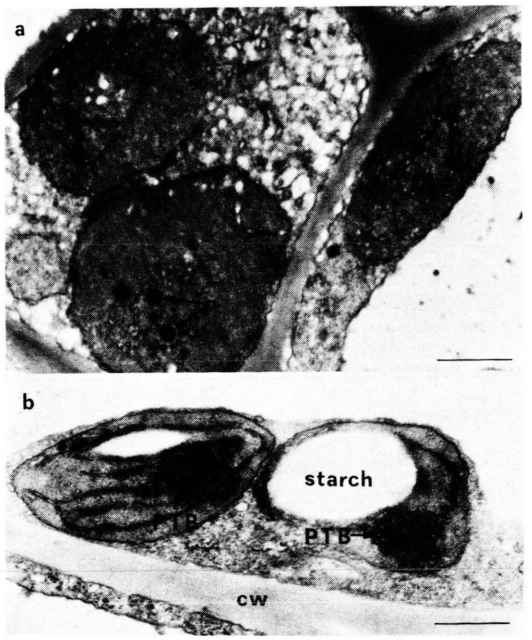


Fig. 4. Chloroplast of a secondary leaf (lower region) of 11 day old barley seedlings. a) control and b) 3 days after treatment with sethoxydim (300 g · ha^{-1}). st = starch; p = plastoglobuli; M = mitochondrion; bar = 0.5 µm.



chloroplast number (about one third and less) per sectioned mesophyll cell of the treated secondary leaf, indicating that sethoxydim not only interferes with chloroplast differentiation, but also with chloroplast replication.

The lowest and youngest part of the secondary leaf blade of treated plants exhibited, instead of mature chloroplasts as in controls, only the early stages of chloroplast biogenesis with vesicles and protubular structures, which are similar to the prochloroplasts of other plants (Fig. 5a). Prochloroplast structures as normal components are found in control plants in the meristem of the shoot apex (Fig. 5b). The shoot apex of sethoxydim treated-barley plants, however, contained only proplastids, that is the undifferentiated plastid form.

These observations emphasize that sethoxydim inhibits all stages of chloroplast biogenesis and differentiation from proplastids *via* prochloroplasts to young chloroplasts and their further development (thylakoid multiplication, grana formation as well as chloroplast division) into the mature chloroplasts of the mesophyll cells of fully expanded leaves.

In maize plants we have studied the influence of sethoxydim on the accumulation of chlorophylls and carotenoids during growth and development of the tertiary leaf. Since maize is more sensitive to sethoxydim than barley, lower doses (16 g, 31 g and 62 g · ha^{-1}) could be applied. With increasing levels of sethoxydim the increase of leaf blade length and of the level of chlorophylls and carotenoids is blocked (Table IV). On the fourth day after treatment the inhibition degree (above the level of 16 g · ha^{-1}) appears to be higher for the leaf length growth than for chlorophyll and carotenoid accumulation. At doses of 32 g · ha^{-1} the treated maize plants recover with time and regain normal leaf growth; white chlorophyll-free leaf zones, however, indicate that chloroplast biogenesis is inhibited longer than are cell multiplication and elongation.

At concentration of 125 g · ha^{-1} or higher doses there occurs no leaf growth and no increase in the levels of chlorophylls or carotenoids. Anthocyanins,

Fig. 5. Plastids from barley seedlings: a) prochloroplast-like plastids from the lower (youngest) region of the secondary leaf of 11 day old seedlings, 3 days after treatment with sethoxydim 300 g · ha^{-1} and b) prochloroplasts from the stem meristem of control plants. P = plastoglobuli; PTB = protubular body, cw = cell wall; bar = 0.5 µm.

Table IV. Addition to leaf length, chlorophyll and carotenoid content of the tertiary leaf of maize seedlings (from day 7 to day 12 after germination) in controls and in maize plants treated with sethoxydim. Mean of 4 determinations, standard deviation ± 7%. The values in parentheses indicate the inhibition in %.

Tertiary leaf	Leaf length [cm]	Chlorophylls [mg/100 leaves]	Carotenoids [μg/100 leaves]
a) Initial value	6.2	2.2	0.56
b) Controls	15.1 (0)	35.4 (0)	5.6 (0)
+ Sethoxydim			
16 g · ha^{-1}	11.0 (27)	25.2 (29)	4.6 (18)
31 g · ha^{-1}	2.8 (82)	11.9 (66)	2.7 (52)
62 g · ha^{-1}	2.0 (87)	7.0 (80)	1.8 (68)

not present in the control maize plants, are accumulated. Treatment on day 7 after germination resulted in about 102 μg cyanidin per secondary leaf which did not increase with a higher herbicide dosis. Induction of cyanidin synthesis and accumulation of cyanidins thus proceed when plant growth (leaf elongation, cell multiplication, thylakoid and pigment formation) is fully blocked by sethoxydim. Both effects (complete stop of growth and differentiation, as well as the induction of cyanidin formation) indicate that sethoxydim interfers in some way (perhaps indirectly) with protein formation. Growth of cells and of cytoplasmic and photosynthetic biomembranes is based on protein formation and requires the aromatic precursors of the shikimate pathway for the synthesis of aromatic amino acids, which are incorporated into proteins. When protein formation is, however, inhibited, the aromatic precursors are available for anthocyanin synthesis.

The results reported here indicate that chloroplast biogenesis and differentiation is a major target for the action of the herbicide sethoxydim. But it is certainly not the only one, there are also hints on its interference with protein formation and also with lipid formation. These additional sethoxydim effects are subject of our present investigations.

Acknowledgements

This work was sponsored by a grant from the Deutsche Forschungsgemeinschaft. I wish to thank Dr. A. W. Alberts, Rahway for a gift of mevinolin, Drs. D. Mangold and G. Retzlaff, Limburgerhof for a gift of sethoxydim, as well as Dr. D. Meier (electronmicrographs), Mr. R. Burgstahler (anthocyanin data) and Mrs. G. Ihrig for assistance.

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