Effects of Pyridazinone Herbicides during Chloroplast Development in Detached Barley Leaves

I. Effects on Pigment Accumulation and Fluorescence Properties

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Pyridazinone Herbicides, Carotenoids, Fluorescence Spectrum

The effects of three differently substituted pyridazinone herbicides on the accumulation of photosynthetic pigments and on the fluorescence properties of greening barley leaves were studied. The two trifluoro-methyl derivatives, SAN 6706 and 9789 caused inhibition in the accumulation of carotenes only after the first 24 h of greening. By the 48th hour of greening no detectable amounts of carotenes were present in the treated leaves. Considerable amounts of xanthophylls however remained present, and no bleaching of the leaves was observed. It is suggested that SAN 6706 and 9789 prevent the formation of the enzyme(s) catalyzing the desaturation of phytoene and phytofluene. Fluorescence spectra indicate that the organization of pigment forms altered seriously in the treated leaves. This experimental system may be useful to study the action of pyridazinone herbicides without causing complete photobleaching of the photosynthetic apparatus. SAN 9785 had only minor effects on pigment accumulation and also on the organization of pigment forms. The possible explanations of the effects observed are discussed.

Introduction

The phytotoxic behaviour of several substituted pyridazinones has been known for about ten years, but their mode of action is still a matter of discussion. It has been demonstrated that pyridazinones inhibit the Hill reaction in isolated chloroplasts when artificial electron acceptors are used [1]. Some evidence suggests however, that these compounds can not penetrate into the chloroplasts under *in vivo* conditions [2, 3]. It is noteworthy that the Hill reaction inhibiting capacity of the pyridazinones is independent of their chemical structure, which does not hold for other herbicidal effects. Two trifluoromethyl derivatives, SAN 6706 and 9789 are reported to be strong inhibitors of carotenoid biosynthesis, leading to the complete absence of all types of coloured carotenoid pigments [4, 5]. The absence of carotenoids results in the photodecomposition of chlorophylls and the desintegration of the chloroplast lamellar structure in light of high intensity [6, 7]. The absence of 70S chloroplastic ribosomes has also been demonstrated to be a consequence of the action of the bleaching compounds [8, 9]. SAN 9785 causes no inhibition of pigment biosynthesis, but leads to great alterations in the lipid- and fatty acid-composition of chloroplasts [10].

In order to gain more information about the herbicidal action of pyridazinone compounds, an investigation has been carried out concerning the effects of the differently substituted derivatives SAN 6706, 9789 and 9785 on the chloroplast development during the greening of etiolated detached barley leaves. This unusual experimental system seems to be very promising to study the herbicidal action of pyridazinones.

Materials and Methods

Plant material

Barley (*Hordeum vulgare* L.) seeds were germinated in the dark for 7 days. The upper 5-cm region of the etiolated leaves was cut off and placed on Petri dishes containing culture medium with and without 4×10^{-4} M herbicide. All operations were carried out under dim green light. The concentration of the herbicides was selected on the basis of previous experiments, so that the herbicides produced total bleaching in barley plants treated from the beginning of germination and kept under continuous illumination. The detached etiolated leaves were incubated in the medium for 24 h in the dark and were then exposed to continuous illumination by white light with intensity of 0.8 mW cm^{-2}.

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Pigment extraction and analysis

Pigments were extracted with ice-cold absolute acetone and were transferred immediately to petroleum ether. Chlorophyll *a*, chlorophyll *b* and the individual carotenoids were separated with thin-layer chromatography on plates of Silica gel G: cellulose powder (1:1) with the solvent mixture benzene:petroleum ether:ethanol:water (5:5:1:0.5) [11]. Quantitative determination of pigments was carried out by using the extinction coefficients after [12].

Fluorescence measurements

Fluorescence spectra of the leaf samples were recorded with a Perkin-Elmer MPF 44/A spectrofluorimeter at 77 K as described in [13].

Results

Accumulation of carotenoids in control leaves

In the dark-grown leaves relatively low amounts of carotenes and neoxanthine are present (Table I, columns 1 and 10) with equally high amounts of lutein + zeanxanthine and violaxanthine (columns 4 and 7). The carotene content is only 10% of the total carotenoids (columns 1 and 13), and the molar ratio of the xanthophyll pigments (lutein + zeaxanthine: violaxanthine:neoxanthine) is 6:6:1. After exposure of the leaves to continuous illumination, the total carotenoid content exhibits a two-fold increase during the first 12 h of greening. By this time the carotene content is 17% of the total carotenoids, and the molar ratio of the xanthophyll pigments is 4.5:3:1. The accumulation rates of the individual carotenoid pigments differ, the largest rates being observed for the carotene (column 1) and neoxanthine (column 10) fractions. During the next 12 h further increases occur. By the end of the first day of greening, the carotene content accounts for the one-fourth of the total carotenoids, and the molar ratio of the xanthophyll pigments is 4:2:1. As the greening proceeds, the accumulation of the carotenoid pigments slows down, and by the end of the second day of greening no further accumulation can be seen. At this time the carotene content accounts for one-third of the total carotenoids, and the molar ratio of the xanthophylls is 2:1:1.

