# COMPARATIVE SPECTROSCOPIC INVESTIGATION OF HERBICIDAL EFFECT UPON PHOTOSYSTEM II PHOTOCHEMISTRY OF CHLOROPLASTS ISOLATED FROM TRIAZINE-SUSCEPTIBLE AND TRIAZINE-RESISTANT BIOTYPES OF *ERIGERON CANADENSIS* L.

by

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I dedicate this thesis to my wife Tahmina Rashid for her strong patience during my absence from her and for her continuous inspiration and encouragement for the progress of my research work.

A. Rashid

C O N T E N T S Page

# ABBREVIATIONS

| | |
|---|---|
| ADRY | = accelerating the deactivation reactions of water splitting enzyme system Y |
| B | = secondary electron acceptor of photosystem II |
| BSA | = bovine serum albumin |
| CF | = coupling factor |
| DBMIB | = 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone |
| DCMU | = 3-(3,4-dichlorophenyl)-1,1-dimethylurea |
| DCPIP | = 2,6-dichlorophenol-indophenol |
| DNOC | = 4,6-dinitro-o-cresol |
| EDTA | = ethylenediamine tetraacetic acid |
| $F_o$ | = the initial chlorophyll fluorescence level |
| $F_I$ | = the intermediate fluorescence level |
| $F_M$ | = the maximum fluorescence level |
| $\Delta F$ | = the variable fluorescence change |
| HEPES | = N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid |
| $I_{50}$ | = concentration of the herbicide giving 50% inhibition |
| KD | = kilodalton |
| LHC | = light harvesting chlorophyll-protein complex |
| ms | = millisecond |
| nm | = nanometer |
| OD | = optical density |
| PQ | = plastoquinone pool |
| PS II | = photosystem II |
| PS I | = photosystem I |
| PC | = plastocyanine |
| P680 | = reaction center of PS II |
| pBQ | = p-benzoquinone |
| Q | = quencher, (primary electron acceptor of PS II) |
| R/S | = $I_{50}$ concentration resistant/$I_{50}$ conc. susceptible |
| SDS | = sodium dodecyl sulphate |
| TL | = thermoluminescence |
| $\lambda$ | = wavelength of radiation |

# 1 REVIEW OF LITERATURE

## 1.1 Inhibition of the electron transport of photosynthesis by herbicides

Photosynthesis is an oxidation-reduction process in which $H_2O$ is oxidized to molecular oxygen and carbon dioxide is reduced to carbohydrate.

$$6\ CO_2 + 12\ H_2O \xrightarrow[\text{chlorophyll}]{\text{light}} C_6H_{12}O_6 + 6\ O_2 + 6\ H_2O$$

$$+ 675\ \text{Kcal/mole hexose}$$

In higher plants the site of photosynthesis is the chloroplasts. Internally the chloroplasts composed of complex membrane structure differentiated into stacked "grana" membrane and unstacked "stroma" membrane. The photosynthetic reactions can be divided into light and dark reactions. The light reaction takes place in the grana and stroma membranes and $CO_2$ fixation takes place in the stroma regions. In photosynthetic electron transport from $H_2O$ to NADP two light reactions co-operate in series and several electron transport components participate which are arranged according to their redox potentials. The electron transport chain from $H_2O$ to PQ (plastoquinone) is called photosystem II

(PS II) and from PQ to NADP is called photosystem I (PS I), Hill and Bendall (1960). The location of electron transport components in the chloroplast membrane is shown in Fig. 1.

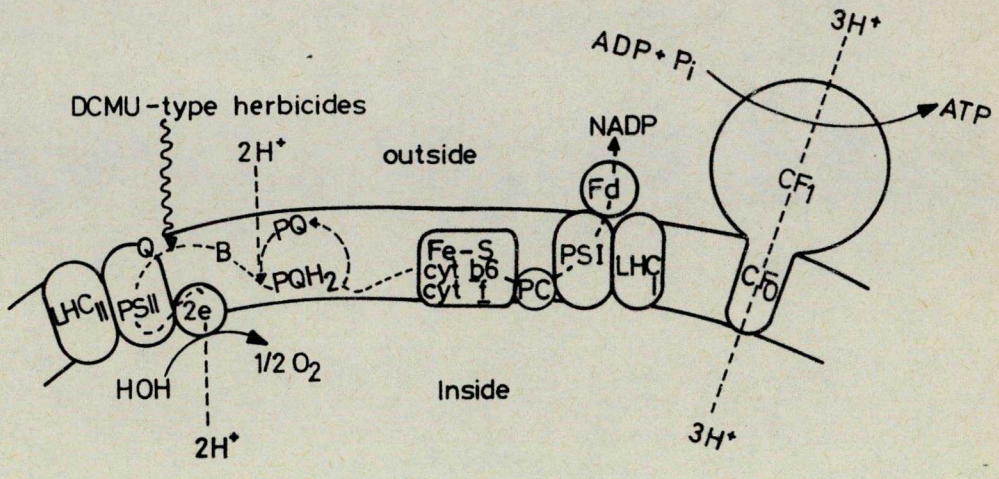


Fig. 1 Membrane structure with electron transport components (Photosynthetic electron flow coupled to ATP formation). The dotted line represents electron transport from $H_2O$ to NADP, the dashed line shows the proton movement as envisaged by the chemiosmotic hypothesis and the solid wavy line represents the inhibitory site of DCMU-type inhibitors.
Abbreviations: LHC, light-harvesting chlorophyll-protein complex; PS, photosystem; Q, primary electron acceptor of PS II; B, secondary electron acceptor of PS II; PQ, plastoquinone pool; Fe-S, Rieske iron-sulphur center; Cyt, cytochrome; PC, plastocyanin; Fd, ferredoxin; NADP, nicotinamide adenine dinucleotide phosphate; CF, coupling factor; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DCMU, dichlorophenyl-dimethylurea

Since the majority of the electron transport inhibitors and herbicides block the electron transport in PS II we deal with the components of PS II in details.

PS II is a large complex most of which is embedded in the photosynthetic membrane. When PS II is fully developed it contains a number of proteins, hundreds of chlorophylls including chlorophyll a (Chl a) and chlorophyll b (Chl b), carotenoids, plastoquinone, cytochrome $b_{559}$, the oxygen evolving enzyme and other electron transport cofactors and chemical constituents (Amesz and Duysens, 1977).

The photochemical and photophysical processes within PS II proceed in the following sequence. First, the antenna chlorophyll molecules absorb photons and the resultant singlet excitation is transferred rapidly via singlet exciton (Knox, 1975; Shipman, 1980) to a special Chl a species (P680) in the reaction center of PS II. When excited by the singlet electronic excitation from antenna, $P680^*$ transfers an electron to a nearby pheophytin a (Pheo) molecule (Klimov et al., 1980).

$P680^+$ $Pheo^-$ Q B

In a second electron transfer step the electron moves from Pheo to Q, a plastoquinone molecule within PS II.

$P680^+$ Pheo $Q^-$ B

Then the electron moves from Q to B, a plastoquinone molecule also within the PS II complex and at the same time $P680^+$ is reduced to P680 by the oxygen evolving enzyme located on the inside of photosynthetic membrane.

P680 Pheo Q $B^-$

The existence and function of B was discovered independently by Bouges-Bocquet (1973) and Velthuys and Amesz (1974) who called it B and R respectively. The electron remains at B for many seconds (Velthuys and Amesz, 1974).

In subsequent photochemical events, a second electron is transferred from $P680^*$ to Q via the pheophytin molecule

$P680^+$ Pheo $Q^-$ $B^-$

and $P680^+$ is reduced to P680 by the oxygen evolving enzyme. The electron then moves from Q to $B^-$

P680 Pheo Q $B^{2-}$

At this point, there is a multi-step reaction in which two protons and two electrons are transferred to a PQ molecule in the pool of PQ carrying electrons between PS II and PS I (Fig. 1).

Studies of electron and proton transfer on the reducing side of PS II have established that Q and B are in a fixed stoichiometry of one each per PS II reaction center and upon reduction to $Q^-$ and $B^-$,

they are not protonated. On the other hand, PQ becomes protonated after getting two electrons from $B^{2-}$ (Haehnel, 1976). The reason for this distinction probably is that Q and B are shielded from the stroma region by a proteinaceous structure. Such a shield was first proposed by Renger (1976). He interpreted the loss of DCMU (dichlorophenyl dimethylurea) inhibition of Hill reaction by mild digestion with trypsin by suggesting that trypsin treatment degrades a proteinaceuos shield covering the lipophilic electron acceptors of light reaction II. He assumed this proteinaceous component to act as an allosteric regulator for electron transport between Q and PQ pool. Moreover, this protein was supposed to contain the binding sites of a large number of inhibitors. In this review the protein is referred to as Q-B protein or herbicide-binding protein.

The herbicidal inhibitors of photosynthesis, according to Moreland (1980) can be divided into five groups:

i. electron transport inhibitors
ii. uncouplers
iii. energy transfer inhibitors
iv. inhibitory uncouplers
v. electron acceptors.

More than 50% of the electron transport inhibitors interact with the Q-B protein of PS II and they are called PS II herbicides (Izawa, 1977; Trebst, 1980). They are conveniently grouped into classes of similar structures:

1. Urea derivatives
2. s-triazines
3. Nitrophenols
4. as-triazinones
5. Uracils
6. Biscarbamates
7. Pyridazinones
8. Hydroxybenzonitriles
9. Anilides and
10. Benzimidazoles.

These inhibitors block the reduction of PQ. Another group of inhibitors also exist which inhibit the oxidation of PQ. This group is termed as DBMIB (dibromothymoquinone) type inhibitors (Trebst, 1980).

Research on the mode of action of herbicides inhibiting photosynthesis has a dual purpose (Van Rensen, 1971).

a/ it yields information on the mechanisms of action of these chemicals, thereby unshielding clues which may lead to the finding of new herbicides

b/ using these chemicals as specific inhibitors, information on the photosynthetic process can be obtained. This review will deal with the PS II inhibitors (urea, triazine and nitrophenol) and to some extent DBMIB-type inhibitors.

