Effects of Pyridazinone Herbicides during Chloroplast Development in Detached Barley Leaves
III. Effects of SAN 6706 on Photosynthetic Activity and Chlorophyll-Protein Complexes

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SAN 6706, Electron Transport, Fluorescence Induction

The photosynthetic activities of barley leaves *in vivo* and of thylakoids, isolated from the leaves, *in vitro* were studied during greening in the presence or absence of 2×10^{-4} M SAN 6706. The degree of chlorophyll bleaching increased from 32% at 24 h of greening to 60% at the final stage. Treated leaves were unable to carry out $^{14}CO_2$-fixation, their fluorescence induction properties showed very limited, if any, photosystem II-activity, and the thylakoids isolated from the leaves were also inactive in mediating ferricyanide Hill reaction. The leaves, however, exhibited light-induced quenching of fluorescence revealed by slow fluorescence induction measurements; and the thylakoids were active in mediating photosystem I-specific *in vitro* Mehler reaction. Thylakoid membranes of the chloroplasts isolated from treated leaves contained CP 1 and LHCP^3 bands as revealed by polyacrylamide gel electrophoresis. From these results it is concluded that i) greening in the presence of SAN 6706 leads to the formation of inactive photosystem-II units; that ii) photosystem-I *per se* is active *in vitro,* and can, possibly, mediate cyclic electron transport *in vivo* coupled to the formation of ATP; and that iii) the presence of xanthophyll pigments is required for the assembly of the light-harvesting complex.

The experiments were repeated with the application of SAN 9789, another pyridazinone compound, and yielded practically the same results as those obtained with SAN 6706. For practical reasons, only the results obtained with SAN 6706 are demonstrated throughout this communication.

Introduction

The pyridazinone herbicides SAN 6706 and 9789 are known to be inhibitors of the photosynthetic electron transport of chloroplasts *in vitro* [1, 2]. Their main phytotoxic action, however, is to induce chlorosis leading to complete photobleaching of the photosynthetic apparatus under *in vivo* conditions when applied in seed-treatments from the onset of germination [3, 4]. It was demonstrated that these compounds are specific and potent inhibitors of the carotene biosynthesis [5, 6], which leads to complete absence of coloured carotenoid pigments. The lack of the protective action of carotenoids results in an over-excitation of chlorophylls which leads to their photobleaching under high light intensities [7, 8].

Little is known about the *in vivo* action of these pyridazinones on the photosynthesis, since the investigations carried out so far induced complete bleaching [9, 10]. Only a few reports appeared (*e.g.* [24]) dealing with objects not completely bleached. Our previous reports [11, 12] demonstrated that greening of detached barley leaves in the presence of pyridazinone compounds proved to be a valuable experimental system, since no complete photobleaching was observed during the 72-hour greening period. While carotenes disappeared during greening, substantial amounts of xanthophylls and chlorophylls remained present in the leaves greened in the presence of 2×10^{-4} M SAN 6706 and 9789.

Abbreviations: SAN 6706, 4-chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-M-tolyl)-3-(2H)-pyridazinone; SAN 9789, 4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-M-tolyl)-3-(2H)-pyridazinone; F_i and F_m, fluorescence intensities at 40 ms and ls, respectively, in fast fluorescence induction measurements; P and T, initial and terminal levels of fluorescence, respectively, in slow fluorescence induction measurements; PS-1, photosystem-I; PS-2, photosystem-II; DCPIP, 2,6-dichlorophenol indophenol; $DCPIPH_2$, chemically reduced form of DCPIP; CPC, chlorophyll-protein-complex; SDS, sodium dodecyl sulphate; CP 1, chlorophyll-protein complex of the reaction centre of PS-1; CP_a, chlorophyll-protein complex of the reaction centre of PS-2; LHCP^3, monomer form of the light-harvesting chlorophyll-protein complex; Q, first stable primary electron acceptor of PS-2; PQ, plastoquinone; P680, reaction centre chlorophyll of PS-2; P700, reaction centre chlorophyll of PS-1; PAGE, polyacrylamide gel electrophoresis.

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In this communication the photosynthetic characteristics of the leaves greened in the presence of the bleaching herbicides are described. It is shown that while the PS-2 units are inactive (similarly to the finding presented in ref. [24]), those of PS-1 display substantial activity *in vitro*. It is also shown that LHCP[3] is present when complete bleaching is prevented.

Materials and Methods

Plant material

Barley (*Hordeum vulgare* L., var. Horpácsi kétsoros) seeds were germinated and were grown for 7 days in the dark. The upper 6-cm part of the etiolated leaves was than cut off and treated with 2×10^{-4} M SAN 6706 as described in [11]. After an additional incubation period (24 h) in the dark, the leaves were exposed to continuous illumination with white light of 0.8 mW cm^{-2} intensity for 3 days.

