Herbicides which Inhibit Electron Transport or Produce Chlorosis and Their Effect on Chloroplast Development in Radish Seedlings I. Chlorophyll a Fluorescence Transients and Photosystem II Activity

K. H. Grumbach

Botanisches Institut der Universität Karlsruhe, Kaiserstraße 12, D-7500 Karlsruhe, Bundesrepublik Deutschland

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Bleaching Herbicides, Photosynthesis, Photosystem II Herbicides, Photosystem II Activity, Chlorophyll Fluorescence

Diuron and bentazon are very strong inhibitors of the photosynthetic electron transport in isolated radish chloroplasts. The chlorosis producing herbicide SAN 6706 also inhibited the photosystem II dependent oxygen evolution. Aminotriazole had no effect. The inhibitor concentration for 50% inhibition of photosystem II activity was 10^{-7} m for diuron and 10^{-4} m for bentazon and SAN 6706 respectively.

Diuron and bentazon quenched the chlorophyll a fluorescence transients in isolated radish chloroplasts drastically, while aminotriazole was not effective. It was of particular interest that the bleaching herbicide SAN 6706 inhibited photosystem II dependent oxygen evolution in a similar concentration as bentazon but had no effect on the chlorophyll a-fluorescence transients suggesting that SAN 6706 is not binding to the same site of the electron transport chain as diuron and bentazon.

Apart from their direct influence on electron transport in isolated photosynthetically active chloroplasts the photosystem II and bleaching herbicides assayed also strongly affected photosynthesis in radish seedlings that were grown in the presence of the herbicides for a long time. As already obtained using isolated chloroplasts, photosystem II dependent oxygen evolution like the chlorophyll a fluorescence transients were strongly inhibited by the photosystem II herbicides diuron and bentazon. A reduction but no inhibition of photosystem II activity was observed in plants that were grown in the presence of aminotriazole. The pyridazinone SAN 6706 was behaving contradictory. In partly green plants photosystem II activity was still maintained and even higher than in untreated plants while in albinistic plants no photosynthetic activity was detected.

Introduction

Among all herbicides which are used in crop protection nearly 50% are affecting the chloroplast. Several herbicides are known as inhibitors of the photosynthetic electron transport [1–4]. Phenylureas (diuron) and benzothiadiazinones (bentazon) are very strong inhibitors [5–8]. Although chemically completely unrelated to the structure of diuron and bentazon many other herbicides like triazines [9], uracils [10] or pyridazinones [11] exhibit a similar inhibitory effect suggesting that various biocides with different chemical structures may bind to the same site of the thylakoid membrane. Only recently it was discovered that there are two different binding sites present close to photosystem II, a 32 KD protein that predominantly binds diuron-type inhibitors and a 41 KD protein that binds phenol-type inhibitors specifically [12, 13].

Apart from inhibiting electron transport many herbicides have more than one site of action. Photosystem II herbicides like diuron or bentazon also alter chloroplast ultrastructure and pigment composition [14–16]. Bleaching herbicides like the pyridazinone SAN 6706 inhibit photosystem II activity but primary interfere with the biosynthesis of carotenoids and their protection of the chlorophylls against photodestruction [17].

Although photosystem II and bleaching herbicides have been studied extensively their primary mode of action is still matter of investigation. Since both processes the absorption of sun light by the pigments of the light harvesting complex and the antennae of the photosynthetic reaction centers as well as the photosynthetic electron transport are very close connected to each other a general overall investigation of the effect of herbicides which in-

Reprint requests to Dr. K. H. Grumbach.

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hibit electron transport or pigment biosynthesis on the development of a green mature chloroplast seemed to be very suitable, not only for the investigation of the herbicide action but also for the understanding of the function and organization of the photosynthetic membrane and its constituents.

In this report the effect of the photosystem II inhibitors diuron and bentazon and the bleaching herbicides SAN 6706 and aminotriazole on the photosystem II dependent oxygen evolution and chlorophyll a fluorescence transients of radish seedlings was investigated. The experiments were performed using plants which were grown in the presence of the herbicides for a long time. The direct influence of all herbicides assayed on the electron transport and the chlorophyll a fluorescence transients of isolated photosynthetically active chloroplasts was also investigated. Experiments that were performed in order to resolve the effects of photosystem II and bleaching on other intraplastidic reactions like pigment and acyllipid biosynthesis, fatty acid composition and chloroplast ultrastructure will be presented in further contributions.