Effect of SAN 9785 on the accumulation of carotenoid pigments

There is a 30% reduction in the total carotenoid content in the dark-grown leaves after incubation with SAN 9785 (columns 13 and 14). The percentage ratio of the carotenes remains unchanged (10%), but the xanthophyll molar ratio is changed to 8:9:1. After exposure of the leaves to light, the accumulation of the carotenoid pigments is similar to that in the control leaves, but the accumulation rates of the individual carotenoids are different. During the first 12 h a large increase can be observed in the carotenes and in the lutein + zeaxanthine fraction, causing a three-fold increase in the total carotenoid content. The xanthophyll molar ratio is changed to 7:3:1. This high accumulation rate slows down during the second day of greening. At this time the amounts of the individual carotenoid pigments equal those in the control, with slightly less carotene content.

Effects of SAN 6706 and 9789 on the accumulation of carotenoid pigments

No appreciable difference in the amounts of the individual carotenoids as compared to the control

Table I. Amounts (in nmol/g fresh weight) of carotenoid pigments in greening barley leaves treated with pyridazinone herbicides at various stages of greening. Each value is the mean of three independent experiments.

Exposure Time [h]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Carotenes			Lutein + Zeax.			Violaxanthine			Neoxanthine			Total		
	Cont.	9785	6706 9789	Cont.	9785	6706 9789	Cont.	9785	6706 9789	Cont.	9785	6706 9789	Cont.	9785	6706 9789
0	5.9	4.5	4.4	23	15	23	23	18	23	3.8	2.0	4.1	56	40	54
12	21	26	14	54	59	61	36	25	47	12	8	10	123	119	132
24	53	43	16	82	73	76	42	36	29	22	15	21	199	166	142
48	67	53	–	83	88	66	41	45	12	40	39	15	230	225	93

can be observed in the dark-grown leaves after the incubation with these pyridazinones. After exposure of the leaves to light, accumulation of pigments takes place during the first 12 h of greening. The accumulation rate of the lutein + zeaxanthine and violaxanthine fractions (columns 6 and 9) exceed the control values, whereas the carotene content reaches only 66% of that in the control (column 3). During the next 12 h of greening the accumulation of pigment show different features: further accumulation can be observed only in the lutein + zeaxanthine and neoxanthine fractions (columns 6 and 12), while the carotene content remains unchanged (column 3), and the violaxanthine content (column 9) decreases considerably. At this time the carotene ratio is 11%, and the molar ratio of xanthophylls is 3.5:1.5:1. During the second day of greening no further accumulation of carotenoid pigments occurs. No detactable amounts of carotenes are present at the end of the second day. The xanthophyll content is also reduced, and their molar ratio is 5:1:1. The two herbicides showed very similar effects at every stage of the greening.

Accumulation of chlorophylls and the effects of pyridazinones

Chlorophyll *a* and chlorophyll *b* are accumulated continuously in the control leaves during greening (Fig. 1). The chlorophyll *a* content of SAN 9785-

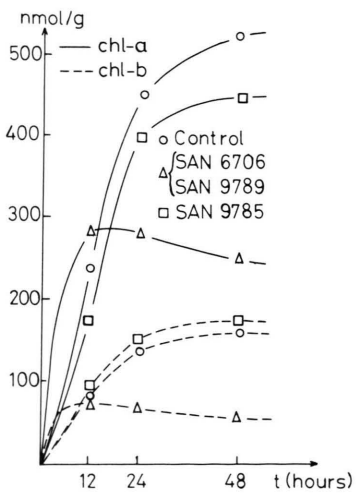

Fig. 1. Time course of the accumulation of chlorophyll *a* and chlorophyll *b* in greening barley leaves treated with pyridazinone herbicides. Values are in nmol/g fresh weight. Each value is the mean of three independent experiments.

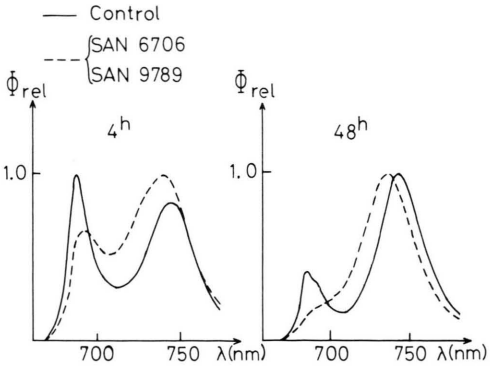

Fig. 2. Fluorescence spectra of barley leaves treated with SAN 6706 and 9789 after 4 h (left) and 48 h (right) of greening measured at 77 K.

treated leaves is reduced in every stage of greening, while the chlorophyll *b* content exceeds the control value. In SAN 6706- and 9789-treated leaves a higher accumulation rate of chlorophyll *a* can be observed in the first 12 h of greening than in the control, whereas the chlorophyll *b* contents are equal. This stimulatory action was observed also in the treated leaves greening under low light intensity [13]. As the greening proceeds, the further accumulation of chlorophylls stops, and a slight destruction occurs, causing a 50% loss in chlorophyll *a* and a 60% loss in chlorophyll *b* by the end of the second day.