Pigment contents of the leaves were analyzed as described elsewhere [11].

The $^{14}CO_2$-fixation activity of the intact leaves was studied as described previously [13].

Fluorescence induction measurements on intact leaves were carried out with a laboratory-built apparatus after a 30-min dark adaptation. A xenon lamp of 650 W was used to produce the actinic beam. Blue actinic light of 5 mW cm^{-2} was transmitted by a Schott BG 12 filter. The opening of the shutter was completed within 2 ms. Fluorescence emitted at 90 °C was detected with a photomultiplier through a red SIF 675 interference filter and recorded with a transient recorder. The dwell time between 1024 samplings was 1 ms and 300 ms in the fast and slow fluorescence induction measurements, respectively. In each experiment, 16 independent curves were recorded and averaged automatically with an averaging unit attached to the transient recorder.

Chloroplasts were isolated from the leaves by grinding in an ice-cold isolation medium containing 0.33 M sorbitol, 10 mM $Na_4P_2O_7$, 4 mM $MgCl_2$, 2 mM ascorbic acid and 0.05% (w/v) bovine serum albumin, pH 6.5, in an Ultra-Turrax homogenizer for 20 s at 8000 rpm. The homogenate was filtered through 8 layers of muslin and centrifuged at $2200 \times g$ for 2 min at 4 °C. The chloroplast pellet was resuspended in ice-cold medium containing 0.05 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 50 mM tricine, pH 7.6. The chlorophyll contents of the samples were determined according to [14].

The activity of electron transport from water to K-ferricyanide (Hill-reaction) was measured with a Clark-type oxygen electrode (Rank Brothers, Bottisham, England) as light-induced oxygen evolution. The 3.1 ml reaction mixture contained 0.33 M sorbitol, 50 mM tricine, 5 mM $MgCl_2$ and 0.5 mM $K_3[Fe(CN)_6]$, 5 mM NH_4Cl, pH 7.8. Samples containing thylakoids equivalent to 40 μg chlorophyll were illuminated with white light of 10 mW cm^{-2} intensity at a constant temperature of 25 °C.

The activity of the photosystem I-dependent electron transport (*in vitro* Mehler reaction) was measured polarographically as light-induced oxygen consumption in the presence of DCPIP/ascorbate and methylviologen. The 3.1 ml reaction mixture contained 0.33 M sorbitol, 50 mM tricine, 5 mM $MgCl_2$, 10 mM methylviologen, 50 μM DCMU, 0.2 mM DCPIP, 6 mM Na-ascorbate, pH 7.6, and thylakoids equivalent to 40 μg chlorophyll [15].

For the analysis of chlorophyll-protein complexes, chloroplasts were isolated from the leaves after 72 h of greening. Isolation of chloroplasts, solubilization of thylakoids membranes and separation of CPCs by SDS-PAGE were carried out essentially according to [16]. The gels were photographed, the relative proportions of the individual CPC bands were determined from densitogram tracings and are given as percentages of the total. The individual bands were cut out of the gels for determination of the Chl-*a*/*b* ratio and for fluorescence measurements. The Chl-*a*/*b* ratios of the CPC bands were determined by measuring the optical densities at 671 and 653 nm, using the integrating sphere of an SZF-18 spectrophotometer according to [17]. Low-temperature fluorescence spectra of the CPC bands were recorded with a Perkin-Elmer MPF 44/A instrument at 77 K. The spectra were corrected for the spectral sensitivity of the apparatus and were normalized to unit area.

Results

Table 1 presents the pigment (total chlorophyll and total carotenoid) contents of the leaves during greening on a dry weight basis. It is seen that about 40% of chlorophylls remained present in the treated leaves after 72 h of greening.

Table I. Pigment content (in μmol/g dry weight) of barley leaves during greening in the presence or absence of SAN 6706. Values are mean ± S.D. of 6 determinations from 3 experiments.