Materials and Methods

Cultivation of plants

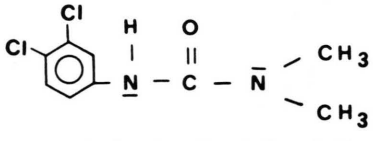
Radish seedlings were grown for 6 days on water (control) or a herbicide solution in fluorescent white light (Osram Fluora lamps, 55 W, 8 W/m²). To result a homogenous uptake and distribution, the herbicide was already applied during soaking. As herbicides we used 10^{-4} and 10^{-3} M 3(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU or diuron; 10^{-4} and 10^{-3} M 3-isopropyl-2,1,3-benzothiadiazinone-(4)-2,2-dioxide, bentazon; 10^{-4} and 10^{-3} M 3-amino-1,2,4-triazole, amitrole and 10^{-5} M 4-chloro-5-(dimethylamino)-2-(3-trifluoromethylphenyl)-3(2H)-pyridazinone, SAN 6706 (Fig. 1).

Isolation of plastids and determination of photosynthetic activity

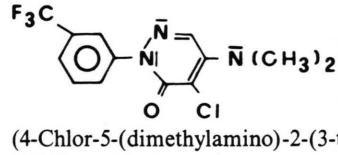
After 6 days growth in the presence of the herbicide, cotyledons were harvested and used directly either for the analyses of chlorophyll fluorescence transients or for the isolation of plastids. Chloroplasts were isolated according to Jensen and Bassham as well as Heber and Santarius [18, 19]. Photosynthetic activity was investigated using DCPIP-photoreduction and chlorophyll fluorescence transients. DCPIP-photoreduction was measured using broken chloroplasts in a ten times diluted suspension solution as described by Heber and Santarius [19].

Measurement of slow and fast chlorophyll a fluorescence transients

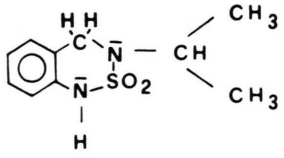
Slow chlorophyll fluorescence transients of the herbicide treated cotyledons were recorded according to Strasser [20]. The cotyledon was exposed to monochromatic red light (λ_{max} 660 nm) from a helium neon laser (4 mW/Typ 134, Spectra Physics). Sample induced fluorescence was passed through different filters (R.G. 665 Schott & Gen., IF 680 Fa.



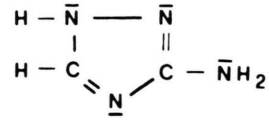
3-(3,4-Dichlorphenyl)-1,1-dimethylharnstoff (DCMU)



(4-Chlor-5-(dimethylamino)-2-(3-trifluormethylphenyl)-3(2H)-pyridazinon) (San 6706)



3-Isopropyl-1,1,3-benzothiadiazinone(4)-2,2-dioxid (Bentazon)



(3-Amino-1,2,4-triazol) (Amitrole)

Fig. 1. Structure of herbicides assayed.

Shimadzu, R.G. 715 Schott and Gen., Neutralfilter NG 9 and NG 11 Schott & Gen.) registrated by a photomultiplier (Typ 7265, Fa. RCA) and recorded.

Besides long term effects of herbicides on the photosynthetic activity of radish seedlings we studied also the direct influence of photosystem II and bleaching herbicides on the oxygen evolution and chlorophyll fluorescence of isolated radish chloroplasts. For this purpose chloroplasts were isolated from 12 days old radish seedlings which were grown in the botanical gardens. The influence of herbicides on DCPIP-photoreduction was assayed using broken chloroplasts. The effect of herbicides on fast chlorophyll fluorescence transients was assayed using broken and intact chloroplasts as well. Fast fluorescence kinetics were recorded exposing 0.5 ml chloroplast solution (30 µg chlorophyll per ml) to monochromatic blue light (λ_{max} 441 nm) from a Helium Cadmium Laser (15 mW, Typ 401, Fa. Liconix). Chlorophyll fluorescence was passed through different filters (R.G. 665 Schott & Gen., IF 680, Fa. Shimadzu, R.G. 715, Schott & Gen., Neutralfilter NG9 and NG11, B.G. 28, Schott & Gen. registrated by a photomultiplier (Typ 7265, Fa. RCA) and stored on the display of an oscilloscop (DN 64, Fa. Telequipment).

