Effect of the Herbicide San 6706 on Biosynthesis of Photosynthetic Pigments and Prenylquinones in *Raphanus* and in *Hordeum* Seedlings

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The pyridazinone herbicide San 6706 blocks chlorophyll and carotenoid formation simultaneously in a correlated way in both *Raphanus* and *Hordeum* seedlings. With decreasing carotenoid content the carotenoid precursor phytoene appears in San 6706-treated plants.

The accumulation of the chloroplast prenylquinones (plastoquinone-9, α-tocopherol, α-tocoquinone and vitamin K_1) is much less affected. This indicates that the biosynthesis of C_{20}- and C_{45}-prenyl chains (geranyl-geraniol, phytol and solanesol) which are incorporated in prenylquinones is not blocked by San 6706.

It is assumed that the block of chlorophyll and carotenoid formation is due to one basic inhibition mechanism. The possibility that San 6706 may interfere with chloroplast ribosome formation, is discussed.

Introduction

Pyridazinone derivatives are effective inhibitors of the photosynthetic electron transport catalyzed by photosystem II, which is thought to be the primary herbicide action[1]. In addition to this the substituted pyridazinone herbicide San 6706, a 4-chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-*m*-tolyl-3(2H)-pyridazinone, as well as some other pyridazinone herbicides are known to inhibit carotenoid synthesis in higher plants[2–5], in algae[5, 7] and in a myxobacterium[8]. In algae and higher plants the inhibition of carotenoid synthesis is paralleled by a block of the chlorophyll formation[2, 5–7]. Carotenoid biosynthesis is inhibited by San 6706 at the desaturation point of phytoene and phytofluene[3, 5, 6]. A simultaneous block of chlorophyll and carotenoid accumulation combined with the appearance of polyenes such as phytoene, phytofluene, ξ-carotene or lycopene has also been reported for some other, structurally different herbicides such as amitrole, dichlormate, pyrichlor and difunone[5, 7, 9–11]. Some authors postulate that the formation of chlorophyll is not inhibited by these herbicides but that the chlorophylls are secondarily bleached out when the carotenoid synthesis has been blocked[2, 6, 7, 9]. It is also assumed that there may be a block in an early step of prenyl chain formation.

Prenylquinones with their isoprenoide side chains belong together with chlorophylls and carotenoids to the group of prenyllipids. By studying the accumulation pattern of the individual chloroplast prenyllipids in pyridazinone-treated plants we wanted to see whether prenylquinone formation is similarly blocked as that of chlorophylls and carotenoids and whether San 6706 blocks prenyl chain formation at an early biosynthetic step. These investigations should also give more information on the regulation of prenyl chain metabolism in plants.

Materials and Methods

Seedlings of *Raphanus sativus* L. (Saxa-Treib) and *Hordeum vulgare* L. (Breun's Villa) were grown under continuous illumination (Fluora lamps, 800 μW/cm², 22 °C. 80% r.h.) in a growth chamber on 10% nutrient solution after van der Crone[12]. San 6706 was given to the nutrient solution (0.1, 0.5, 1 and 10 μmol/l) on sowing. The seedlings were germinated for 2 days in darkness, then illuminated and harvested after 3 and 8 days *(Raphanus)* or 7 and 10 days *(Hordeum)*. Cotyledons *(Raphanus)* and primary leaves + coleoptiles *(Hordeum)* were macerated in an ultrathurrax homogenizer with methanol, repeatedly extracted with mixtures of methanol and petroleumether (b.p. 40 – 60 °C) and the prenyllipids transferred to petroleumether.

The chlorophylls were determined after Ziegler and Egle[13] and the carotenoids after Hager and Meyer-Bertenrath[14] using an extinction coefficient $E_{1\,\mathrm{cm}}^{1\%}$ of 2500. For phytoene the coefficient given by

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Davies[15] was used. The level of prenylquinones was measured spectrophotometrically after thin layer chromatography[16] or by high pressure liquid chromatography[17]. The identification and quantitative determinations were performed on a Beckman spectrophotometer (model DB – GT). The results represent mean values from 3 separate determinations.