Urea and triazine herbicides were introduced between 1950 and 1960. Both groups contain large numbers of active chemicals. Their common basic chemical structure is a =C-N-group (Trebst and Harth 1974). The site and mode of action are the same for all members of these chemical groups. The differences in activity are caused by the various lipophilic side chains. In photosynthesis research, DCMU and atrazine, are the best known urea and triazine herbicides respectively. They are both specific inhibitors, acting at very low concentration.

Wessels and Van der Veen (1956) were the first to show that DCMU inhibits the Hill reaction in isolated chloroplasts. The stimulation of fluorescence by DCMU was explained by Duysens and Sweers (1963) by assuming that this inhibitor prevents the reoxidation of Q. By studying various parts of electron transport pathway the site of inhibition of these herbicides was located between Q and the PQ pool. PS I dependent electron transport is inhibited only at very high concentration. Rosenberg et al.

(1972) reported DCMU action on the oxidizing side of PS II from their experiment with flash-induced oxygen evolution measurement. Witt (1973) and Garab et al. (1976) reported also the DCMU action on the reaction center of PS II (P680).

The study of the mode of action of these herbicides was greatly stimulated after the introduction of a new technique by Tischer and Strotmann (1977). After a radiolabelled herbicide was bound to isolated chloroplasts, it was investigated if other (non-radiolabelled) herbicide could replace the labelled one. By this replacement technique they showed that phenylureas, triazines, pyridazinones and biscarbamates compete for the same binding site. Moreover, the relative concentration of specific binding sites was 1 per 300-500 chlorophyll molecules i.e, about 1 per electron transfer chain.

Laasch et al. (1981, 1982) reported the high affinity (specific) binding behaviour of DCMU, atrazine and dinoseb to thylakoid and algal cell. They reported the 1 : 1 stoichiometry between bound herbicide and electron transport chain suggesting the 300-500 chl/inhibitor binding site. In addition to the high affinity binding low affinity (unspecific) binding was also observed in thylakoids and

algal cells, which was not related to inhibition. Unspecific binding was found to be irreversible in contrast to the easily reversible specific binding in which complete mutual displacement of bound diuron type herbicides by either diuron type or phenol type herbicides was possible. The mechanism of binding of all PS II herbicides at the specific binding site was found to be competitive.

A characteristic of the herbicidal inhibitors is the reversibility of their effects. Van Rensen and Van Steekelenburg (1965) found that the inhibition of oxygen evolution in algae by DCMU and simeton can easily be removed by washing. Izawa and Good (1965) showed that DCMU is reversibly bound to chloroplasts. This means that only weak bonds are involved in the interaction of herbicide and the receptor molecule in the thylakoid membrane. Tischer and Strotmann (1979) measured the $\Delta H^{o}$(enthalpy) for binding of metribuzin and found to be equal to -50 KJ/mole. According to Shipman (1981), this binding energy is much too small for covalent binding to a protein, and it is also not consistent with hydrogen bonding. He suggested that polar components of the herbicides bind via coulombic interactions at or near to a highly polar protein site, probably a protein salt bridge or the terminus of an $\alpha$-helix on the Q-B protein.

It has been confirmed by experiments with the water soluble enzyme, trypsin that the binding site of these herbicides is of a proteinaceous in nature. Regitz and Ohad (1975) and Renger (1976) demonstrated that DCMU sensitivity of Hill reaction was removed by proper treatment of chloroplasts with trypsin. These observations were extended by Böger and Kunert (1979) and by Rensen and Kramer (1979) to the triazine and phenolic herbicides. It has been considered that trypsin treatment affects the Q-B protein in such a way that Q becomes accessible to the electron acceptor ferricyanide, thus inducing a short-circuit electron transport circumventing the site of action of the herbicides. However, it was also found that trypsin treatment removes the receptor sites of the herbicides (Trebst, 1979; Tischer and Strotmann, 1979; Steinback et al. 1981).

The presence of bicarbonate is a requirement for electron transport in the isolated broken chloroplasts. Govindjee and Van Rensen (1978) reported that the electron transport is inhibited from Q to PQ pool in the absence of bicarbonate. Since this is also the site of action of herbicides inhibiting PS II, there might be an interaction of bicarbonate and these herbicides. Van Rensen and Vermaas (1981) indeed demonstrated that DCMU and simeton influence bicarbonate

binding and that DNOC (dinitro-o-cresol) is a competitive inhibitor of bicarbonate binding. $CO_2$ depletion of chloroplasts causes a decrease in affinity of $C^{14}$-atrazine to the membranes indicating a close spatial location of the binding sites for atrazine and bicarbonate (Khanna et al. 1981).

Schemes visualizing the binding sites of herbicides to the Q-B protein have been proposed by Trebst and Draber (1979) Fig. 2, and Pfister and Arntzen (1979).

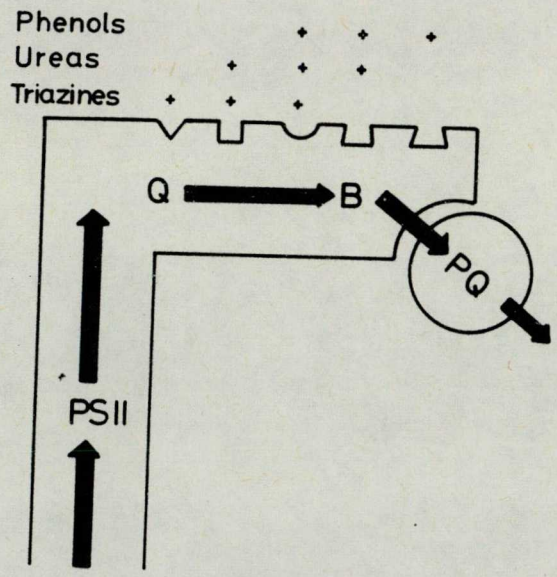


Fig. 2 Model representing the different but overlapping binding areas for urea, triazine and phenolic herbicides on the acceptor side of photosystem II. Conformational change by herbicide binding induces inhibition (Trebst, 1980).

Vermaas and Van Rensen (1981) have incorporated, in addition, two binding sites for bicarbonate. It is assumed that the binding of a herbicide and also the absence of bicarbonate causes a conformational change of Q-B protein. This change in conformation would have two consequences: 1/ the binding of another herbicide or bicarbonate is impaired and 2/ electron transport from Q to B is inhibited.

How does the conformational change of the Q-B protein caused by the binding of a herbicide or the absence of bicarbonate affect electron transport from Q to B? Velthuys and Amesz (1974) proposed that DCMU lowers the mid-point potential of B i.e, DCMU makes B harder to reduce. Thus the equilibrium of $Q^-B \rightleftarrows QB^-$ is shifted to the left in the presence of herbicides. Since the B semiquinone ($B^-$) is stable in hydrophobic environment for at least some seconds, Shipman (1981) rationalized that the Q-B protein must provide a stabilization of B in its semiquinone anion form. This stabilization can be effected by protonation of the protein or by relaxation of the protein conformation when B is reduced. Hence inhibitor binding could affect $Q^-B \rightleftarrows QB^-$ equilibrium by affecting the means of stabilization of $B^-$ by the Q-B protein. Moreover, Shipman (1981) suggested that B might be displaced by inhibitors from its binding site. Renger et al.

(1981) proposed that the Q-B protein is composed of at least two distinguishable protein units, one enwraping Q, the other B. By analogy with electron transport between the redox active heam groups of cytochrome c and cyt c peroxidase, they suggested that the Q-B protein mediates electron transport between Q and B via an "electron channel" established by a specific arrangement of functional amino acid residues. Binding of an inhibitor could cause a structural modification of the protein units in the area forming the electron tunnel. In this way the kinetics of electron transport between Q and B are changed.

Lavergne (1982) and Laasch et al. (1983) reported that the inhibitor (DCMU) competes with plastoquinone for binding to a common site on the acceptor side of PS II. They reported that binding of inhibitors to "even state" of the center (QB) is rapid in comparison to "odd state" ($QB \rightleftharpoons Q^{-}B$), in a pH range 6-8. The lower the pH the slower is the kinetics of binding.

Velthuys (1982) proposed that PQ binds reversibly to the PS II reaction center and reduced PQ binds reversibly to the Rieske iron sulfur center. Since B is of a plastoquinone in nature, he suggested that it exchanges rapidly (except while semire-

duced) with free PQ diffusing in the membrane. This implies that B binding site is frequently left vacant, resulting in the possibility that other compounds can bind. Thus herbicides could bind instead of B and in this way electron transport is inhibited from Q to B.

Most recently Vermaas et al. (1983) reported the interaction of herbicides and azidoquinones at a PS II binding site in the thylakoid membrane. They found that 6-azido-$Q_oC_{10}$ could replace the native plastoquinone at B and could accept electrons from the primary electron acceptor Q. In this way it allows the electron transport through PS II to the plastoquinone pool. The synthetic azidoquinone was also found to compete with the PS II herbicides ioxynil and atrazine for binding, an observation strongly favors the hypothesis that PS II herbicides block electron transport by replacing the native quinone (B) which acts as the second electron carrier on the reducing side of PS II. Covalent linkage of 6-azido-$Q_oC_{10}$ to its binding environment by UV irradiation had greatly reduced herbicide binding affinity but did not lead to a loss in herbicide binding sites. Therefore, they reported that covalent attachment of 6-azido-$Q_oC_{10}$ had allowed some freedom of quinone head-group movement such that the herbicide could enter the binding site. This indicates that the protein determinants

which regulate quinone and herbicide binding are very closely related but not identical.

Among the phenolic herbicides, DNOC, dinoseb and ioxynil are well known inhibitors. DNOC was the first organic chemical ever used as a herbicide (about 1930). Because of its similarity to 2,4-dinitrophenol, DNOC was considered to act by uncoupling of oxidative phosphorylation, untill Kerr and Wain (1964) reported that it inhibits the Hill reaction. Van Rensen et al. (1977, 1978) and Van Rensen and Hobe (1979) demonstrated that at low concentrations DNOC inhibits photosynthetic electron transport at the same site as DCMU while at high concentration PS I dependent electron transport is uncoupled. Because of this dual effect, inhibition and uncoupling, Moreland (1980) classified the phenolic herbicides as inhibitory uncouplers.

