Herbicides which Inhibit Electron Transport or Produce Chlorosis and Their Effect on Chloroplast Development in Radish Seedlings II. Pigment Excitation, Chlorophyll Fluorescence and Pigment-Protein Complexes

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Bleaching Herbicides, Carotenoids, Chlorophylls, Chlorophyll Fluorescence, Photosystem II Herbicides, Pigment-Protein-Complexes

DCMU, bentazon, amitrole and SAN 6706 affected the formation of the pigment-protein complexes and caused drastic alterations in the absorption of light and in the transfer of the absorbed energy in the antennae systems. Bentazon and DCMU, photosystem II inhibitors, did not change the pigment absorption and fluorescence excitation spectra. After application of both herbicides the long wavelength fluorescence emission band at 740 nm was reduced similar as in young developing leaves. Although DCMU and bentazon inhibit the photosynthetic electron transport at the same site, bentazon mainly suppressed the formation of the photosystem I complexes CP1a and CP1 while DCMU mainly reduced the photosystem II complex CPa. Bentazon specifically enhanced the formation of LHCP3. This may be important for the increased grana stacking in plastids from bentazon treated plants.

The bleaching herbicides amitrole and SAN 6706 inhibited the formation of carotenoids leading to an accumulation of lycopene, phytofluene and phytoene, while the accumulation of chlorophylls was suppressed. This bleaching effect was most pronounced during growth under higher intensities of light. In weak light (100 lux) amitrole reduced the long wavelength fluorescence maximum but the fluorescence excitation was not affected. With amitrole at 2000 lux and SAN 6706 at 100 lux the long wavelength emission band was further decreased and the fluorescence excitation spectra point to a less efficient energy transfer to chlorophyll *a*. The fluorescence spectra changed due to herbicide treatment resembled those of not yet fully developed leaves. In contrast to the photosystem II herbicides the bleaching herbicides amitrole and SAN 6706 had a similar effect on the formation of pigment-protein complexes. After growth at 2000 lux both herbicides suppressed the formation of the photosystem I complex CP1a and the photosystem II complex CPa. At 100 lux only the formation of CP1a was affected.

Except for DCMU all herbicides assayed primarily changed the formation of photosystem I.

Introduction

Within the chloroplast there are several targets of herbicide action. Herbicides like diuron and bentazon inhibit electron transport specifically at the donor side of photosystem II [1 – 5]. Bleaching herbicides like amitrole and SAN 6706 primarily interfere with the biosynthesis of carotenoids [5 – 9]. Photosystem II and bleaching herbicides are therefore very useful tools for the investigation of the development of the thylakoid membrane and its constituents.

It is well known that the combined action of the light harvesting pigments and the electron transport chain is most important for the performance of photosynthesis. This strong interaction of the pigment protein complexes, the photosynthetic reaction centers and the electron transport chain has been demonstrated extensively by several groups [10 – 12]. Therefore any change in the organization of the electron transport components or the orientation of the chlorophyll and carotenoid proteins in the thylakoid membrane may induce drastic alterations in the absorption of sun light, the transfer of the absorbed

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)1,1-dimethylurea = diuron; bentazon, 3-isopropyl-2,1,3-benzo-thiadiazinone-(4)-2,2-dioxide = basagran; amitrole, 3-amino-1,2,4- triazole; SAN 6706, 4-chloro-5-(dimethylamino)-2-(α,α,α- trifluoro-*m*-tolyl)-3(2H)-pyridazinone; PAGE, polyacrylamide gel electrophoresis; Tris/HCl, Tris(hydroxymethyl)aminomethane adjusted with HCl to the pH indicated; SDS, sodium dodecylsulfate; TEMED, N,N,-N′,N′-tetramethylethylenediamine; $Na_2 \cdot EDTA \cdot 2\,H_2O$, sodium ethylenediamine tetraacetate = Titriplex III; MBA, N,N′-methylenebisacrylamide; PER, ammoniumperoxydisulfate = $(NH_4)_2S_2O_8$; LHCP, light harvesting chlorophyll *a/b* protein complex; CP1a and CP1, chlorophyll-protein complexes of photosystem I; CPa, chlorophyll-protein complexes of photosystem II; FP, free pigments.

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energy from the light harvesting pigments to the photosynthetic reaction centers and the conservation of energy in the electron transport.

In this communication the effect of the bleaching herbicides SAN 6706 and amitrole and the photosystem II herbicides DCMU and bentazon on the formation of subthylakoid particles was studied by using SDS-polyacrylamide gel electrophoresis. The ability of the developed plastid to absorb light and to transfer the absorbed energy was investigated by recording absorption and fluorescence emission and excitation spectra. The consequences of the herbicide effect on the pigment protein composition of the thylakoid membrane and the capability of the plastid to perform photosynthesis will be discussed.