Fluorescence of the developing leaves

The formation of photosynthetically competent pigment forms was studied by low-temperature fluorescence measurements. The development of the final shape of the fluorescence spectrum of green leaves was completed in 48 h of greening (Fig. 2). No significant difference between the control and SAN 9785-treated leaves was observed during greening, indicating that this compound exerts no interference on chloroplast development. This is not the case for SAN 6706 and 9789, for these herbicides caused marked alterations in the fluorescence spectra in all stages of greening. It seems that the early phase of greening proceeds more rapidly in the treated leaves, as the spectrum shows a more developed state after 4 h of greening. A 5 nm shift of the long-wave maximum of the spectrum towards shorter waves can be observed, the shift reaching 10 nm as the greening proceeds. This change is

accompanied by a gradual reduction in the fluorescence intensity of the short-wave band: in the final stage of greening no discrete fluorescence bands can be distinguished in this region, and the whole short-wave band tends to disappear from the spectrum.

Discussion

SAN 6706 and 9789 are known to be effective inhibitors of carotene biosynthesis. This inhibitory action has been shown to be independent of the light intensity used, and also occurs in darkness [4]. Under our experimental circumstances, however, only very slight change in the carotene content of dark-grown leaves was observed after treatment with these compounds. A similarly slight change was detected in SAN 9785-treated leaves. Since this compound is not an inhibitor of carotenogenesis, we conclude that the two bleaching compounds had no appreciable effect on carotene biosynthesis in the dark. Several possibilities exist to explain this finding: 1) The herbicide concentration used in our experiments was insufficient for the inhibitory action to be exerted. In other experimental systems (growing intact barley plants from the beginning of germination in the presence of the same concentration of the inhibitors) however, we observed a total loss of all coloured carotenoids in the dark. 2) Practically no further accumulation of carotenes takes place during the 24 h incubation period, so an inhibitory action, even if it exists, can not be observed. This would mean that the turnover rate of carotenes is very small, which is not very likely. 3) The penetration of the herbicides from the medium to the leaves, and within the leaves to the functionally active sites, is very low. This can explain the fact that, during the first day of greening under continuous illumination, carotene biosynthesis and accumulation do occur in the treated leaves too, but at a considerably lower rate. This accumulation stops and all carotenes disappear by the end of the second day of greening. It is a likely assumption that the disappearance of the carotenes coincides with the time by which a sufficient amount of inhibitors is accumulated in the leaves. 4) Carotene biosynthesis proceeds via enzymatical desaturation of colourless precursor molecules, *e.g.* phytoene and phytofluene to carotenes. SAN 6706 and 9789 are reported to inhibit this desaturation step [14]. The delay in the inhibitory action on carotene biosynthesis in our case can be explained, if we assume that this inhibitory action results from interference of the chemicals with the *de novo* biosynthesis of the enzyme(s) catalyzing the desaturation reaction and not from the direct action on the function of these enzymes.

It is worth mentioning that, once the carotene content stops accumulating, an additional effect of SAN 6706 and 9789 on the xanthophyll content can be observed. It seems that the zeaxanthine → violaxanthine transformation is inhibited, but the violaxanthine → neoxanthine transformation can proceed. This experimental system provides a useful tool for studying the biosynthesis of xanthophylls *per se*.

Chlorophylls are known to be photodecomposed under high light intensity in the absence of coloured carotenoids and in the presence of molecular oxygen [6, 7]. In our case, however, the situation is different, because after two days of greening considerable amounts of xanthophylls are present in the SAN 6706- and 9789-treated leaves, and xanthophylls have been demonstrated to exert effective protection against chlorophyll photobleaching [15]. In our experiments parallel to the disappearance of the carotenes the chlorophyll accumulation stopped, but appreciable degradation did not occur. Our results favour the idea that pyridazinones can interfere with chlorophyll biosynthesis. This includes the possibility that these compounds affect the synthesis of proteins necessary for the formation of chlorophyll-protein complexes. It is interesting in this respect that the low-temperature fluorescence spectra of SAN 6706- and 9789-treated leaves in the final stage of greening are very similar to those obtained after treatment with a chloroplast protein synthesis inhibitor, lincomycin [16]. A similar change in the spectrum was detected following the application of benzonitrile herbicides [17], indicating that this symptom can be considered as a general response of plants to different herbicides and inhibitors.

Acknowledgements

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