Reimer et al. (1979) and Trebst et al. (1979) found that phenolic herbicide like ioxynil, dinitrophenols, halogen and alkyl substituted nitrophenols all inhibit electron transport at the same site as DCMU. Though acting at the same site, the phenolic herbicides do not have the same basic chemical structure of ureas and triazines; interaction with the receptor site in the thylakoid membrane is also different (Van Rensen et al. 1978). Trebst and Draber

(1979) studied the structure activity relationships of a large number of halogenated nitro- and dinitrophenols. The activity of ureas and triazines is related to lipophilicity and electronic parameters, whereas with the phenolic herbicides activity is best related to steric parameters.

Evidence that urea and phenolic herbicides act at the same site was supported by the displacement of $C^{14}$ labelled metribuzin from thylakoid membranes by bromonitrothymol and ioxynil (Reimer et al. 1979). In an extensive study of displacement of urea and phenolic herbicides, Oettmeier and Masson (1980) showed that the ureas interfere noncompetitively but the phenolic herbicides interfere competitively with specific binding site of the phenolic herbicide, i-dinoseb. They concluded that the binding sites of both types of herbicides are not identical although they are located on the same protein.

This interpretation is in accordance with the previouly found differences between the urea and triazine inhibitors versus the phenolic herbicides. Böger and Kunert (1979) found that during short-term treatment of isolated chloroplasts with trypsin, inhibition by ureas and triazines is reversed, while the phenolic herbicide showed increased inhibitory activity. Vermaas and Van Rensen (1981) also reported

from their studies with interaction of herbicides with bicarbonate that phenolic herbicide bind to a site on the Q-B protein different from that of the ureas and triazines. It has been reported by Croze et al. (1979), Steinback et al. (1981), Mullet and Arntzen (1981) and Pfister et al. (1981) that the urea and triazine binding site in the chloroplast membrane is composed of 32-34 KD (kilo dalton) polypeptide. On the other hand, Oettmeier et al. (1980, 1982) reported that the phenolic herbicide binding site in the membrane is composed of a 41 KD polypeptide.

They isolated the herbicide binding proteins by photoaffinity labelling techniques. An azido-group has been introduced into a phenolic herbicide, azido-i-dinoseb-$H^3$ labelled (Oettmeier et al. 1980) and the similar azido-group has been introduced into s-triazine, azido-triazine-$C^{14}$ labelled (Pfister et al. 1981). After irradiation of thylakoid membrane with either visible or UV light it was found that azido-atrazine was bound to a 32 KD protein and azido-i-dinoseb to a 41 KD protein. This observation indicates that two different polypeptides, 32 and 41 KD are involved for binding urea and phenolic herbicides respectively. From the trypsin treated experiment, Oettmeier et al. (1982) proposed a model

(Fig. 3) which explains that the 32 KD polypeptide is surface exposed and the 41 KD polypeptide is located deeper in the membrane.

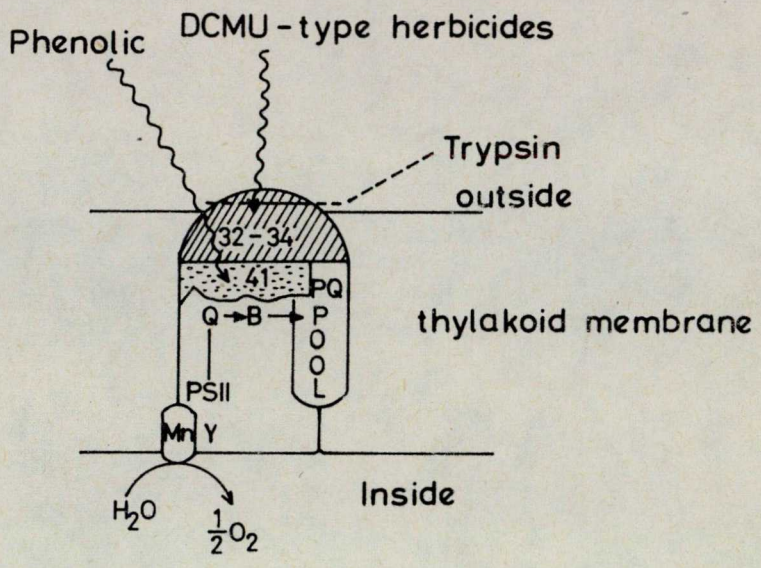


Fig. 3 Model indicating the location of two herbicide -binding proteins at the reducing side of photosystem II. 32-34 and 41 represent (in kilo daltons) the corresponding proteins for binding DCMU-type and phenolic herbicides respectively.

Mild trypsin treatment completely removes the sensitivity to diuron and atrazine but sensitivity towards phenolic herbicides remains unchanged.

Most recently Oettmeier et al. (1983) reported that PS II particles prepared from the thylakoid of spinach chloroplasts and Chlamydomonas reinhardii CW 15 were sensitive to phenolic herbicide dinoseb but insensitive to atrazine and diuron. Photoaffinity

labeling revealed that azido-atrazine labeled a 32 KD polypeptide both in spinach as well as in Clamydomonas thylakoids but this label did not react with PS II particles preparations. Azido-dinoseb, however, were found to label both the thylakoid membrane and the PS II particles, predominantly polypeptides in the 40-53 KD molecular region. Since the latter polypeptides are probably part of the reaction center of PS II, they suggested that phenolic herbicides have their inhibition site within the reaction center complex. Therefore, they concluded that the atrazine binding 32 KD polypeptide is either absent or functionally inactive in PS II particles whereas phenol inhibitor binding are not.

## 1.2 Herbicide resistance in plants

Resistance to herbicides has been anticipated for a long time especially in corn fields where triazine herbicides have been applied for several years. Ryan (1970) reported resistance to atrazine in the weed Senecio vulgaris L.. Resistance of crop plants to herbicides is usually due to various mechanisms which prevent the herbicide reaching its site of action in the plants. However, atrazine resistance in Senecio is caused by a change in the receptor site

within photosynthetic apparatus. Later this mechanism has been also found in other weeds. To-date 30 common annual weed species in 18 genera including 23 dicot and 7 monocot (Table 1.) previously susceptible to triazine have been found to be resistant (LeBaron and Gressel, 1982).

Table 1. List of triazine-resistant weed species reported from field observations

| Genus | Species |
|---|---|
| Broadleaf Species | |
| Amaranthus | arenicola |
| Amaranthus | hybridus |
| Amaranthus | lividus |
| Amaranthus | powellii |
| Amaranthus | retroflexus |
| Ambrosia | artemissifolia |
| Atriplex | patula |
| Bidens | tripartita |
| Brassica | campestris |
| Chenopodium | album |
| Chenopodium | ficifolium |
| Chenopodium | missouriense |
| Chenopodium | polyspermum |
| Chenopodium | strictum |
| Erigeron | canadensis |
| Galinsoga | ciliata |
| Kochia | scoparia |
| Polygonum | convolvulus |

| Genus | Species |
|---|---|
| Polygonum | lapathifolium |
| Polygonum | persicaria |
| Senecio | vulgaris |
| Solanum | nigrum |
| Stellaria | media |
| Grass Species | |
| Bromus | tectorum |
| Echinochola | crus-galli |
| Panicum | capillare |
| Poa | annua |
| Setaria | faberi |
| Setaria | lutescens |
| Setaria | viridis |

According to Pfister and Arntzen (1979) many species of atrazine resistant weed biotypes have been found to share the following common features:

a/ they were discovered in areas where triazine herbicides had been used repeatedly,

b/ resistance to triazine is extreme; it is not due to a minor shift in herbicidal response,

c/ no changes in herbicide uptake, translocation or metabolism - as compared to susceptible biotypes - can be detected,

d/ resistance is selective for only certain classes of photosynthetic herbicides,

e/ chloroplasts isolated from triazine resistant weeds display high preferential resistance to the triazine in assays of PS II partial reactions,

LeBaron and Gressel (1982) added some more points:

f/ species that have developed resistance to triazines were normally very sensitive and easy to control with these herbicides prior to the resistance,

g/ triazine resistant biotypes have generally shown cross resistance to all s-triazine herbicides,

h/ there also seems to be related degrees of resistance in the resistant biotypes to the asymmetrical triazines, ureas and many other N-containing photosynthetic inhibitors,

i/ the resistant biotypes seem to develop an even greater degree of sensitivity than the triazine-susceptible biotypes to herbicides having other modes of action (eg. dinoseb and ioxynil),

j/ the $I_{50}$ values (herbicide concentration which inhibits 50% of the electron transfer phenomenon) for each triazine herbicide is quite constant between all sensitive plants. Between resistant weeds the $I_{50}$ values vary with the species,

k/ a very low frequency of the triazine-resistant biotype was present among the species population when tri-

azine herbicide was first applied,

l/ there is no evidence that the herbicides were the direct causes of resistance or that they have had any mutation effect on the natural susceptible population

m/ once resistant plants have developed and infested an area with resistant seeds, it is virtually impossible to iradicate the source of resistance.

Variability in the sensitivity of photosynthesis to s-triazine has been demonstrated in ten different weed species (Table 2.) by Pfister and Arntzen (1979).

Table 2. List of weed species showing variability in the sensitivity of photosynthesis towards triazines.

| Genus | Species |
|---|---|
| Ambrosia | artemisiifolia |
| Amaranthus | retroflexus |
| Brassica | campestris |
| Chenopodium | album |
| Chenopodium | strictum Roth va. glaucophyllum (Allea) Wahl |
| Poa | annua |
| Polygonum | lapathifolium |
| Senecio | vulgaris |
| Solanum | nigrum |

They have reported that the mechanism for resistance to triazine has resulted from specific genetic mutations or subtle modifications of the biochemistry in photosynthetic apparatus.

It is generally accepted that PS II herbicides with chemically different structure have a common target of inhibition in the photosynthetic electron transport chain (Trebst and Draber, 1979). The mechanism that produced herbicide resistance has been investigated extensively by many authors.