Results and Discussion

In *Raphanus* seedlings, the expansion growth of the cotyledons is promoted by treatment with San 6706. The surface area of cotyledons is 20% higher than in the control plants. At a medium concentration of 0.5 to 1 μM San 6706 green and pigment-free cotyledon zones alternate, at higher concentrations (10 μM) the cotyledons appear uniformly white. Anthocyanins (pelargonidin), which are formed in epidermal and subepidermal layers of cotyledons and hypocotyl[18], are still to be seen.

The accumulation of chlorophylls and carotenoids is successively inhibited in a correlated way by increasing concentrations of the herbicide San 6706 (Table I). The formation of prenylquinones, however, is much less affected by San 6706. The inhibition degree for prenyllipid formation is age-dependent. At medium San 6706 concentrations (0.5 and 1.0 μM) the inhibition degree of chlorophyll and carotenoid synthesis decreases with increasing age of *Raphanus* seedlings while that of prenylquinones rises (Table II). Using high levels of San 6706 (10 μM), which cause the formation of albino cotyledons, the inhibition percentage is about the same in 3 and 8 d old *Raphanus* plants.

Table I. % Inhibition of prenyllipid synthesis in 3 d old *Raphanus* seedlings by increasing concentrations of the herbicide San 6706. The prenylquinone fraction comprises total plastoquinone-9, α-tocopherol and α-tocoquinone.

San 6706 [μmol]	Chlorophylls [%]	Carotenoids [%]	Prenylquinones [%]
0.1	9	4	8
0.5	40	47	4
1.0	93	94	14
10.0	97	97	40

Hordeum seedlings behave similarly to *Raphanus* plants by treatment with San 6706. Higher San 6706 concentrations induce the formation of pigment-free albino leaves and coleoptiles. The younger, lower part of the primary leaf is more sensitive to San 6706 application than the oldest part, the leaf tip. The latter (in about the first 2 cm region) may still contain some chlorophylls and carotenoids even at a 10 μM San 6706 concentration. The width of the primary leaf appears to be a little larger at 0.1 μM and somewhat smaller at 10 μM San 6706.

Table II. Inhibition percentage for chlorophyll, carotenoid and lipoquinone synthesis induced by San 6706 treatment (1 μM) in *Raphanus* seedlings of different age.

	3 d [%]	8 d [%]
Chlorophylls	93	69
Carotenoids	94	70
Total plastoquinone-9	23	46
α-Tocopherol (+ α-tocoquinone)	3	17

In contrast to *Raphanus* plants a low level of San 6706 (0.1 μM) induces in *Hordeum* seedlings a promotion of prenyllipid synthesis, which gives higher levels for chlorophylls (+15%), carotenoids (+20%), total plastoquinone-9 (+40%) and α-tocopherol + α-tocoquinone (+47%) than in the 7 d old control plants. This San 6706 promotion effect is due to an enhanced leaf development in the first days and can no more be seen in the 10 d old plants. With higher concentrations of San 6706 (1 and 10 μM) one obtains an increasing inhibition of prenyllipid formation. As in *Raphanus* seedlings the formation of chlorophylls and carotenoids is blocked in a correlated way (Table III). Again the accumulation of the prenylquinones is less affected than that of the photosynthetic pigments. In *Raphanus* plants the formation of plastoquinone-9 is inhibited to a higher degree than that of α-tocopherol.

Table III. % Inhibition of prenyllipid accumulation by the herbicide San 6706 in 8 d old *Raphanus* and 7 d old *Hordeum* seedlings with indication of standard deviation.

	Raphanus		*Hordeum*	
	1 μM	10 μM	1 μM	10 μM
Chlorophylls	69 ± 4	98 ± 4	39 ± 3	91 ± 6
Carotenoids	70 ± 5	92 ± 3	35 ± 6	93 ± 5
Plastoquinone-9	46 ± 3	65 ± 4	11 ± 3	61 ± 5
Phylloquinone (vitamin K_1)	20 ± 6	62 ± 7	22 ± 5	60 ± 6
α-Tocopherol (+ α-tocoquinone)	17 ± 3	19 ± 3	5 ± 3	56 ± 4

The formation of phylloquinone is also less affected than that of chlorophylls and carotenoids (Table III).