Results

Photosystem II activity of cotyledons from radish that was grown for 6 days in the presence of photosystem II and bleaching herbicides

The activity of photosystem II was investigated either by measuring DCPIP-photoreduction or by recording chlorophyll a fluorescence transients using isolated chloroplasts or intact herbicide treated cotyledons (Table I, Figs. 2–4). As shown in Table I diuron and bentazon inhibited photosystem II activity in the herbicide treated cotyledons and their chloroplasts, while the bleaching herbicides SAN 6706 and aminotriazole showed no inhibition. Variable fluorescence which is regarded as a very sensitive indicator of the activity of photosystem II was completely inhibited in cotyledons from plants that were grown in the presence of 10^{-4} M diuron for 6 days (Fig. 2). On the other hand plastids isolated from these cotyledons still retained 50% of the

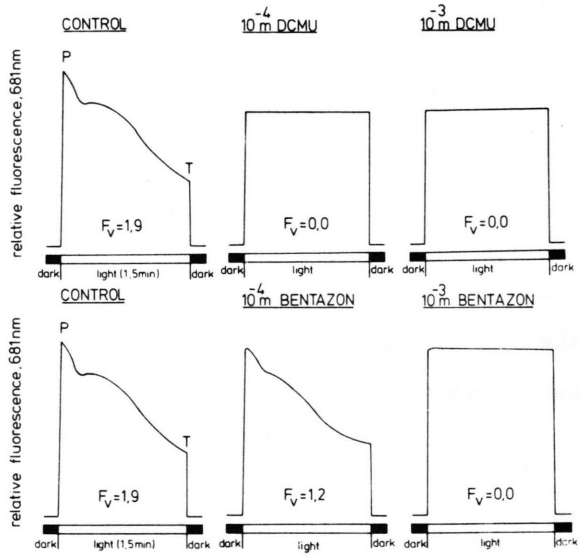


Fig. 2. Slow chlorophyll fluorescence transients of radish cotyledons after 6 days growth in the presence of photosystem II herbicides.

Table I. Photosystem II activity of plastids that were isolated from plants after 6 days growth in the presence of photosystem II or bleaching herbicides. Photosystem II activity is based on three independent chloroplast isolations ± SD. Fluorescence data that were recorded using intact cotyledons are means of three independent experiments with six replications ± SD.

Photosynthetic activity	Herbicide assayed							
	Control	10^{-4} M DCMU	10^{-3} M DCMU	10^{-4} M Bentazon	10^{-3} M Bentazon	10^{-4} M Amitrole	10^{-3} M Amitrole	10^{-5} M SAN 6766
µmol O_2-evolved per mg chlorophyll × h	82.2 ± 15.2	46.4 ± 5.1	0.0	55.0 ± 4.2	0.0	51.0 ± 4.2	26.7 ± 3.9	250.0 ± 50.5
Maximum fluorescence	10.8 ± 0.8	8.2 ± 1.4	8.0 ± 1.6	10.5 ± 0.7	10.3 ± 1.1	11.0 ± 0.5	4.9 ± 0.6	2.6 ± 0.4
Variable fluorescence	1.9 ± 0.1	0.0	0.0	1.2 ± 0.1	0.0	1.7 ± 0.1	0.1 ± 0.02	0.0

photosystem II dependent oxygen evolution as compared to the untreated cotyledons (Table I). This difference might be explained by the partly removal of the photosystem II inhibitor from its binding site during chloroplast isolation. However, with increasing inhibitor concentrations photosystem II activity was inhibited. As compared to diuron a ten times higher concentration was nessessary for the complete inhibition of the photosystem II activity by bentazon supporting that diuron is a much more powerful inhibitor than bentazon (Table I, Fig. 2).

While diuron and bentazon inhibited photosynthetic electron transport, and the chlorophyll a fluorescence transients drastically the bleaching herbicides SAN 6706 and amitrole had only indirect effects on both processes (Fig. 3). SAN 6706 and amitrole primary inhibited carotenoid biosynthesis leading to albinistic or brown-red cotyledons. Even at an unphysiological high concentration of amitrole (10^{-3} M), still a reasonable activity of photosystem II could be detected supporting that aminotriazole is only affecting electron transport and photosystem II activity indirectly.