Radosevich and Appleby (1973) have shown that the differences in resistance found in common groundsel (Senecio vulgaris L.) biotypes were not attributed to variation in germination time, rooting depth, or morphology resulting in differences in exposure to the herbicides. They also demonstrated that uptake and eventual metabolism of the triazines was identical for susceptible and resistant groundsel biotypes. A mechanism of resistance other than that associated with metabolism of the herbicide as reported for other triazine-resistant species was operative in the groundsel biotype. Radosevich and Devilliers (1976) reported that 10 μM atrazine inhibited photosynthesis in isolated chloroplasts of susceptible biotype but could not inhibit in the resistant ones of common groundsel. In comparative studies, inhibition of photosynthetic electron transport in chloroplasts isolated from resistant biotypes has required up to 1000-fold higher concentrations of triazine than those needed to inhibit electron transport in chloroplast isolated from susceptible

biotypes (Arntzen et al. 1979; Pfister et al. 1979; Mechado et al. 1978). They clearly demonstrated that the resistance was associated with the reducing side of PS II.

Recent studies on the nature of herbicide resistance as mentioned above, have focused primarily upon the structure and function of atrazine binding protein. This protein of the chloroplast thylakoid from various weed species was identified by the covalent binding of an $C^{14}$-azido-analog of atrazine (Pfister and Arntzen 1979; Pfister et al. 1980). Azido-s-triazines have the same structure-activity relationships as other substituted s-triazines. Thus it may be presumed that azido-atrazine and atrazine act at the same site. Azido-atrazine has the added advantage that in the presence of UV light, the activation of the azido group causes the atrazine to bind covalently to proteins. However, in those studies $C^{14}$-azido-atrazine was shown to bind to a 32 KD polypeptide of susceptible but not resistant chloroplast membrane. Analysis of the atrazine binding protein by SDS-polyacrylamide gel electrophorosis has failed to demonstrate any protein modifications between biotypes that might account for the mechanism of the type of triazine resistance. Since the 32 KD polypeptide is an integral component of the PS II structural complex within the membrane, it has

been suggested, therefore, that though 32 KD protein is present in resistant chloroplast, a genetic alteration within that protein might be responsible for the loss of atrazine binding capacity in the resistant chloroplast (Arntzen et al. 1979). This interpretation has recently been questioned by Gressel (1982). He found that chloroplast from Spirodela depleted of 32 KD polypeptide did not show more tolerance towards atrazine. Therefore, he argued that the interpretation which has been given by Arntzen's group from photoaffinity labeling experiments, that the azido group was bound to the same polypeptide as the herbicidal moiety of triazine; a 32 KD protein, has led to a erroneous conclusion. He proposed an alternative suggestion which explains that as the azido group is on the part of the herbicide directly opposite the part of the structure required for herbicide activity (Gabbott, 1969), the azido-atrazine might be acting as a bifunctional reagent. According to him, it is most probable that the ring with the azide is perpendicular to the plane of the electrostatically bound part of the molecule: the two side chains bound at the 4 and 6 positions and the adjacent part of the ring. The herbicidally active site of atrazine may bind (loosely) to its target protein in thylakoids of sensitive plants and UV radiation causes most of the azide moiety to bind primarily to

an adjacent or apposing 32 KD protein. Only the covalent part of the bifunctional azido-atrazine would remain bound to the 32 KD protein and the binding to the herbicide target site would be cleaved upon detergent disruption of thylakoid.

He concluded that the best acceptable hypothesis based on all the evidence to date is that the 32 KD protein is not the herbicide binding protein but is located very near the triazine binding site in PS II.

The triazine resistant weed biotypes that have appeared around the world differ from the wild type biotypes in ways other than just the ability to bind triazines (LeBaron and Gressel, 1982). There are a very significant quantitative and qualitative difference in thylakoid lipid composition. Pillai and John (1981) have shown that the lipid composition of resistant chloroplast is different from susceptible one, especially the amount of unsaturated fatty acids is higher in resistant chloroplast. He concluded that the partition into the thylakoid membrane to reach the binding site may be different for a herbicide in susceptible and resistant chloroplast.

Recently Burke et al. (1982) studied the lipid composition, ultrastructure and quantum requirements of photosynthesis in chloroplasts isolated from triazine susceptible and triazine resistant biotypes of

Brassica campestris. They reported that in general phospholipids but not glycolipids in chloroplasts from the triazine resistant biotype had a higher linolenic acid concentration and lower levels of oleic and linoleic fatty acids, than chloroplast from triazine-susceptible plants. Chloroplast from triazine-resistant biotypes has a 1.6-fold higher concentration of t-$\Delta$ 3 hexadecenoic acid with concomitantly lower palmitic acid concentration in phosphatidyglycerol. Phosphatidyglycerol previously has been hypothesized to be a boundary lipid for PS II. They also reported that chloroplasts from the triazine resistant biotypes had a lower chlorophyll a/b ratio and exhibited increased grana stacking. Quantum requirement for whole chain electron transport at limiting light intensities was lower for the susceptible biotype than for the triazine resistant biotype. They concluded that although the level of chlorophyll a/b light harvesting complex associated with PS II was greater in resistant biotype, the increased levels of light harvesting complex did not increase the photosynthetic efficiency enough to overcome the rate limitation that is inherited concomitantly with the modification of the s-triazine binding site.

Genetic investigations into the inheritance of the thylakoid-modified atrazine resistant and unmodified susceptible biotypes of many weed species have been done by several scientists.

Machado and Bandeen (1977) performed an experiment with Chenopodium album to determine the genetic aspects of triazine resistance in this species. C. album is hexaploid, mainly self pollinated with some cross pollination, and has minuscule flowers that are laborious to hand emasculate. Simulated wind pollination progeny and parental populations were treated with atrazine. All the progeny responded similarly to their female parent, which might mean that either no cross pollination, some cross pollination with cross incompatibility, or cross pollination and uniparental inheritance were involved. Subsequent work by Warwick and Black (1980) confirmed the uniparental inheritance through the female parent in this species.

Machado et al. (1978) investigated the inheritance of triazine resistance in wild Brassica campestris - a diploid species (n = 10), self incompatible, cross pollinated with large flowers suited to hand emasculation in the bud stage. They made reciprocal crosses between susceptible and resistant biotypes and treated the $F_1$ progeny with 3 kg/ha atrazine post emergence. All the $F_1$ seedlings with the atrazine susceptible

biotype as female parent had phytotoxic symptoms within a week and later died. None of the $F_1$ seedlings with the atrazine-resistant biotype as female parent had any symptoms of phytotoxicity. They therefore, reported that the inheritance of resistance to herbicides was uniparental female and this uniparental effect might controll by maternal cytoplasmic DNA present in the chloroplasts and mitochondria.

Machado et al. (1978) further studied the Hill reaction using isolated chloroplasts from atrazine susceptible and atrazine resistant biotypes of wild Brassica campestris. They carried out this experiment to evaluate the effect of atrazine on these genotypes. Atrazine was found to inhibit the photochemical activity in chloroplasts isolated from the atrazine-susceptible biotypes and $F_1$ hybrid, where susceptible biotype was used as female parent. The atrazine resistant biotype and $F_1$ hybrid, where resistant biotype was used as female parent were not affected by $10^{-4}$M atrazine. From this experiment they concluded that a differential resistance to atrazine does exist at chloroplast level and could be involved with a cytoplasmic components in wild Brassica campestris. This aspect has been elaborated by Darr et al. (1981) in the same species. They used three biotypes of B. campestris and $F_1$ progeny from six reciprocal crosses to determine whether atrazine resistance

by intact seedling correlates with inherited resistance to triazine at the level of chloroplast membrane components. Analysis of chlorophyll fluorescence after short flash excitation revealed that the Q to B electron transport in chloroplasts from progeny of reciprocal crosses between triazine-resistant and susceptible biotypes showed similar kinetic characteristics. For any given sample the kinetics of electron transfer paralleled that of the maternal parent indicating the inheritance of atrazine resistant character through uniparental female parent. Therefore, they suggested that the structural gene encoding the 32 KD polypeptide identified as triazine receptor (Pfister et al. 1981) is maternally inherited. They further demonstrated that although on the basis of these data the effect of maternal nuclear DNA can not be conclusively eliminated, these observations suggest that the 32 KD protein is encoded for by chloroplast DNA.

Solymosi (1981) studied the inheritance of triazine resistance in Amaranthus retroflexus and observed uniparental inheritance through the female parent using cotyledon size as a nuclear marker.

Beversdrof et al. (1980) carried out a series of repeated back crosses in order to incorporate the cytoplasm of the triazine resistant bird's rape (2n = 20) into B. napus cv "Tower" (2n = 38) and B. campestris

cv "Torch" and "candle" (2n = 20). Three 38-chromosome progeny were identified among back cross I ($BC_I$) progeny of bird's rape X Tower (recurrent pollen parent). All $BC_I$ progeny of all crosses between bird's rape (female) and cultivated oil seed rape (recurrent pollen parent) were resistant to post emergence application of several triazines, including atrazine, cyanazine and metribuzin. In similar crosses using cultivars as recurrent female parents, $BC_I$ progeny were susceptible to triazines which also indicated that the resistance was inherited cytoplasmically through female parent.

While studying the chloroplast genome Bogorad et al. (1980) found that these organelles make a number of proteins in response to light exposure. Among the proteins was one with a molecular weight of 34 KD which is subsequently processed to form a molecular weight of 32 KD. The gene for this protein was designated as photogene 32.

Most recently the Arntzen and Bogorad's group (article published in "SCIENCE" 1 april, 1983, vol. 220 no. 45926) showed that the product of photogene-32 and the atrazine-binding are one and the same molecule, a big boost to the research because Lee McIntosh of Bogorad's group had already cloned the gene and reported that it is easier to sequence genes than proteins. McIntosh and Hirschberg have determined the

nucleotide sequence of the normal photogene-32. They have also cloned and sequenced the mutant gene from herbicide resistant plants. They have shown that there is only one change in the mutants, a substitution of an adenine for a guanine. This changes a single amino acid in the protein, but the alteration is enough to produce a product to which atrazine can no longer bind.