The inhibition degree for chlorophyll and carotenoid synthesis does not decrease as in *Raphanus* plants but rises with increasing age of barley seedlings (7 d → 10 d) for both 1 and 10 μM San 6706. The inhibition percentage for the prenylquinones, in turn, increases at 1 μM and decreases at 10 μM San 6706 with rising age of barley seedlings.

Table IV. Accumulation of phytoene in barley (100 primary leaves) and *Raphanus* plants (200 cotyledons) treated with the herbicide San 6706 (μg per 100 plants).

	Control	+ San 6706 1 μM	 10 μM
Raphanus:			
Carotenoids	*1215*	*294*	*61*
Phytoene	0	46	78
$\frac{\text{Phytoene}}{\text{Carotenoids}}$	–	0.16	1.28
Hordeum:			
Carotenoids	*650*	*506*	*31*
Phytoene	0.5	53	29
$\frac{\text{Phytoene}}{\text{Carotenoids}}$	–	0.10	0.95

It has been demonstrated before that San 6706 causes inhibition of carotenoid synthesis with the simultaneous accumulation of colourless polyenes such as phytoene and phytofluene [5, 6]. Phytoene has also been found in *Raphanus* and barley plants treated with San 6706 (Table IV). Phytofluene could not be detected or at the most, in trace amounts. The absorption spectra and maxima for phytoene corresponded to those given by other authors [6, 7, 15].

The differential inhibition degree of the single prenyllipids changes the ratios of the individual components. The higher chlorophyll a/b ratios as well as the lower ratio for chlorophyll a to prenylquinones speak for a high light intensity adaptation of the remaining photosynthetic apparatus as compared to the control plants (Table V). Similar changes in the prenyllipid ratios combined with a higher photosynthetic activity have also been found at high light intensities, in sun leaves and in blue light as compared with low light intensities, shade leaves and red light respectively (see l. c. [19]). From this it appears that San 6706 not only blocks pigment formation but also has an influence on the size and composition of the few photosynthetic units still being formed.

The accumulation of the prenylquinones being little or much less affected than the formation of the photosynthetic pigments indicates that the early steps in prenyl chain biosynthesis are not or little influenced by San 6706. The synthesis of geranylgeraniol and of phytol, which are incorporated in α-tocopherol, α-tocoquinone and vitamin K_1 still proceeds at good rates under the influence of San 6706. The inhibition of chlorophyll synthesis, as described here, can thus not be due to a missing geranylgeraniol production (Fig. 1). The formation of the C_{45} solanesyl chain is also less inhibited as can be seen from the plastoquinone-9 accumulation.

The simultaneous block of carotenoid and chlorophyll synthesis by San 6706 and other herbicides (Fig. 1) raises the question whether there exists one basic inhibition mechanism which blocks the biosynthesis of both prenyllipid classes. So far it has been postulated that the primary site of action of San 6706 [2, 6, 7] as well as of amitrole [9], dichlormate [9], pyrichlor [9] and difunone [7] is the block of carotenoid biosynthesis, which secondarily would result in a photooxidation of chlorophyllls. This photo-bleach-

Raphanus:	a/b	x/c	$\frac{a+b}{x+c}$	a/PQ-9	a/α-T	a/α-TQ	a/K_1
Control	*3.1*	*2.9*	*5.4*	*15*	*12*	*270*	*302*
0.5 μM	3.2	3.0	4.9	13	9	169	
1.0 μM	3.3	3.5	5.3	8.7	4.7	72	122
10.0 μM	*3.5*	*6.6*	*1.4*	*1.1*	*0.3*	*49*	*3.0*
Hordeum:	a/b	x/c	$\frac{a+b}{x+c}$	a/PQ-9	a/α-T	a/α-TQ	a/K_1
Control	*3.5*	*2.4*	*5.2*	*18*	*32*	*230*	*379*
0.1 μM	3.6	2.4	5.1	15	30	210	
1.0 μM	3.7	2.5	5.5	12	20	178	223
10.0 μM	*3.7*	*2.7*	*6.4*	*4*	*6*	*37*	*97*

Table V. Changes of prenyllipid weight ratios in *Raphanus* and *Hordeum* seedlings induced by San 6706 treatment.