As compared to amitrole treated plants the cotyledons from seedlings that were grown in the presence of SAN 6706 were white and the amount of chlorophyll was far below the spectrophotometric detectibility. However, as can be deduced from the

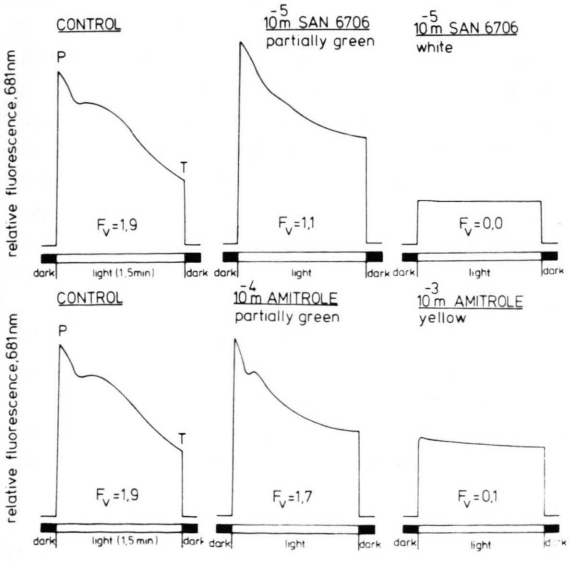


Fig. 3. Slow chlorophyll fluorescence transients of radish cotyledons after 6 days growth in the presence of bleaching herbicides.

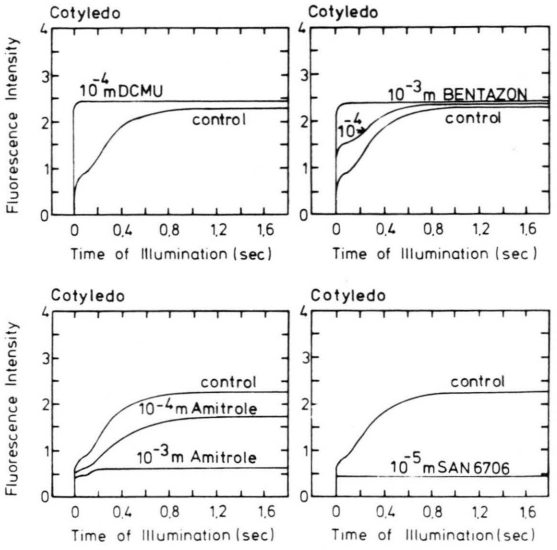


Fig. 4. Fast chlorophyll fluorescence transients of radish cotyledons after 6 days growth in the presence of photosystem II or bleaching herbicides.

chlorophyll fluorescence (Table I, Fig. 3) there was still a very small amount of chlorophyll contained in the cotyledons but no transient in the chlorophyll fluorescence could be detected (Fig. 3).

In the plastids that were isolated from a large batch of SAN 6706 treated white cotyledons a significant amount of oxygen evolution could be traced. On a chlorophyll basis the photosystem II activity was even higher in the SAN treated plastids as compared to the untreated green chloroplasts (Table I). This may be due to the existence of a very small amount of plastids that were still photosynthetically active and the fact that some of the chlorophyll was already lost via photodestruction.

Similar results as obtained from the slow chlorophyll fluorescence transients and photosystem II dependent oxygen evolution were also received by recording the chlorophyll a fluorescence transients in a higher resolution. This is shown in Fig. 4. Diuron and bentazon quenched the chlorophyll fluorescence transient drastically while aminotriazole was ineffective. Only the relative amount of the emitted fluorescence was drastically reduced because of the loss of chlorophylls. In the SAN-treated white cotyledons a very small amount of chlorophylls could still be detected by their fluorescence but no chlorophyll a fluorescence transient was detected.