Duration of greening (h)		0	24	48	72
Total chlorophyll	Control	–	5.6 ± 0.7	6.6 ± 0.7	7.2 ± 0.7
	SAN 6706 treated	–	3.8 ± 0.4	3.4 ± 0.4	2.9 ± 0.3
Total carotenoids	Control	0.5 ± 0.2	2.0 ± 0.2	2.3 ± 0.2	2.5 ± 0.3
	SAN 6706 treated	0.6 ± 0.2	1.7 ± 0.2	1.1 ± 0.2	0.8 ± 0.2

Table II. Values of $^{14}CO_2$-fixation activity of barley leaves after 72 h of greening in the presence or absence of SAN 6706. The values are mean ± S.D. from at least 60 determinations from three experiments.

	nmol $CO_2 \cdot cm^{-2} \cdot h^{-1}$	%	nmol CO_2 (μmol Chl)$^{-1} \cdot h^{-1}$	%
Control	395.8 ± 81.6	100	1992 ± 364	100
SAN 6706 treated	71.4 ± 12.8	1.5	43 ± 11	2.1

The *in vivo* photosynthetic capacities of barley leaves greened in the presence or absence of SAN 6706 were investigated by fluorescence induction in the course of greening and by $^{14}CO_2$-fixation measurements at the final stage. Leaves greened in the presence of SAN 6706 were practically unable to fix CO_2 (Table II), since their fixation rates did not exceed several % of the control, regardless of whether it was related either to unit leaf area or to the same chlorophyll content. Table III presents the ratios $(F_m\text{-}F_i)/F_m$ and (P-T)/P, calculated from fast and slow fluorescence induction curves, respectively. Fast fluorescence induction curves were recorded in the time range of ~ 1 s, F_i and F_m denote the fluorescence intensities at 40 ms and 1 s, respectively. The $(F_m\text{-}F_i)/F_m$ ratio increased progressively in the course of greening of untreated leaves. The fluorescence induction curves of the leaves greened in the presence of SAN 6706 exhibited a fast rise of fluorescence, leading to high F_i values. The corresponding $(F_m\text{-}F_i)/F_m$ ratios were found to be around 3% of the control. The fluorescence yield at the F_m level (maximum fluorescence) was considerably higher in SAN 6706-treated leaves at every stage of greening. For illustration, the fluorescence induction curves after 72 h of greening are shown in Fig. 1A.

Table III. Values of $(F_m\text{-}F_i)/F_m$ and (P-T)/P ratios, calculated from fast and slow fluorescence induction curves, respectively, during greening of detached barley leaves in the presence or absence of SAN 6706. F_i and F_m denote the fluorescence intensity at 40 ms, and 1 s illumination, respectively. P and T denote the initial and terminal (around 5 min) fluorescence level, respectively, in slow fluorescence induction measurements. Values are mean ± S.D. from at least 45 determinations from three experiments.

Parameter	Duration of greening [h]	Control	SAN 6706 treated
$\frac{F_m\text{-}F_i}{F_m}$	24	0.403 ± 0.017	0.015 ± 0.007
	48	0.449 ± 0.021	0.017 ± 0.008
	72	0.485 ± 0.020	0.015 ± 0.007
$\frac{P\text{-}T}{P}$	24	0.475 ± 0.028	0.358 ± 0.024
	48	0.563 ± 0.030	0.416 ± 0.030
	72	0.604 ± 0.022	0.465 ± 0.028

The slow fluorescence induction curves of the leaves greened in the presence or absence of SAN 6706 were recorded in the time range of 5 min. Control leaves exhibited quenching of fluorescence from the initial P to a terminal T level. This quenching is expressed as (P-T)/P and is presented in Table III. It is seen that fluorescence quenching was also observed with the leaves greened in the presence of SAN 6706. The extent of this quenching showed progressive development in the course of greening, similarly to the control, the actual values being about 25% less (Table III). For illustration, Fig. 1B de-

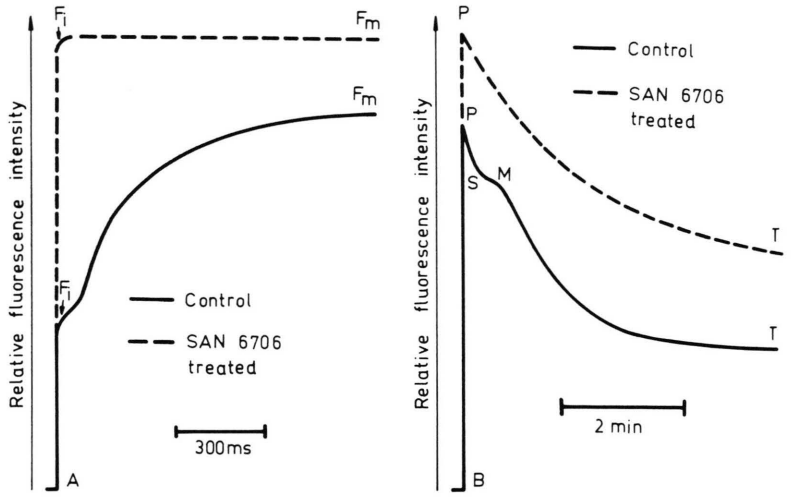

Fig. 1. Fast (A) and slow (B) fluorescence induction curves of barley leaves after 72 h of greening in the presence or absence of SAN 6706. The characteristic points of the curves are indicated.

monstrates the slow fluorescence induction curves of intact leaves at the final stage of greening. It is well seen that the SMT transient is absent from the treated leaves.