Materials and Methods

Cultivation of plants

Radish seedlings (*Raphanus sativus* L., cv. Saxa Treib) were grown for six days in continuous white light (fluorescent lamps: OSRAM 'Fluora' 65 W/77 R, 2000 lux, 7.5 $Jm^{-2} s^{-1}$) on tap water at 21 ± 2 °C

Table I. Solutions for the isolation and solubilization of chloroplasts as well as for the polyacrylamide gel electrophoresis. Buffers and gel solutions were prepared using bidistilled water.

	molecular weight	(density)
Isolation buffer (pH 7.5)		
0.06 M K_2HPO_4	174	
0.04 M KH_2PO_4	136	
0.35 M sorbitol	182	
0.01 M $Na_2 \cdot EDTA \cdot 2\ H_2O$	372	
5 mM $MgCl_2 \cdot 6\ H_2O$	203	
36 mM 2-mercaptoethanol (2.5 ml/l)[a]	78	(1.11)
Solubulization buffer		
0.10 M Tris/HCl (pH 6.8)	121	
2.71 M glycerol (200 ml/l)	92	(1.25)
0.28 M 2-mercaptoethanol (20 ml/l)[a]	78	(1.11)
Separation gel (11.85% polyacrylamide)		
0.35 M Tris/HCl (pH 8.8)	121	
1.67 M acrylamide	71	
0.02 M MBA	154	
3.5 mM SDS	288	
1.8 mM PER[2]	228	
1.66 mM TEMED (0.25 ml/l)	116	(0.77)
Stacking gel (4.9% polyacrylamide)		
0.13 M Tris/HCl (pH 6.8)	121	
0.73 M acrylamide	71	
8.6 mM MBA	154	
3.5 mM SDS	288	
6.8 mM PER[b]	228	
4.6 mM TEMED (0.694 ml/l)	116	(0.77)
Anode buffer		
12.5 mM Tris/HCl (pH 8.3)	121	
0.10 M glycine	75	
catode buffer		
25 mM Tris/HCl (pH 8.3)	121	
0.20 M glycine	75	
3.5 mM SDS	288	

[a] Added directly before use.
[b] Freshly prepared.

and 60 ± 10% relative humidity. Cultivation of plants under low intensity light was performed using fluorescent lamps (PHILIPS TL 25 W/25, 100 lux, 0.33 $Jm^{-2} s^{-1}$). The herbicides applied were present in the water during soaking and germination. As herbicides DCMU and bentazon (10^{-3} M each), amitrole or SAN 6706 (10^{-4} M each) were used.

Absorption and fluorescence spectra

Pigments were extracted from the cotyledons using cold acetone and transfered into light petrol. The absorption spectra were measured using diethyl ether as solvent. Fluorescence emission and excitation spectra of the cotyledons were determined at room temperature during the steady state period of the fluorescence induction kinetic. During the measurement all spectra were automatically corrected for changes in the intensity of the excitation light.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of SDS solubilized thylakoids was carried out according to Hrkal [13]. The cotyledons were grinded in an isolation buffer (Table I). After filtration through a nylon cloth the plastids were isolated by centrifugation for 10 min at 1500 × *g*, resuspended in destilled water and recentrifuged for 20 min at 6000 × *g*. Aliquots of the thylakoids were mixed with a solubilization buffer (Table I) and incubated with SDS for 3 to 4 min at 4 °C. SDS was added to the thylakoids of the herbicide treated plants in the same concentration as to the thylakoids of the untreated plants (chlorophyll/SDS = 1/40 w/w, final concentration: 0.6% SDS). Electrophoresis was carried out in glass tubes (0.4 × 12 cm) containing a 11.85% polyacrylamide separation gel and a 4.9% polyacrylamide stacking gel using a vertical separation system (DESAGA 'Havana'). Gels and buffers were prepared as described in Table I. In the beginning of electrophoresis the current was set to 1 mA per tube. When the particles had passed the stacking gel, the separation of the thylakoid subparticles was performed by increasing the current to 2 mA per tube. Plastid isolation, solubilization and SDS-PAGE were carried out at 4 °C. After their separation the pigment-protein complexes were localized using a scanning densitometer (RFT, Model 2950) by measuring the absorption at 663 nm (log transmittance mode).

The presented absorption and fluorescence spectra as well as the pattern of the pigment-protein complexes of the SDS-solubilized thylakoid membranes are representatives of six replications from three to five independent cultivations.