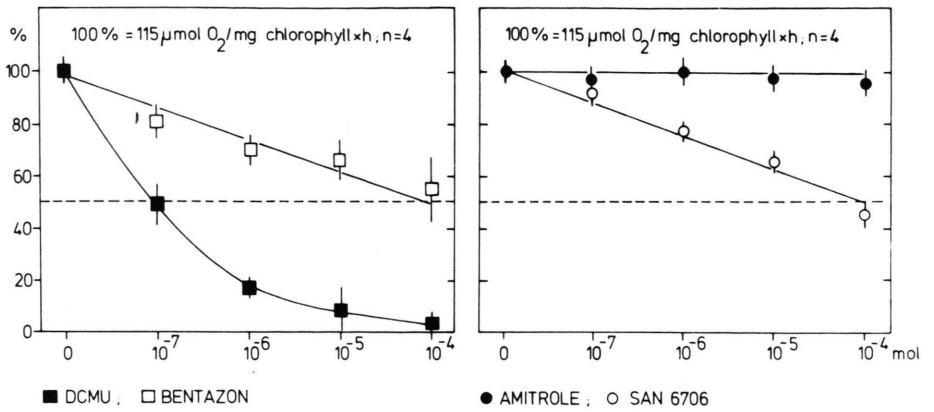


Fig. 5. Inhibition of the photosystem II dependent oxygen evolution in isolated radish chloroplasts in the presence of photosystem II or bleaching herbicides.

The direct influence of photosystem II and bleaching herbicides on the photosystem II dependent oxygen evolution and the chlorophyll a fluorescence transients in isolated chloroplasts

If chloroplast development has to take place in the presence of a herbicide the action of the photosystem II and bleaching herbicides that were assayed on various physiological and biochemical reactions in the chloroplast can be quite different. In order to resolve this question a comparative analysis of diuron, bentazon, amitrole and SAN 6706 on the photosystem II activity of isolated photosynthetically active chloroplasts was performed.

In agreement with the results shown in Table I diuron and bentazon inhibited the photosystem II dependent oxygen evolution in isolated chloroplasts (Fig. 5), but a much lower concentration was actually needed. From the investigation of the concentration dependency it was observed that diuron inhibited the photosystem II activity already at a final concentration of 10^{-7} M up to 50% while for the same inhibition of bentazon a 1000 times more concentrated solution was needed.

In contrast to diuron and bentazon, amitrole did not inhibit electron transport in the isolated chloroplasts although the photosynthetic activity was reduced in the chloroplasts that were isolated from the herbicide treated cotyledons. This is explained by the fact that amitrole interferes with the biosynthesis of carotenoids during chloroplast development by inhibiting the cyclization of lycopene to α- and β-carotene and therefore may disturbe the assembly of the thylakoid membrane and the interaction of the light-harvesting complex and the photosynthetic reaction centers with the electron transport chain.

As compared to amitrole the bleaching herbicide SAN 6706 was behaving completely different. In isolated chloroplasts SAN inhibited the photosystem II dependent oxygen evolution with a similar inhibition kinetic as the photosystem II inhibitor bentazon suggesting that SAN may also bind to the same site of the electron transport chain as bentazon and diuron [21]. On the other hand plastids that were isolated from the white cotyledons which were grown for 6 days in the presence of SAN 6706 were photosynthetically very active. In contrast to amitrole that has no direct influence on the photosystem II activity, SAN 6706 seems to inhibit electron transport very well but only in the isolated disrupted chloroplasts, suggesting that SAN can hardly enter the chloroplast and under *in vivo* conditions effects photosystem II activity only indirectly. Further support of this assumption was also given by Ridley (personal communication).

Besides the analysis of the photosystem II dependent oxygen evolution fast chlorophyll a fluorescence transients were also recorded in order to confirm the inhibition site of the herbicides assayed in electron transport. Chloroplasts were isolated according to Jensen and Bassham and Heber and Santarius [18, 19], supplied with the different herbicides and the chlorophyll fluorescence transients recorded. Diuron and bentazon inhibited the photosystem II dependent chlorophyll a fluorescence tran-