The activities of the photosystems were studied by *in vitro* measurements on isolated, osmotically shocked chloroplasts. Table IV presents the values of electron transport rates for PS-2 and PS-1, measured as Hill and Mehler reactions, respectively, in the presence of the uncoupler NH_4Cl. Thylakoids isolated from the leaves greened in the presence of SAN 6706 were found to be inactive in PS-2 specific Hill reaction. On the other hand, their PS-1 activity, measured as the rate of electron transport from $DCPIPH_2$ to methylviologen was substantial during the overall course of greening when expressed on a chlorophyll basis.

Table IV. Rates of Hill reaction from water to K-ferricyanide and of *in vitro* Mehler reaction from $DCPIPH_2$ to methylviologen of chloroplasts isolated from barley leaves during greening in the presence or absence of SAN 6706. Values are expressed in μmol O_2 evolved (mg chlorophyll)$^{-1}$ h^{-1} for Hill reaction and in μmol O_2 taken up (mg chlorophyll)$^{-1}$ h^{-1} for Mehler reaction. Each value is the mean ± S.D. of nine determinations from three experiments. FeCy = K-ferricyanide, MV = methylviologen.

Reaction	Duration of greening [h]	Control	SAN 6706 treated
$H_2O \rightarrow FeCy + NH_4Cl$	24	84 ± 9	0
	48	126 ± 14	2.1
	72	154 ± 13	4.8
$DCPIPH_2 \rightarrow MV$	24	327 ± 28	432 ± 36
	48	264 ± 30	448 ± 40
	72	239 ± 22	436 ± 32

In order to investigate the formation and properties of the chlorophyll-protein complexes, detailed CPC analyses were carried out at the final stage of greening. The chlorophyll-protein complexes could be solubilized from the thylakoids of SAN 6706-treated leaves only with high detergent concentration (SDS : chlorophyll 30 : 1 weight ratio), while those from untreated leaves were easily solubilized with SDS-chlorophyll 5 : 1 detergent concentration. It was necessary, therefore, to check the effect of the high detergent concentration on the solubilization characteristics of the untreated samples, too. The high detergent concentration caused the disappearance of the discrete forms of the more aggregated LHCP bands ($LHCP^1$ and $LHCP^2$), and also led to a higher amount of free chlorophylls (Table V). Samples treated with SAN 6706 are characterized by the total absence of CP_a and by the very limited amount of CP 1. Only the $LHCP^3$ band and free chlorophylls could be distinguished in the gels after electrophoresis. Investigation of the spectral properties of these bands revealed that the CP 1 band of the treated samples lacked the characteristic fluorescence emission at 723 nm when studied *in situ*. Instead, a large fluorescence peak appeared at 677 nm with a small shoulder at 730 nm (Fig. 2).

Table V. Chlorophyll-*a*/*b* ratios and relative proportions (in % of total) of chlorophyll-protein complexes isolated from thylakoids of barley leaves after 72 h of greening in the presence or absence of SAN 6706. Values are means of two determinations. FP = free pigment, LHCP = light harvesting chlorophyll-protein complex, chl = chlorophyll.

Treatment (SDS:chl ratio)	Parameter	CP 1	LHCP[1]	LHCP[2]	CP_a	LHCP[3]	FP
	Chl-*a*/*b*	16.1	1.6	1.7	3.9	1.3	1.8
Control (5:1)	%	24.1	9.3	4.2	7.6	31.6	23.2
	Chl-*a*/*b*	22.2	–	–	4.5	1.2	1.8
Control (30:1)	%	18.1	–	–	4.9	31.2	45.8
	Chl-*a*/*b*	21.7	–	–	–	1.7	2.1
SAN 6706 (30:1)	%	2.1	–	–	–	34.6	61.3