Results

Pigment absorption spectra

The 6-day-old radish seedlings showed morphological differences between herbicide treated and untreated plants. Cotyledons from seedlings that were grown in the presence of DCMU or bentazon were green and exhibited the same characteristics in their pigment absorption spectra as untreated plants. There was a main absorption maximum at 428 and at 660 nm and a minor one at 448 nm (Fig. 1).

Cotyledons from radish that was grown under normal greening conditions (2000 lux) in the presence of high concentrations of amitrole looked yellow. The absorption maxima of the pigment extract lay at 438, 467, 498 and 660 nm, with the 660 nm peak being decreased compaired to the controls. Two additional absorption maxima appeared in the UV region at 344 and 364 nm (Fig. 2). After growth under light of low intensity (100 lux) the amitrole treated seedlings looked green but the pigment absorption spectrum was similar to that after amitrole treatment at 2000 lux, with two maxima in the blue slightly shifted (Fig. 2).

Cotyledons from plants that were grown in the presence of SAN 6706 were green when grown at 100 lux, but rather white when grown at 2000 lux. SAN 6706 did not affect the pigment absorption spectra of the plants illuminated with low intensity light, whereas after cultivation at 2000 lux absorption maxima at 415, 435 and 466 nm appeared and the absorption at 660 nm was strongly decreased (Fig. 3).

Both amitrole and SAN 6706 drastically change the pigment pattern of the plants. This is shown by the changed absorption ratios $A\,470/A\,660$ and $A\,448/A\,660$ (Table II).

Fluorescence emission spectra of the cotyledons

At room temperature the *in vivo* fluorescence emission spectra (Fig. 4) of cotyledons from untreated plants exhibited emission bands at 695 and

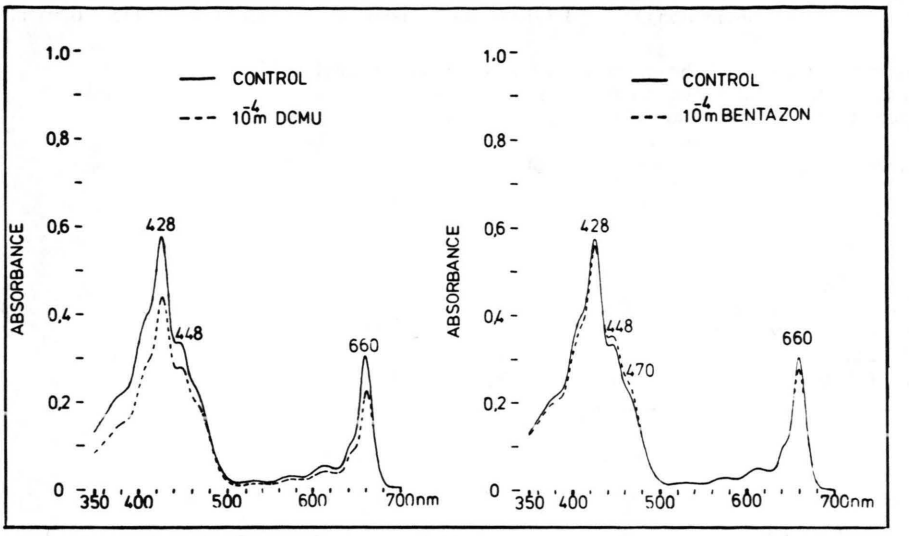

Fig. 1. Absorption spectra of plastid pigments extracted from radish cotyledons that were grown for 6 days in continuous white light in the presence of the photosystem II herbicides DCMU and bentazon. As solvent diethylether was used.