Arntzen and Duesing identified a "plastome mutator" gene in a strain of atrazine-resistant black night shade. They reported that it is a nuclear gene that increases the frequency of mutation in the chloroplast genome by 100-1000 fold. They also postulated that the plastome mutator gene may have facilitated the development of atrazine resistant weeds in the regions where prolonged application of herbicide provided the pressure for selection of the trait.

## 2 STATEMENT AND OBJECTIVES OF THE PROBLEM

In recent years there have been several reports (Radosevich et al. 1977; Pfister and Arntzen, 1979; LeBaron and Gressel, 1982) of weed biotypes which have developed a high degree of resistance to triazines (Table 1.). Arntzen and coworkers [1979] have established that resistance of mutant weeds towards triazine herbicides is due to a significantly decreased binding of the herbicides to thylakoid membranes of the mutants as compared to the wild types. Thus photosynthetic electron transport in chloroplasts from mutant plants is not inhibited at a concentration which would lead to complete inhibition in chloroplasts from the wild types. Furthermore, differential effects of other chemical groups of herbicides have also been reported (Pfister and Arntzen, 1979; Oettmeier et al. 1982): they are however not fully understood. We have therefore, made and attempt to investigate the effect of three representative herbicides (DCMU, atrazine and DNOC) from three different herbicidal families (urea, triazine and nitrophenol herbicides respectively) on photosynthetic electron transport system of chloroplasts isolated from triazine-susceptible and triazine-resistant biotypes of Erigeron canadensis L..

The biotypes of E. canadensis L. were selected because of the fact that this species has not yet been

exploited and characterized. Although it has been reported that this weed species has developed resistance to triazines and causes a serious problems in agricultural crop production. Investigation of the triazine resistance of this species has recently got immense importance from agricultural point of view.

Therefore, we designed to compare the effects of urea-type (DCMU and atrazine) and phenolic (DNOC) herbicides in susceptible and resistant chloroplasts of this species. We planned to determine the $I_{50}$ value (herbicide concentration which inhibits 50% of the electron transport phenomenon) of the above mentioned herbicides and to evaluate subsequently the R/S ratio ($I_{50}$ concentration resistant/$I_{50}$ concentration susceptible) of this species. Furthermore, we planned to characterize the modifications occurring in the redox state of the primary acceptor, Q and secondary acceptor, B due to the mutation-induced alterations in the Q-B herbicide binding complex of the chloroplast membrane. The variation in the electron transfer rates from Q to B was also one of the objectives of our investigations.

In the investigation of this problem we used a rarely applied technique, thermoluminescence with the hope that it will help to reveal newer aspects of the herbicide resistance in weed species.

# 3 MATERIALS AND METHODS

## 3.1 Plant materials and herbicides used

Plant materials:

Green leaves of the following plants were used for experimental purposes:

1. Erigeron canadensis* L. (susceptible and resistant biotypes)
2. Amaranthus retroflexus L. (susceptible and resistant biotypes)

Herbicides:

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), a triazine herbicide,

DCMU N-(3-4-dichlorophenyl)-N-N-dimethylurea, a urea herbicide.

DNOC (4,6-dinitro-o-cresol), a nitrophenol herbicide.

## 3.2 Isolation of chloroplasts

Green leaves of the above mentioned plants (plants grown under ordinary green house conditions) were collected, thoroughly washed in running tap water, cut into small pieces after removing the mid ribs and then grinded in a mortar very gently with isolation buffer (Table

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*The seedlings of Erigeron canadensis L. were kindly supplied by Dr. Endre Lehoczki, Department of Biophysics, József Attila University, Szeged, Hungary.

3.). The resultant slurry was sequeezed through two layers of nylon cloth (63 μ pore diameter). The filtrate was then centrifuged at 3000 rpm in a K-23 centrifuge for 3-4 minutes. The supernatant was discarded and the pellet was gently resuspended in 1 ml of suspension medium (Table 4.). All procedures were carried out at 0-4°C and the resultant chloroplast pellet was kept on ice.

Table 3. Components of isolation medium (pH 6.5), Reeves and Hall (1973).

| | | |
|---|---|---|
| D-sorbitol | 0.4 M | 7.29 g/100 ml |
| NaCl | 10 mM | 0.0585 g/100 ml |
| $MgCl_2$ | 5 mM | 0.1015 g/100 ml |
| $MnCl_2$ | 1 mM | 0.0198 g/100 ml |
| EDTA | 2 mM | 0.0585 g/100 ml |
| Ascorbic acid | 2 mM | 0.0350 g/100 ml |
| BSA | 0.40% | 0.40 g/100 ml |
| HEPES | 5 mM | 0.9760 g/100 ml |

Table 4. Components of suspension medium (pH 7.5), Reeves and Hall (1973).

| | | |
|---|---|---|
| D-sorbitol | 0.4 M | 7.29 g/100 ml |
| NaCl | 10 mM | 0.0585 g/100 ml |
| $MgCl_2$ | 5 mM | 0.1015 g/100 ml |
| $MnCl_2$ | 1 mM | 0.0198 g/100 ml |
| EDTA | 2 mM | 0.0585 g/100 ml |
| HEPES | 50 mM | 1.1920 g/100 ml |

## 3.3 Quantitative determination of chlorophyll contents in the isolated sample

In a test tube the following components were thoroughly mixed and then centrifuged at 3000 rpm for 2-3 minutes in a T-32 C centriguge.

0.025 ml chloroplast suspension
0.575 ml distilled water
2.40 ml acetone
3.00 ml acetone (80%)

After centrifugation the supernatant was used for spectrophotometeric determination of Chl a and Chl b at absorption maxima 663 nm and 644 nm respectively. The following formula was used for calculation of chlorophyll contents in the sample (French, 1960).

$$(20.2 \times OD_{644\ nm} + 8.02 \times OD_{663\ nm}) \times 240$$

$$= \mu g\ chl/ml\ of\ the\ sample,$$

where OD is optical density.

## 3.4 Measurement of photosynthetic oxygen evolution

The rate of photosynthetic oxygen evolution was measured by using a Clark-type oxygen electrode (Delieu and Walker, 1972) in a temperature controlled cuvette (Rank Brothers, Cambridge, UK) at + 25°C. Saturating white light was provided by a 650 W iodine tungsten

lamp (Fig. 4). The measurement was taken in an assay medium (Table 5.).

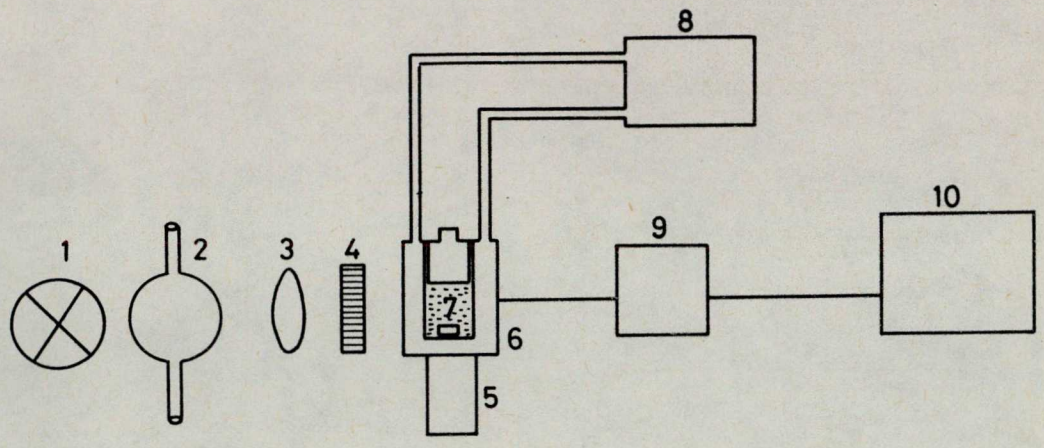


Fig. 4 Block diagram of the "Clark type" oxygen electrode with illumination attachment. 1- Tungsten lamp 640 W, 220 V, 2 - Water container (heat cooler), 3 - Lens, 4 - Neutral optical filter, 5 - Magnetic stirrer, 6 - Sample holder, 7 - Sample, 8 - Thermostat, 9 - Amplifier, 10 - X-Y recorder

Table 5. Components of assay medium (pH 7.5) Reeves and Hall (1973)

| | | | |
|---|---|---|---|
| D-sorbitol | 100 mM | 1.822 | g/100 ml |
| $MgCl_2$ | 4 mM | 0.081 | g/100 ml |
| NaCl | 20 mM | 0.116 | g/100 ml |
| $K_2HPO_4$ | 10 mM | 0.174 | g/100 ml |
| EDTA | 2 mM | 0.058 | g/100 ml |
| HEPES | 50 mM | 1.192 | g/100 ml |

Chloroplasts containing 50 µg clorophyll and 2.5 mM pBQ (parabenzoquinone) were used in a final volume of 3 ml aliquot to perform the oxygen evolution measurement. The role of pBQ in the assay medium was to act as an electron acceptor of PS II. Herbicides of different concentrations were added directly to the measuring medium. The signals of oxygen evolution in control and herbicide treated samples were recorded in a XY potentiometric recorder. The rate of electron transport was given in $\frac{\mu M \text{ oxygen evolved}}{\text{mg chlorophyll x h}}$

## 3.5 Measurement of flash-induced 515 nm absorption change

1.2 ml of chloroplast suspension having optical density 0.5 to 1.00/cm was used for the measurements. The chloroplasts suspension was placed into a cuvette with an optical pathlength of 1 cm and illuminated through a horizontal light guide of xenon flashes (General Radio, Stroboslave, 8 µs, energy 0.5 J) perpendicular to the measuring beam. Actinic light was passed through a Calflex C (Balzers) filter and a Scott RG 630-2 mm filter (Fig. 5). Flashes were fired at an interval of 4 sec. The measuring light passed through a Zeiss SPM 2 monochromator (bandwidth 4 nm) and after passing the sample the light was led through a light guide to an EMI 9558 B photomultiplier pro-

tected by Corning 4-96 filter. The intensity of the measuring beam was about $3 \times 10^{-3} J\ m^{-2} s^{-1}$ at 515 nm, 100 to 200 signals were collected in an Intertechnique SA 40 multichannel analyser. The measurement were taken in the time region of 200 ms and at a temperature of + 20°C for both control and herbicide treated samples. The signals were fed to a XY recorder.