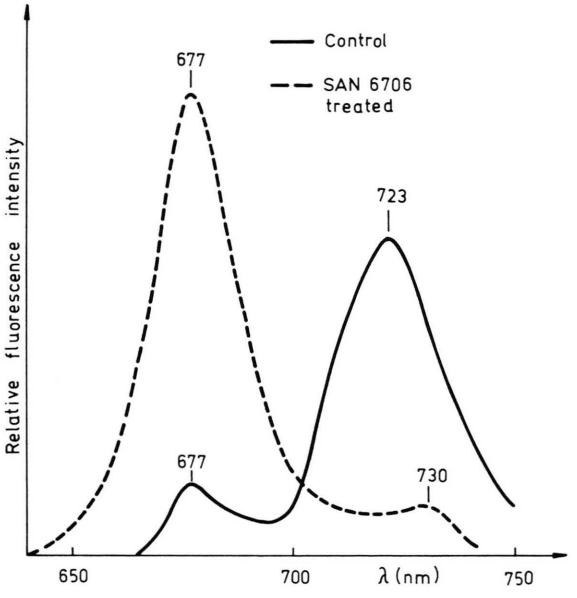

Fig. 2. Low temperature fluorescence spectra of the CP 1 bands isolated from thylakoids of barley leaves after 72 h of greening in the presence or absence of SAN 6706. Excitation was at 440 nm.

Discussion

The results presented in this communication demonstrate that barley leaves greened in the presence of the pyridazinone herbicide SAN 6706 are unable to carry out photosynthetic CO_2 assimilation. Their fast fluorescence induction tracings reveal that i) the light utilization efficiency by PS-2 photochemistry is very low, and ii) the electron transport from Q to the PQ pool proceeds at practically zero rate. Isolated chloroplasts are also inactive in mediating PS-2 specific Hill reaction, indicating a serious impairment of PS-2 function. The inactivation of PS-2 reaction centres was reported for SAN 9789 in *Scenedesmus* [24]. Previous investigations with greening barley [18] have shown that the primary photochemical function of PS-2 (charge separation by the reaction centre) proceeds at a much lower rate than in the control. Analysis of the chlorophyll-protein complexes of the thylakoids showed that chloroplasts from leaves greened in the presence of SAN 6706 practically do not contain the CP_a complex, which is thought to be the chlorophyll-protein complex of P680, the reaction centre chlorophyll of PS-2 [19]. These results indicate that not only the number of PS-2 units but their functional activity is greatly decreased during greening in the presence of SAN 6706. By the 48th hour of greening, these leaves do not contain any detectable amounts of carotenes, while substantial quantities of xanthophylls and chlorophylls remain still present (Table I, see also ref. [11]). As concerns the functional role of β-carotene in the PS-2 complex, it has been established that the presence of β-carotene is not an absolute requirement for PS-2 function, but is required for full activity [20]. In our case the very low or zero level of PS-2 activity coincided with the lack of β-carotene and of the CP_a band. From this we conclude that the presence of β-carotene is required for the maintenance of the structural integrity of PS-2 units.

The rate of the *in vitro* Mehler reaction (mediated by PS-1 solely) declined during greening in untreated samples when expressed on a chlorophyll basis. This decline (which was reported to be closely related to the developmental status of PS-1, [21]) was absent from the treated samples, which showed high PS-1 activity at every stage of greening, indicating a higher

proportion of P700 related to the total chlorophyll content. Though the analysis of the chlorophyll-protein complexes showed only very limited presence of CP 1 (the chlorophyll-protein complex of P700 [19]), we think that the high detergent concentration resulted in over-solubilization of the CP 1 band. This action is observed also with the spectral characteristics of the CP 1 band (Fig. 2). The lack of the 723 nm emission and the appearance of that at 677 nm may possibly resulted from the destruction of the native complex during the solubilization and electrophoretic procedures, as was observed also by other authors [22].

One of the main points to be discussed is the presence of the fluorescence quenching from P to T in the treated leaves. Since electron transport from PS-2 is negligible, but the PS-1 *per se* is not inactive, this quenching may well be the consequence of cyclic electron transport around PS-1. Since this electron flow is also coupled to ATP formation [15], the degree of fluorescence quenching could demonstrate the generation of the high-energy state of thylakoids required for photophosphorylation [23]. The degree of this fluorescence quenching showed progressive development in the course of greening, similarly to the control (Table I), suggesting that the extent of the quenching is correlated to the developmental status of the chloroplasts. A part of this quenching can very possibly be attributed to a reversible photobleaching of chlorophyll during the measurements, as it was shown for SAN 9789-treated *Scenedesmus* [24] and barley [18]. The CPC-profile of the treated samples contained considerable amounts of the monomer $LHCP^3$. It was demonstrated that these chloroplasts does not contain carotenes at the end of greening, but considerable amounts of xanthophylls and chlorophylls are present. The presence of $LHCP^3$ clearly demonstrate the important role of xanthophylls in the assembly of the monomer form of the light-harvesting complex. It has been reported [25] that bleached leaves (induced by SAN 9789) contain neither CP_a, nor LHCP. This is readily interpreted by the lack of all carotenoid pigments.

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