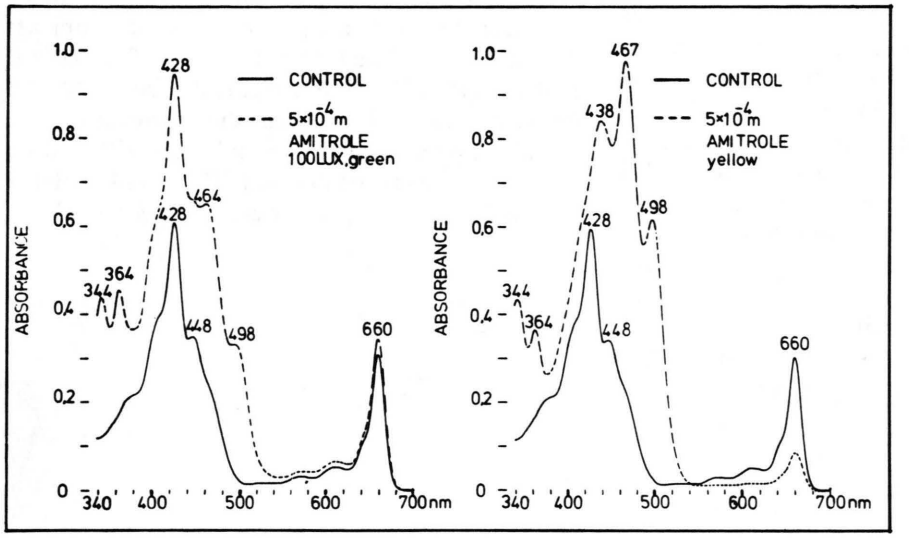

Fig. 2. Absorption spectra of plastid pigments extracted from radish cotyledons that were grown for 6 days in continuous white light in the presence of the bleaching herbicide amitrole. As solvent diethylether was used.

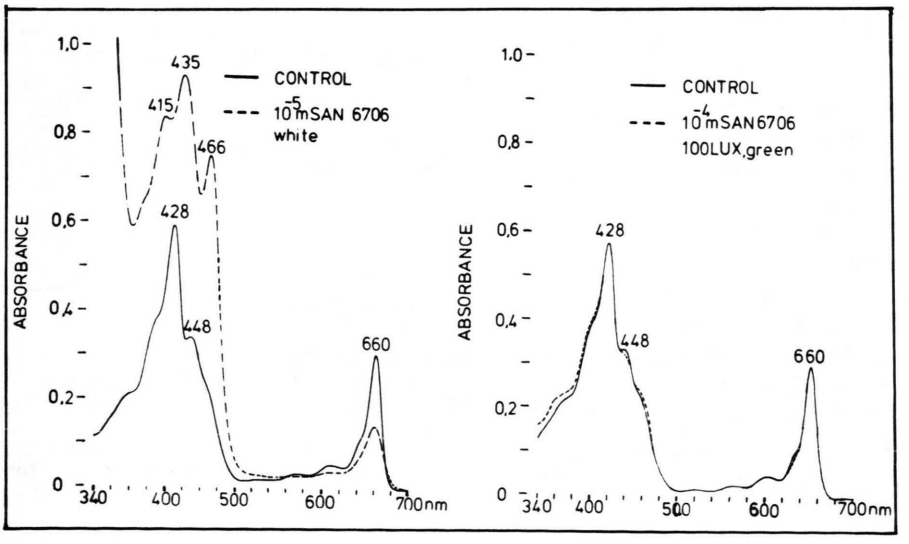

Fig. 3. Absorption spectra of plastid pigments extracted from radish cotyledons that were grown for 6 days in continuous white light in the presence of the bleaching herbicide SAN 6706. As solvent diethylether was used.

Table II. Positions of the absorption maxima of the pigment extract from radish cotyledons after 6 days growth in continuous white light in the presence of photosystem II or bleaching herbicides. All Spectra were recorded using diethylether as solvent.

Growth condition	Position of absorption maxima	$\frac{A_{470}}{A_{660}}$	$\frac{A_{448}}{A_{660}}$
Control	428, 448, 660	0.70	1.10
DCMU	428, 448, 660	0.86	1.25
Bentazon	428, 448, 660	0.74	1.25
Amitrole (100 Lux)	344, 364, 428, 464, 498, 660	1.85	1.94
Amitrole (2000 Lux)	344, 364, 438, 467, 498, 660	11.87	10.00
SAN 6706 (100 Lux)	428, 448, 660	0.43	1.07
SAN 6706 (2000 Lux)	415, 435, 466, 660	5.03	5.65

740 nm. In seedlings grown in the presence of DCMU or bentazon the fluorescence emission at 740 nm was significantly decreased. These plants showed no variable fluorescence (Kautsky effect). This was also the case for the seedlings grown in the presence of amitrole under low intensity light. The decrease in the fluorescence emission at 740 nm was even higher in the yellow cotyledons from radish that was grown in the presence of amitrole under light of higher intensity (2000 lux). Similar results were obtained with cotyledons of plants treated with SAN 6706 under 100 lux. A slight shoulder around 740 nm remained always visible. After six days of growth in the presence of SAN 6706 in light of higher intensity the white cotyledons emitted no and the slightly green cotyledons only very little fluorescence.