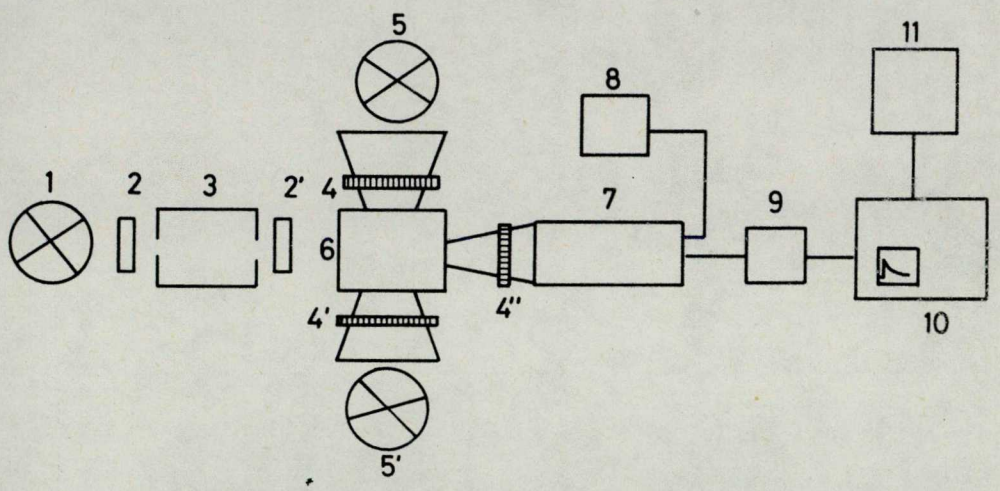


Fig. 5 Block scheme of 515 nm absorption charge set up. 1 - Tungsten lamp, 2,2' - lenses 3 - Zeiss SPM 2 monochromator, 4,4' - Schott RG 630-2 optical filter + light guide ($\lambda >$ 650 nm), 4''- Corning 4-96 optical filter ($\lambda <$ 600 nm), 5,5'- Flash light, 6 - Sample, 7 - Photo-multiplier EMI 9558 B, 8 - High voltage power supply, 9 - Differential amplifier, 10 - Multi-channel analyzer ICA 70 KFKI, 11 - XY recorder

## 3.6 Measurement of fluorescence induction

2.5 ml of chloroplast suspension having the same optical density and taken in a similar cuvette as in the case of 515 nm absorption change measurement was used for this experiment. Light from a 450 W high pressure xenon arc lamp was filtered through a Corning 4-96 glass filter and focused onto the chloroplasts sample (Fig. 6).

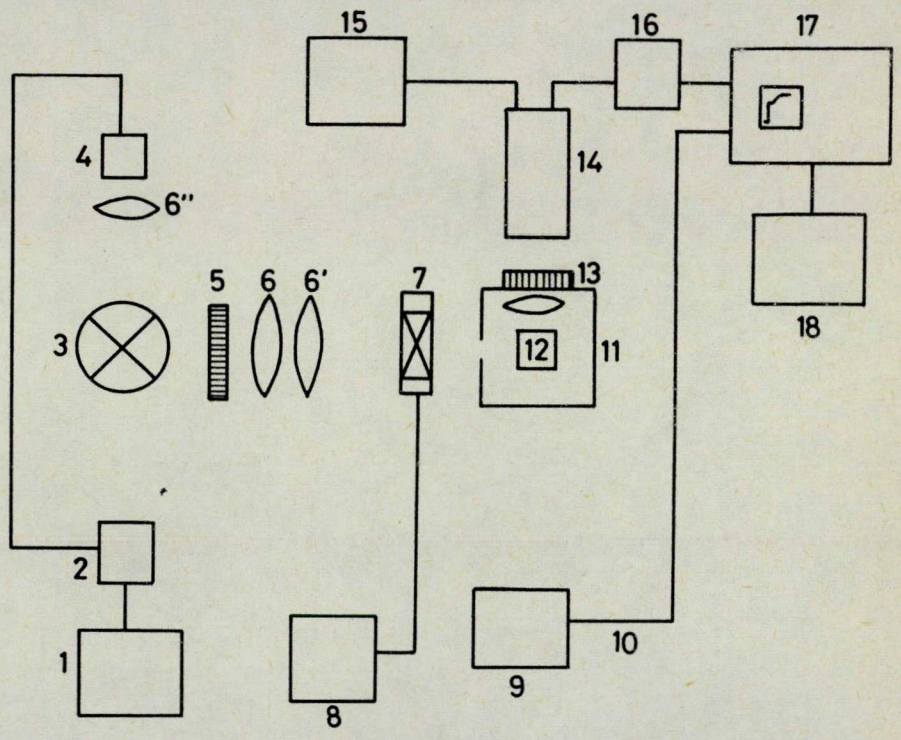


Fig. 6 Block scheme of fluorescence induction set up 1 - XE lamp power supply, 2 - XE light stabilizer, 3 - XE arc lamp 640 W, 220 V, 4 - light sensor, 5 - Corning 4-96 optical filter $\lambda$=350-600 nm, 6,6';6''- Lenses, 7 - Electronic shutter, 8 - Shutter driver, 9 - Timing unit, 10 - Trigger pulse, 11 - Sample holder, 12 - Sample, 13 - Corning 2-64 optical filter $\lambda >$ 620 nm, 14 - Photomultiplier, 15 - High voltage power supply, 16 - Differential amplifier, 17 - Multichannel analyzer ICA 70 KFKI, 18 - XY recorder

The intensity of the homogeneous excitation beam was 16 $W.m^{-2}$. Fluorescence light from the sample was detected by an EMI 9558 B photomultiplier perpendicular to the excitation beam. The scattered exciting light was blocked by a Corning 2-64 glass filter set in front of the photomultiplier. The fluorescence transients were collected in a multichannel analyser (ICA 70 KFKI). The time resolution with the optical shutter used (Wincent Assoc., type L2175) was 10 ms. Addition of herbicides and plotting of the signals were carried out as in the case of 515 nm absorption change measurements.

## 3.7 Measurement of thermoluminescence

For the measurement of thermoluminescence (TL) the isolated chloroplast pellets suspended in suspension medium were broken in liquid nitrogen and were diluted to a chlorophyll concentration of 125 µg/ml. 0.4 ml aliquots of the broken chloroplast suspension were used for the measurement of TL. The measurement of glow curve (the intensity of the TL light plotted against temperature) was carried out in the temperature region from - 60°C to + 80°C using an apparatus as shown in Fig. 7.. The light emission of the sample was measured by a red sensitive photomultiplier (EMI 9558 B) and the signal was amplified through a differential amplifier

and fed to a PM 8120 XY recorder. The temperature of the sample holder was monitored using a platinum resistor thermometer placed below the samples.

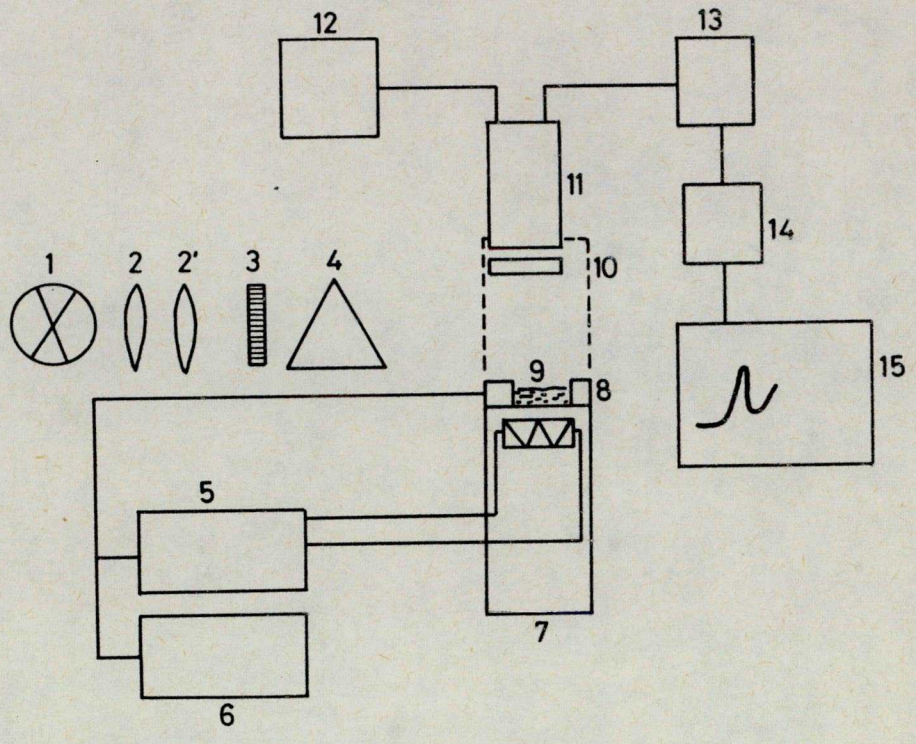


Fig. 7 Block scheme of thermoluminescence set up 1 -halogen lamp 650 W, 220 V, 2,2' - lenses, 3 - Neutral optical filter, 4 - Prism, 5 - Temperature programmer, 6 - Temperature display. 7 - Liquid nitrogen holder, 8 - Sample holder, 9 - Sample, 10 - Heat filter glass, 11 - Photomultiplier EMI 9558 B, 12 - High voltage power supply, 13 - Differential amplifier, 14 - Filter, 15 - XY recorder

Samples were illuminated with white light from a NARVA halogen lamp of 650 W for 2 minutes at - 60°C. The exciting light was passed through a heat absorbing water filter (thikness 10 cm) and a Balzer neutral density filter giving an illumination intensity of

10 W $m^{-2}$. In flash experiments samples were excited with saturating flashes provided by a xenon flash tube (General Radio, Stroboslave, 3 μs, 0.5 J) and fixed at 1 sec intervals. In both flash and continuous illumination experiments the samples were heated in darkness at a rate of 20°C/min in order for best resolution of the peaks (Sane et al. 1977). Herbicides of different concentrations were mixed with the samples before putting into the sample holder.