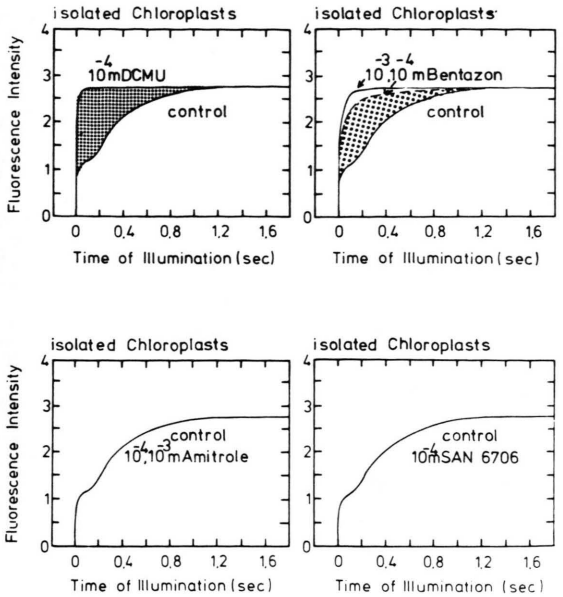


Fig. 6. Fast chlorophyll fluorescence transients of isolated radish chloroplasts in the presence of photosystem II or bleaching herbicides.

sient while amitrole had no effect (Fig. 6). Again the pyridazinon herbicide SAN 6706 was behaving very contradictory. Although it inhibited the photosystem II dependent oxygen evolution like bentazon no effect on the chlorophyll fluorescence transients was obtained even after using higher concentrations (10^{-4} M). This observation may well raise the question whether SAN is bound to the same binding site of the thylakoid membrane as the photosystem II inhibitors for it is well known that diuron and bentazon inhibit electron transport at the oxidizing side of the plastoquinone-9 pool and therefore quench the chlorophyll fluorescence transient drastically.

Discussion

It has been recognized for a long time from studies with various herbicides including photosystem II and bleaching herbicides that how the lethal effect of a herbicide is expressed in higher plants during growth depends on the particular site at

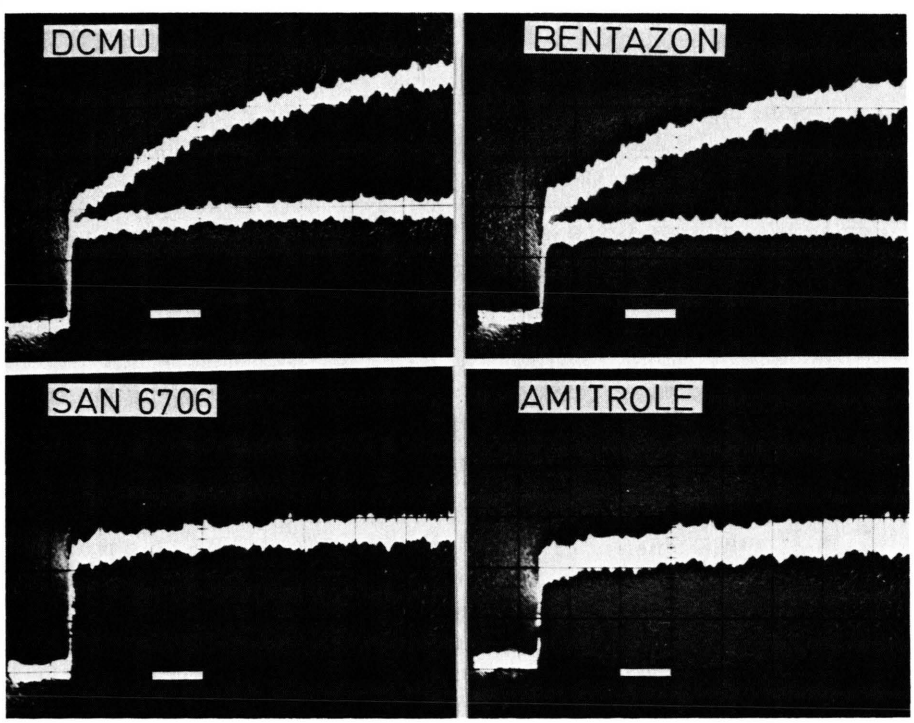


Fig. 7. High resolution of the chlorophyll a fluorescence transients of isolated intact or broken chloroplasts in the presence of photosystem II or bleaching herbicides. White bar represents 5×10^{-3} sec.