Fluorescence excitation spectra of the cotyledons

At room temperature the fluorescence excitation spectra (Fig. 5) of cotyledons from untreated plants are characterized by a prominent peak at 470 nm, a small shoulder at 483 nm and broader shoulders at 440 and 420 nm. The fluorescence excitation spectra of DCMU and bentazon treated seedlings did not differ from those of the untreated plants. This was also found with the cotyledons from radish grown in the presence of amitrole under low intensity light (100 lux). In plants that were grown under light of higher intensity (2000 lux) in the presence of amitrole or under low intensity light in the presence of SAN 6706 a short wavelength band at 440 nm appeared in addition to that at 470 nm. The fluorescence of cotyledons from SAN 6706 treated plants grown at 2000 lux (white cotyledons) could not be detected.

SDS-PAGE of SDS-solubilized thylakoids

After SDS-PAGE separation of SDS-solubilized thylakoids the following pigment-protein complexes were detectable (Fig. 6): (start) CP1a, CP1, LHCP1, LHCP2, CPa, LHCP3, free pigments (front) [10]. All herbicides assayed changed the pigment-protein complex composition (Fig. 6 and Table III), but no new complex could be detected. Thylakoids isolated from DCMU treated plants were enriched in LHCP1

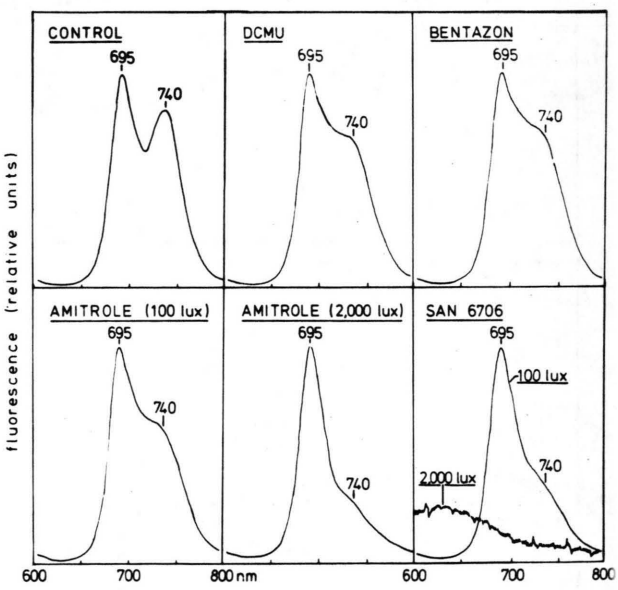

Fig. 4. Fluorescence emission spectra of radish cotyledons measured at room temperature. All spectra are adjusted to the same height at the 695 nm maximum. The radish seedlings were grown for 6 days under continuous white light in the presence of DCMU, bentazon, amitrole or SAN 6706. The light intensity used during growth was 2000 lux. One set of amitrole and SAN 6706 treated plants was grown at 100 lux.