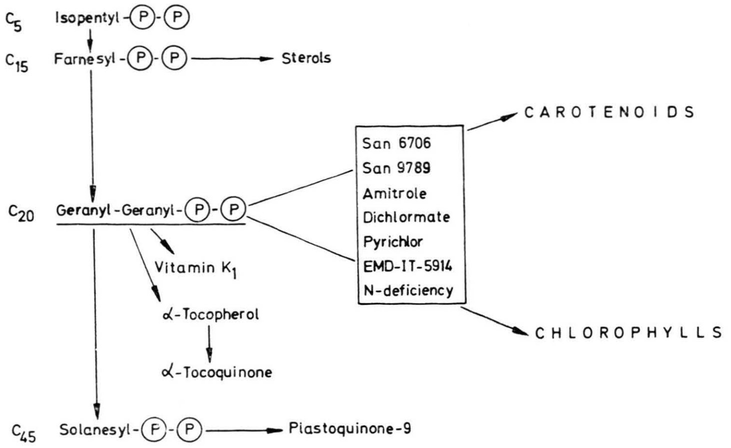

Fig. 1. Scheme for the formation of photosynthetic prenyllipids with indication of the inhibition site of some herbicides.

ing hypothesis, however, is unlikely for several reasons.

Using different concentrations of San 6706 and different incubation times we always find a correlated inhibition of chlorophyll and carotenoid formation for both *Raphanus* and *Hordeum* plants. This has now been confirmed in wheat seedlings [5]. Also, if photo-bleaching of the chlorophylls (porphyrin ring) would occur, one should detect an increased concentration of the remaining phytol. This is not the case [5]. The finding that under certain conditions *e. g.* in darkness a herbicide induced block of carotenoid biosynthesis can soon be localized but not (or not yet?) in chlorophyll formation [6] could be due to differences in half life times of either the enzymes and/or the pigments involved. In fact, in *Chlorella* suspensions under photosynthetic conditions, it has been demonstrated that the turnover rates of carotenes are faster than those of chlorophylls [20].

Furthermore in two cases a double inhibition effect in the chlorophyll and in the carotenoid metabolism has been demonstrated, which speaks against the photo-bleaching hypothesis. Thus, difunone not only blocks the desaturation step of carotenoid synthesis [7], but also affects the formation of the porphyrin ring by a specific inhibition of the synthesis of the enzyme porphobilinogenase [11]. Amitrole treatment, in turn, not only accumulates the carotenoid precursor ξ-carotene but also results in the formation of 'false' chlorophylls such as geranylgeraniol- and dihydrogeranylgeraniol-chlorophylls [5]. The situation with San 6706 resembles very much the early steps of nitrogen deficiency, which reduces chlorophyll formation by interference with the porphyrin ring synthesis and also inhibits, in a correlated way, the accumulation of carotenoids [12]. The formation of prenylquinones in turn is not or much less affected as has been shown here too after San 6706 treatment. In addition to this, San 6706 not only blocks the formation of chlorophylls and carotenoids but also that of other thylakoid lipids such as galactolipids [21] and the major chloroplast fatty acid linolenic acid [22], an observation which cannot be explained by a photooxidation mechanism.

Taken together the data mentioned here point to a common basic inhibition mechanism for San 6706 and other herbicides which results in a block of both chlorophyll and carotenoid formation. If so, what could this primary point of interaction be? It has been shown that San 6706 reduces the number of chloroplast ribosomes [2] but leaves those of the cytoplasm unaffected. Chloroplast ribosomes are missing in amitrole [23–25] and dichlormate [26] treated plants. It is of interest in this respect that the plant growth regulator glyphosine, which induces chlorosis, also acts via a reduction of the level of chloroplast ribosomes and chloroplastic RNA [27]. Thus the central inhibition mechanism for the action of San 6706 and other herbicides could be their interference with the formation of chloroplast ribosomes. As a consequence this would result in a simultaneous synthesis inhibition of chloroplastic enzymes needed for both, carotenoid and chlorophyll biosynthesis.

That the formation of chloroplastic prenylquinones is much less affected by San 6706 treatment, as has been shown here, could mean that the enzymes for prenylquinone synthesis possess either a longer half life time or are not formed with the help of chloroplast ribosomes. There are, indeed, some recent observations from which it appears that prenylquinones or their precursors (*e. g.* prenyl chains) may be synthesized outside the chloroplast and become transported into chloroplasts only in the final stage of biosynthesis [19, 28–30].

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