# 4 EXPERIMENTAL RESULTS

## 4.1 Differential effects of PS II herbicides (DCMU, DNOC and atrazine) on the electron transport chain in isolated chloroplasts of triazine-susceptible and triazine-resistant biotypes of Erigeron canadensis L., investigated by photosynthetic oxygen evolution measurements

PS II mediated electron flow from $H_2O \longrightarrow$ pBQ which gives rise to oxygen evolution was monitored at various herbicide concentrations in isolated chloroplasts of triazine-susceptible and triazine-resistant biotypes of Erigeron canadensis L. and Amaranthus retroflexus L.. Virtually no differences were observed in the effectiveness of DCMU, and DNOC in inhibiting electron transport in the susceptible versus resistant biotypes. The $I_{50}$ concentrations of DCMU and DNOC for susceptible and resistant biotypes of E. canadensis L. were 100 nM and 3 µM respectively (Fig. 8 and 9).

On the other hand, a large variation in the $I_{50}$ concentration of atrazine (Fig. 10) was observed in susceptible versus (vs) resistant biotypes of E. canadensis L. ($I_{50}$ = 200 nM vs 150 µM). In A. retroflexus L. also a large difference in the $I_{50}$ concentra-

tion of atrazine towards susceptible versus resistant biotypes was observed ($I_{50}$ = 200 nM vs 200 $\mu$M).

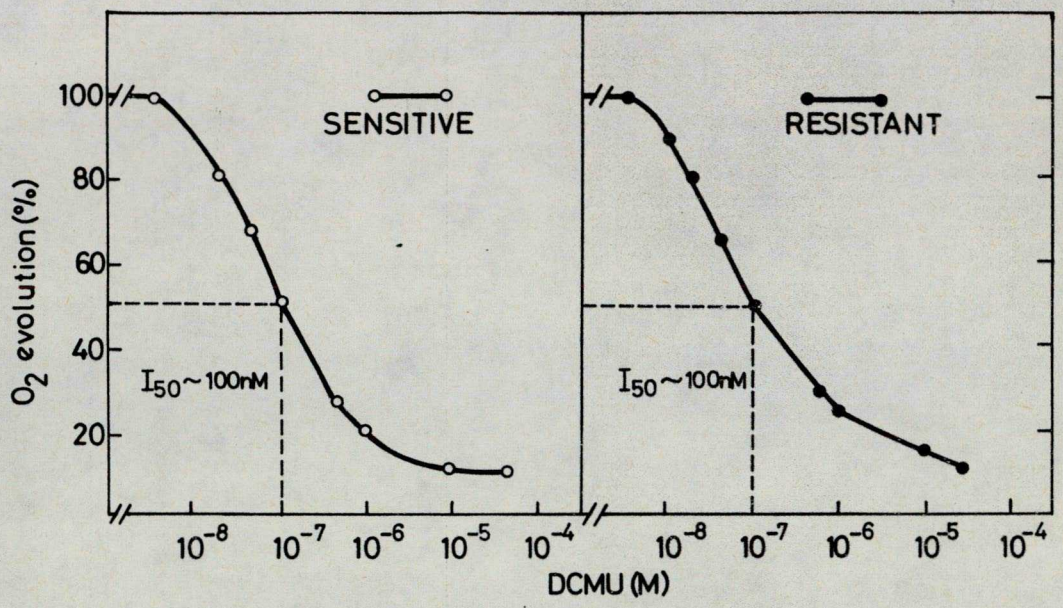


Fig. 8 Inhibition of photosynthetic oxygen evolution ($H_2O \longrightarrow$ pBQ) in isolated susceptible and resistant chloroplasts of Erigeron canadensis L. by DCMU

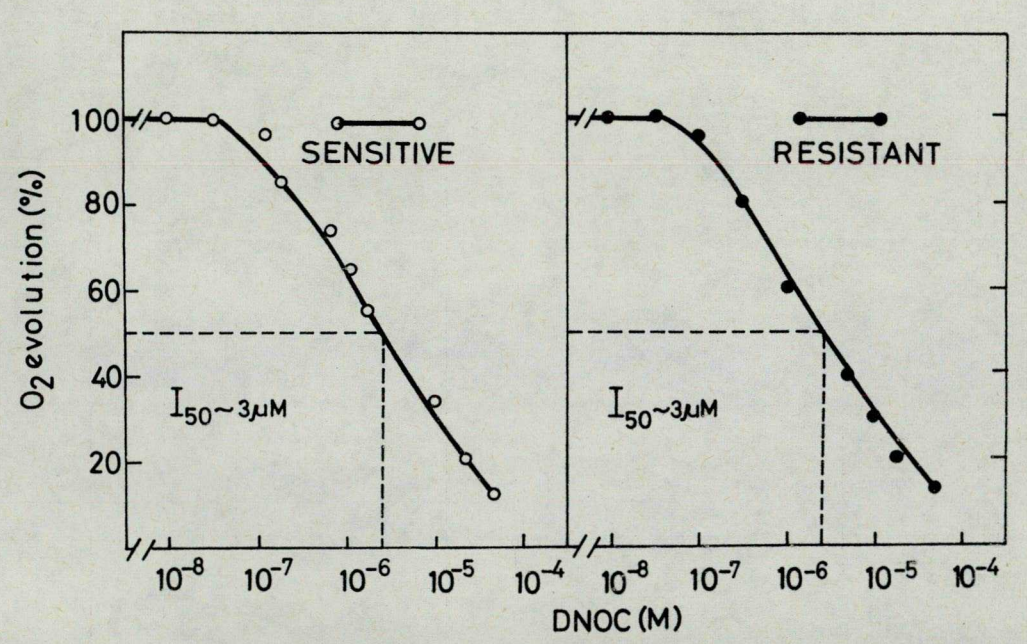


Fig. 9 Inhibition of photosynthetic oxygen evolution ($H_2O \longrightarrow$pBQ) in isolated susceptible and resistant chloroplasts of E. canadensis L. by DNOC

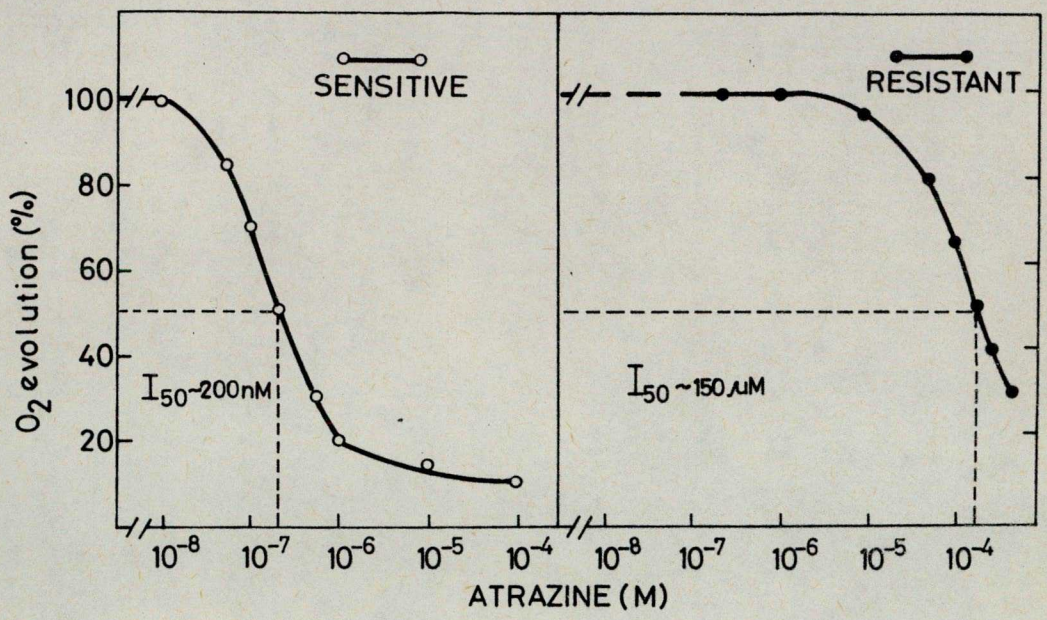


Fig. 10 Inhibition of photosynthetic oxygen evolution ($H_2O \longrightarrow$ pBQ) in isolated susceptible and resistant chloroplasts of E. canadensis L. by atrazine

The comparison of $I_{50}$ concentration of atrazine towards susceptible and resistant biotypes of both E. canadensis L. and A. retroflexus L. is presented in Table 6.

Table 6. $I_{50}$ concentration for inhibition of photosynthetic oxygen evolution ($H_2O \longrightarrow$pBQ) in isolated chloroplasts from susceptible and resistant biotypes of E. canadensis L. and A. retroflexus L.. Resistance ratio (R/S)=$I_{50}$ concentration resistant/$I_{50}$ concentration susceptible

| Species | Biotype | Atrazine $I_{50}$ conc (M) | R/S |
|---|---|---|---|
| E. canadensis L. | susceptible chloroplasts | $2 \times 10^{-7}$ | $7.5 \times 10^{2}$ |
| | resistant chloroplasts | $1.5 \times 10^{-4}$ | |

| Species | Biotype | Atrazine $I_{50}$ conc (M) | R/S |
|---|---|---|---|
| A. retroflexus L. | susceptible chloroplasts | $2 \times 10^{-7}$ | $10^3$ |
| | resistant chloroplasts | $2 \times 10^{-4}$ | |

From the table, it is evident that susceptible biotypes of both species have the same $I_{50}$ concentration of atrazine (200 nM) whereas it is much higher for the resistant ones. The $I_{50}$ concentrations of resistant biotypes of E. canadensis L. and A. retroflexus L. were 150 μM and 200 μM respectively.

## 4.2 Flash-induced 515 nM absorption change measurements in isolated chloroplasts of triazine-susceptible and tiazine-resistant biotypes of E. canadensis L.