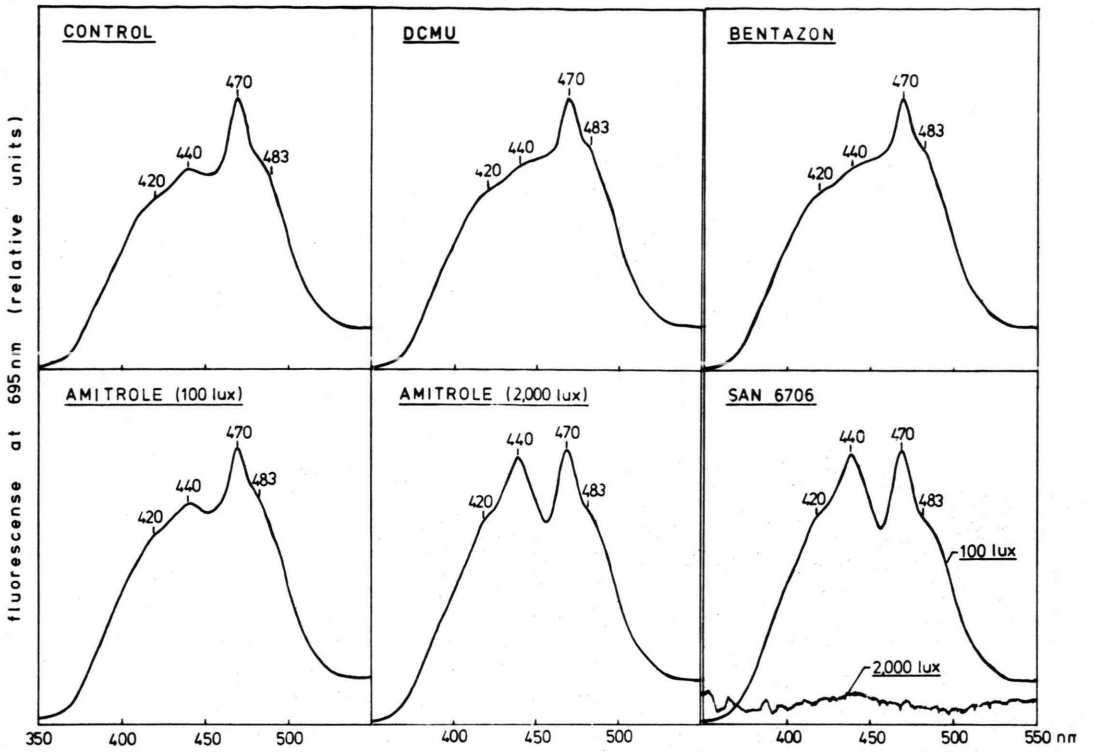

Fig. 5. Fluorescence excitation spectra of radish cotyledons measured at room temperature for the 695 nm fluorescence (short wavelength emission maximum). All spectra were adjusted to the same height at 470 nm. Otherwise as for Fig. 4.

but contained much less CPa besides lower amounts of CP1a and CP1. Thylakoids that were developed under the influence of bentazon contained markedly less CP1a and CP1 but relatively more LHCP3 and free pigments than the controls. A decrease in the amount of CP1a and CP1 and a relative increase of LHCP3 was also found in the thylakoids from radish that was grown in the presence of amitrole under low light intensity (100 lux). Thylakoids from amitrole treated plants grown in light of higher intensity (2000 lux) showed a drastical increase of free pigments. The amount of LHCP3 was relatively diminished as was that of CP1a, CPa and CP1. A higher free pigment content was also found in plants grown

Table III. Pigment-protein complex composition (area % of gel scans) of SDS-solubilized thylakoids from radish cotyledons after 6 days of growth under continuous white light in the presence of DCMU, bentazon, amitrole and SAN 6706.

Herbicides assayed	Pigment-protein complexes						Free Pigments	Sum
	Photosystem I		Light harvesting complexes			Photosystem II		
	CP1a	CP1	LHCP1	LHCP2	LHCP3	CPa		
Control	16	20	3	1	35	12	13	100
DCMU	12	15	8	2	39	7	17	100
Bentazon	4	9	5	3	44	12	23	100
Amitrole (100 lux)	3	11	5	3	48	12	18	100
Amitrole (2000 lux)	1	13	5	2	30	5	44	100
SAN 6706 (100 lux)	1	21	8	3	28	19	20	100
SAN 6706 (2000 lux)	0	7	1	1	43	3	45	100