which a physiological reaction is inhibited in a plant cell and its compartments. In the cytoplasm herbicides interfere with the biosynthesis of ribonucleic acids, proteins or secondary plant products that maintain plant growth. In the chloroplast there are two main targets of herbicides action. One target is represented by the electron transport chain with its electron carriers and enzymes which are involved in phosphorylation and NADP photoreduction. Herbicides that interrupt electron transport and finally inhibit photophosporylation and NADP-photoreduction are very powerful inhibitors because of the immediate loss of biochemically available energy. Another main target of herbicide action is the biosynthesis of chlorophylls and carotenoids that are contained in the light-harvesting complex and the antennae of the photosynthetic reaction centers. Herbicides that interfere with the formation of chlorophylls and carotenoids induce a strong chlorosis and disturbe the protection mechanism that dissipated the excess light energy by passing it on to harmless reactions.

As shown in Table I and Figs. 1–6 diuron and bentazon inhibited photosynthetic electron transport, but diuron was confirmed as a much more powerful inhibitor than bentazon. Apart from diuron bentazon also binds to the same site of the electron transport chain. Convincing proof for the binding and inhibiting site of both herbicides was given by the demonstration of the quenching of the chlorophyll a fluorescence transients in isolated chloroplasts (Fig. 6).

As compared to the photosystem II herbicides the action of the bleaching SAN 6706 and aminotriazole on the photosystem II activity was contradictory. SAN 6706 inhibited the photosystem II dependent oxygen evolution but did not quench the chlorophyll a fluorescence transients in isolated chloroplasts. On the other hand the photosystem II activity was much higher in plastids that were isolated from plants which were grown for 6 days in the presence of SAN 6706 although the SAN treated plants looked white and contained only a very small amount of chlorophyll (Table I, Fig. 3). This observation most likely suggests that SAN under *in vivo* conditions can not enter the chloroplast and may influence the photosystem II activity only indirectly by inhibiting pigment biosynthesis. The photosynthetic activity may therefore increase in SAN bleached chloroplasts on a chlorophyll basis due to their lower chlorophyll content.

In contrast to SAN 6706 aminotriazole did not inhibit photosystem II activity directly neither in isolated chloroplasts nor under *in vivo* conditions in the intact plant. The slight inhibition of the photosynthetic activity in radish that was grown for 6 days in the presence of the herbicide probably depends on the interference of amitrole with the biosynthesis of chlorophylls and carotenoids. Even under unphysiological high concentrations of amitrole (10^{-3} M) the photosynthetic activity was still maintained suggesting that this herbicide does not inhibit photosystem II activity directly. In order to exclude any specific binding site of aminotriazole and SAN 6706 in the electron transport chain near photosystem II the effect of both herbicides on the reduction of the primary acceptor Q of photosystem II was reinvestigated and compared with the effect of diuron and bentazon using chloroplasts isolated according to Tischer and Strotmann [21]. This is shown in Fig. 7. Diuron and bentazon inhibited the oxidation of the reduced primary electron acceptor Q leading to an increase in the chlorophyll fluorescence. In contrast to diuron and bentazon the bleaching herbicides aminotriazole and SAN 6706 did not interfere with the reduction of the quencher Q suggesting that both herbicides are not inhibiting non cyclic electron transport close to photosystem II. Aminotriazole and SAN 6706 may rather interfere with the photosystem II activity only indirectly via their effect on pigment biosynthesis.

As compared to the electron transport inhibitors diuron and bentazon the interaction with the function of carotenoids in protecting the chlorophylls against photooxidative destruction seems to be the primary effect of the bleaching herbicides and particularly SAN 6706. β-carotene which is contained in both photosynthetic reaction centers but predominantly in photosystem I particles is supposed to represent the main carotenoid that is involved in the dissipation of the excess light energy. Inhibition of carotenoid biosynthesis by bleaching herbicides may therefore disturbe this protection mechanism leading to a photooxidative damage of the photosynthetic reaction centers. Only recently it was reported by Ridley [22] that the cyclic electron transport around photosystem I is probably the main protection mechanism of the photosynthetic apparatus for the harmless dissipation of the excess light energy. This means that either an inhibition of the

electron transport by photosystem II herbicides or a destroyment of the protecting mechanism by inhibiting β-carotene biosynthesis under the influence of bleaching herbicides may result in a photooxidative damage of the reaction centers and the degradation of the chloroplast.

Acknowledgements

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