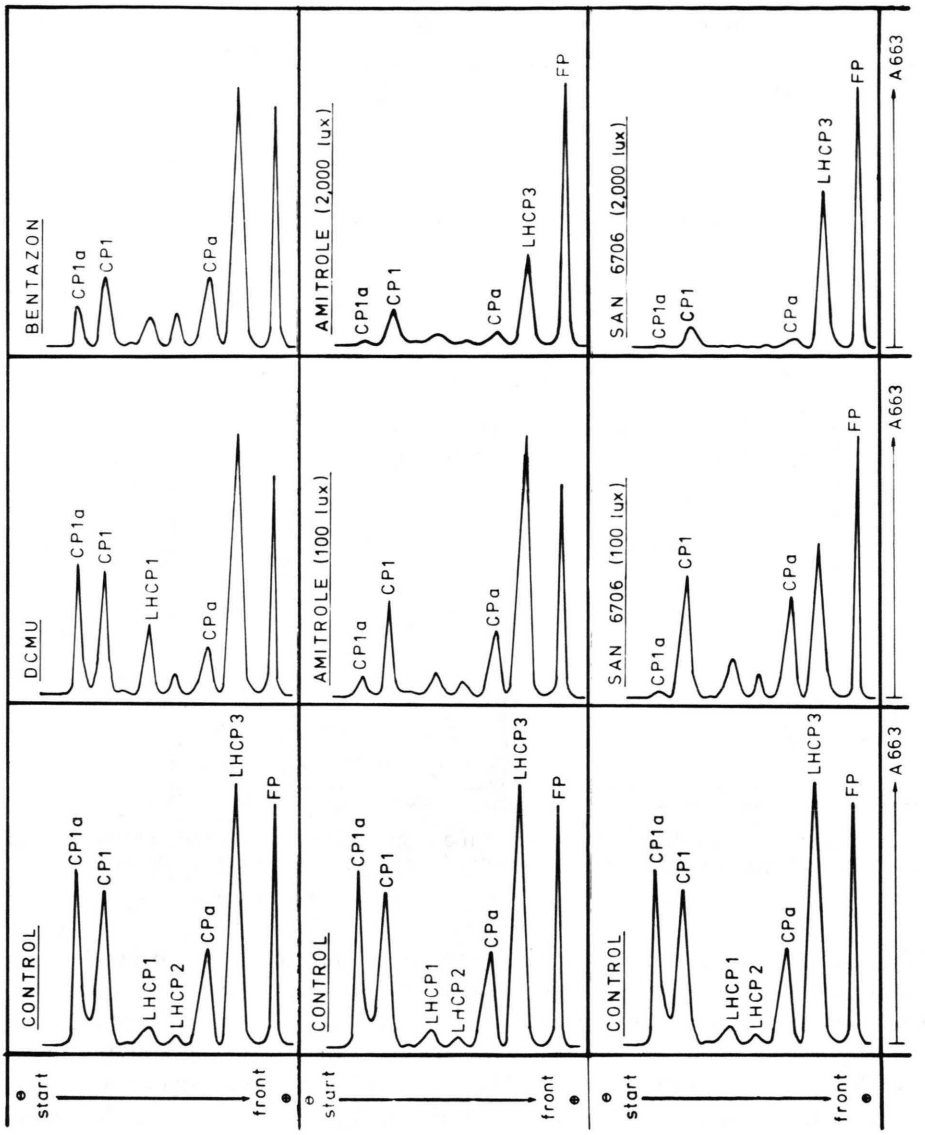

Fig. 6. Densitometric gel scans at 663 nm of SDS solubilized thylakoids isolated from radish seedlings after separation by SDS-PAGE. All scans are adjusted to the same maximum height. Otherwise as for Fig. 4.

Table IV. Inhibitory effects of herbicides on the amounts of chlorophyll-protein complexes from 6-day-old radish seedlings grown in continuous white light. The cultivation was performed at 2000 lux, except for amitrole green and SAN 6706 green (100 lux each).

Thylakoid Particles	Chlorophyll Protein Complexes	Herbicide assayed					
		DCMU	Bentazon	Amitrole yellow	Amitrole green	SAN 6706 white	SAN 6706 green
PS I	CP1a	○	●	●	●	●	●
	CP1	○	●	○	○	○	
PS II	CPa	●		●		●	
Antenna	$LHCP_3$			○			○

● Main inhibitory effect.
○ Minor inhibitory effect.

in the presence of SAN 6706. This effect occured predominantly after cultivation at 2000 lux but also at 100 lux. Gel scans of SDS-solubilized thylakoids of SAN 6706 treated plants grown at higher light intensity could only be obtained from plants which still contained trace amounts of pigments. In thylakoids that developed under low intensity light SAN 6706 suppressed the formation of CP1a and LHCP3 but relatively enhanced the content of CPa. Under light of higher intensity, apart from the free pigments, mainly LHCP 3 but also CP1 were found in reasonable amounts.

Discussion

The application of the photosystem II inhibitors DCMU [1, 2] and bentazon [3, 4] during growth of radish seedlings did not affect plant habitus and the absorption spectrum of the extracted pigments (Fig. 1), although in these plants photosynthesis was blocked as could be demonstrated by the absence of the variable fluorescence (Kautsky effect) [5, 14]. Both herbicides induced a decline of the 740 nm long wavelength fluorescence band as compared to the controls (Fig. 4). Similar emission spectra as obtained from DCMU and bentazon treated plants were also found during greening in younger seedlings that were not fully developed [15]. The decreased 740 nm fluorescence emission may be an indication for (a) a smaller amount of long wavelength chlorophyll forms (photosystem I [16]) or (b) an diminished energy transfer to these chlorophylls which would result in a higher fluorescence emitted by the short wavelength chlorophyll forms (photosystem II, light harvesting chlorophyll *a*/*b*-protein complex [16]).

The fluorescence excitation spectra were unaffected by DCMU and bentazon (Fig. 5) supporting that the transfer of energy from β-carotene or chlorophyll *b* to chlorophyll *a* [17] is not influenced by the herbicide treatment. Besides the effect on fluorescence emission, DCMU and bentazon changed the composition of the pigment-protein complexes (Fig. 6, Table III). Under the influence of DCMU mainly less photosystem II complex (CPa [18]) was formed, whereas bentazon predominantly reduced the formation of photosystem I complexes CP1a and CP1 [19]. Although DCMU and bentazon inhibit the photosynthetic electron transport at the oxidizing side of the plastoquinone pool [1 – 4] the main effect on the formation of pigment-protein complexes was completely different (Table IV). This indicates that the inhibition of photosynthesis is not primarily involved in the changes of the formation of pigment-protein complexes but rather changes in the pigment concentration (for bentazon: [20 – 22]). The slight increase of the LHCP3 induced by bentazon treatment may be explained by an enhanced formation of grana stacks [22]. A correlation between grana stacking and LHCP formation has been suggested [23 – 25].

During cultivation at 2000 lux the bleaching herbicides amitrole and SAN 6706 changed the pigment composition as can be seen from the absorption ratios carotenoids/chlorophyll *a* (*A* 470/*A* 660) and chlorophyll *b*/chlorophyll *a* (*A* 448/*A* 660) (Table II). In amitrole treated plants less chlorophyll is contained, the carotenoid biosynthesis is inhibited and lycopene and phytofluene are accumulated [7, 9]. This can be seen in the absorption spectra of the pigment extract as a decline of the chlorophyll a peak (660 nm) and the appearance of maxima in the blue (lycopene) and the UV region (phytofluene).

In SAN treated plants the pigment composition was not affected when the plants were grown at 100 lux, but after cultivation at 2000 lux (almost) no chlorophylls and only traces of carotenoids were found (Fig. 3). This may be explained by the inhibition of carotenoid biosynthesis which leads to the bleaching of chlorophylls in light of higher intensity [6, 26].

Although bleaching herbicides strongly affect pigment accumulation, photosynthesis was still detectable as long as pigments were present [5]. Like after application of DCMU and bentazon, the fluorescence emission spectra of leaves from amitrole or SAN 6706 treated plants showed a decreased 740 nm long wavelength band (Fig. 4). Here again the long wavelength chlorophyll forms were accumulated in smaller amounts or received less energy from the short wavelength chlorophyll forms as can be expected from greening leaves of not yet fully developed plants which exhibit similar fluorescence emission spectra [15]. In amitrole treated plants this effect on fluorescence emission was most pronounced after growth at 2000 lux. Seedlings grown in the presence of SAN 6706 showed already a very low 740 nm emission when cultivated at 100 lux; in the

white cotyledons of SAN 6706 treated plants grown at 2000 lux no fluorescence was detectable.

A decrease of the long wavelength fluorescence maximum was also described for chloroplasts of SAN 9789 treated wheat [27]. But the reported concomittant shift of the fluorescence band from 740 to 720 nm could not be detected in our SAN 6706 treated plants.

The fluorescence excitation spectra of amitrole treated plants grown in weak light resemble those of the controls demonstrating that the energy transfer to chlorophyll *a* is not affected. Application of amitrole in stronger light (2000 lux) or of SAN 6706 in weak light (100 lux) reduced the energy transfer from chlorophyll *b* to chlorophyll *a* (Fig. 5). This is evident from the decrease of the 470 nm (chlorophyll *b* [17]) maximum. The increased fluorescence excitation at 440 nm reflects the direct excitation of chlorophyll *a*. Like for the fluorescence emission the excitation spectra of the herbicide treated plants with peaks at 440 and 470 nm resemble those of a greening but not yet fully developed leaf [15].

During growth under weak light amitrole reduced the formation of the photosystem I complexes whereas with SAN 6706 the amount of CP1a and LHCP3 was diminished (Fig. 6, Table III). Obviously the lack of LHCP3 affects the fluorescence characteristics more drastically than a reduced amount of photosystem I and II complexes because fluorescence spectra showed stronger changes after SAN 6706 than after amitrole treatment. The observation that in SAN treated plants only one pigment-protein complex remained [27] could not be confirmed by our investigations.

The high amounts of free pigments after SDS-PAGE of thylakoids from amitrole and SAN 6706 treated plants grown at 2000 lux indicate that the pigment containing membranes are rather unstable because free pigments are reported to appear only during solubilization with SDS [28]. That the bleaching effect of SAN 6706 and amitrole is only expressed under light of higher intensities and not under weak light [6, 9, 26] or in total darkness [8] has already been reported.

All herbicides tested mainly reduce the formation of the antenna systems of photosystem I and II. Except for DCMU, photosystem I is the primary target for the herbicide action (Table IV). This is consistant with the data recently reported by Ridley [29]. The formation of the light harvesting chlorophyll *a*/*b*-protein complexes was reduced only by the bleaching herbicides. The effect of the herbicides on the formation of pigment-protein complexes and in addition on chlorophyll and carotenoid accumulation may disturb the transfer of energy in the antenna similar as it is established for young developing leaves. While in greening leaves of untreated plants fully functioning thylakoid membranes will be formed in the herbicide treated plants any further development is suppressed.

Acknowledgement

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