Photochemical charge separation in the reaction centers induces a potential difference across the membrane. In the present experiment, the relative amplitude of the flash-induced 515 nm absorption change was measured by using different concentrations of PS II herbicides (DCMU, DNOC and atrazine) in isolated triazine-susceptible and triazine-resistant E. canadensis L. chloroplasts. The amplitude of the signals were

found to decrease linearly with the increase in concentrations of DCMU and DNOC in both susceptible and resistant biotypes (Fig. 11 and 12).

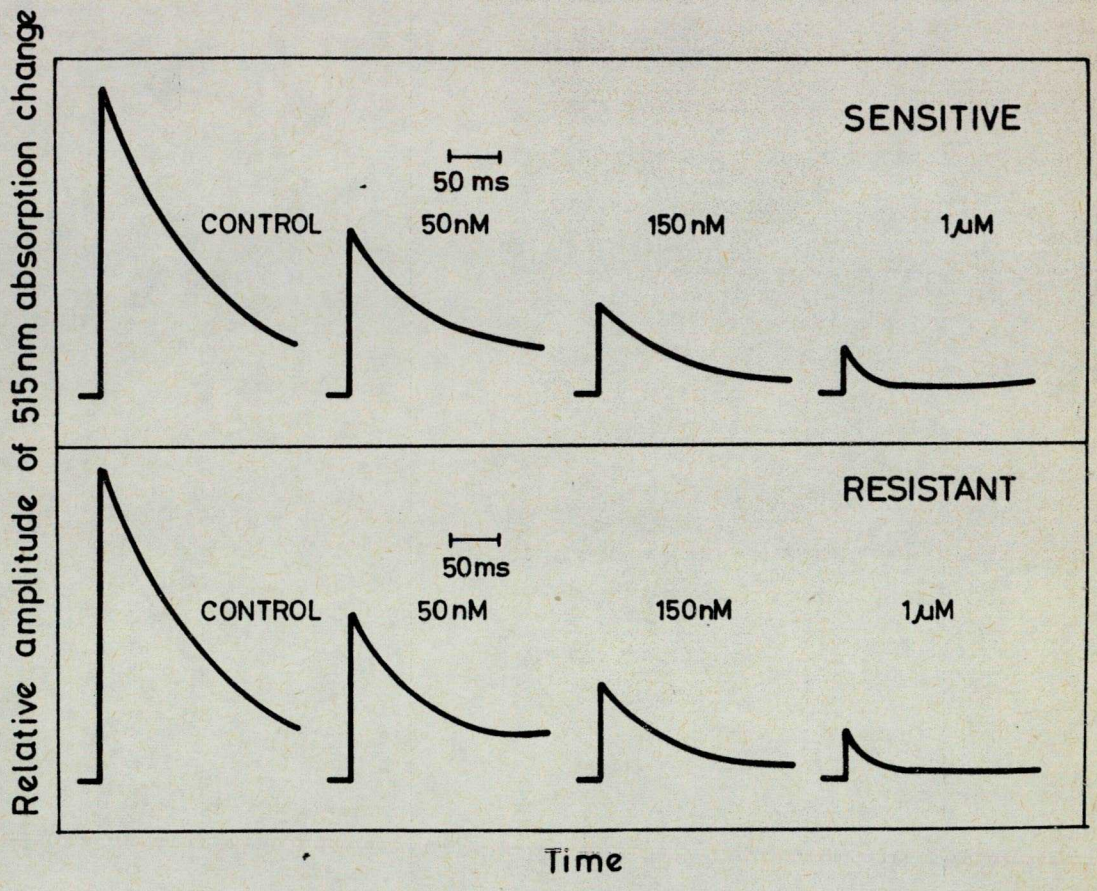


Fig. 11 Effect of DCMU on the amplitude of the signals of flash-induced 515 nm absorption change in the isolated susceptible and resistant chloroplasts of E. canadensis L.

DCMU at a concentration of 1 μM and DNOC at a concentration of 100 μM completely abolished the 515 nm absorption change in both cases. The approximate $I_{50}$ concentrations for DCMU and DNOC treated samples were 100 nM and 3 μM respectively for both biotypes.

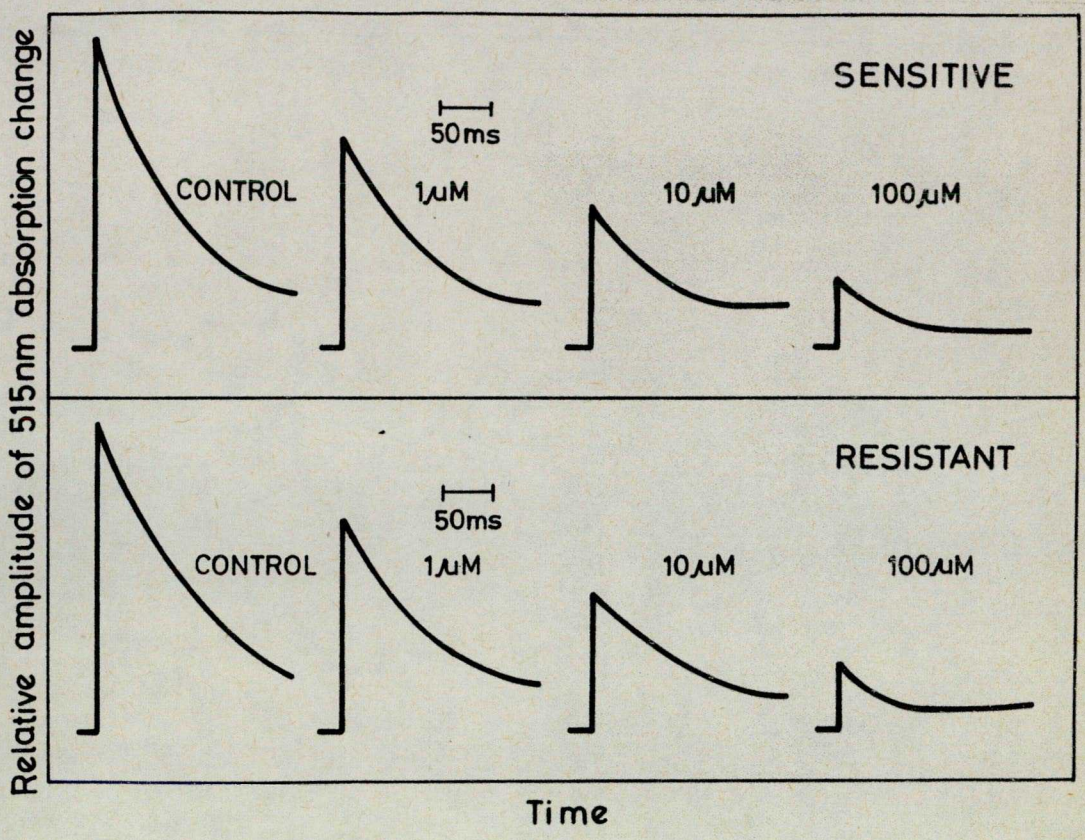


Fig. 12 Effect of DNOC on the amplitude of the signals of flash-induced 515 nm absorption change in the isolated susceptible and resistant chloroplasts of E. canadensis L.

On the other hand, both biotypes showed great variation in the sensitivity towards atrazine. 5 µM atrazine almost completely abolished the signal in the case of the susceptible biotype whereas a similar concentration did not change the signal at all in the case of resistant one (Fig. 13). The $I_{50}$ concentrations were 300 nM for susceptible biotype and 200 µM for resistant one (Fig. 14).

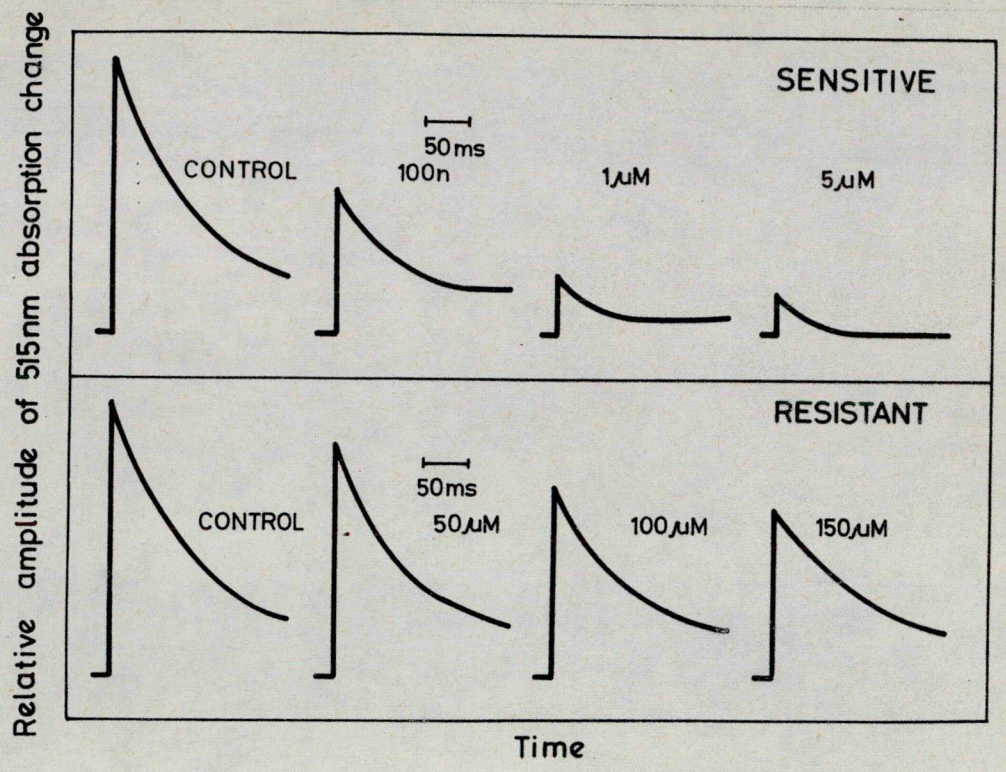


Fig. 13 Effect of atrazine on the amplitude of the signals of flash-induced 515 nm absorption change in the isolated susceptible and resistant chloroplasts of E. canadensis L.

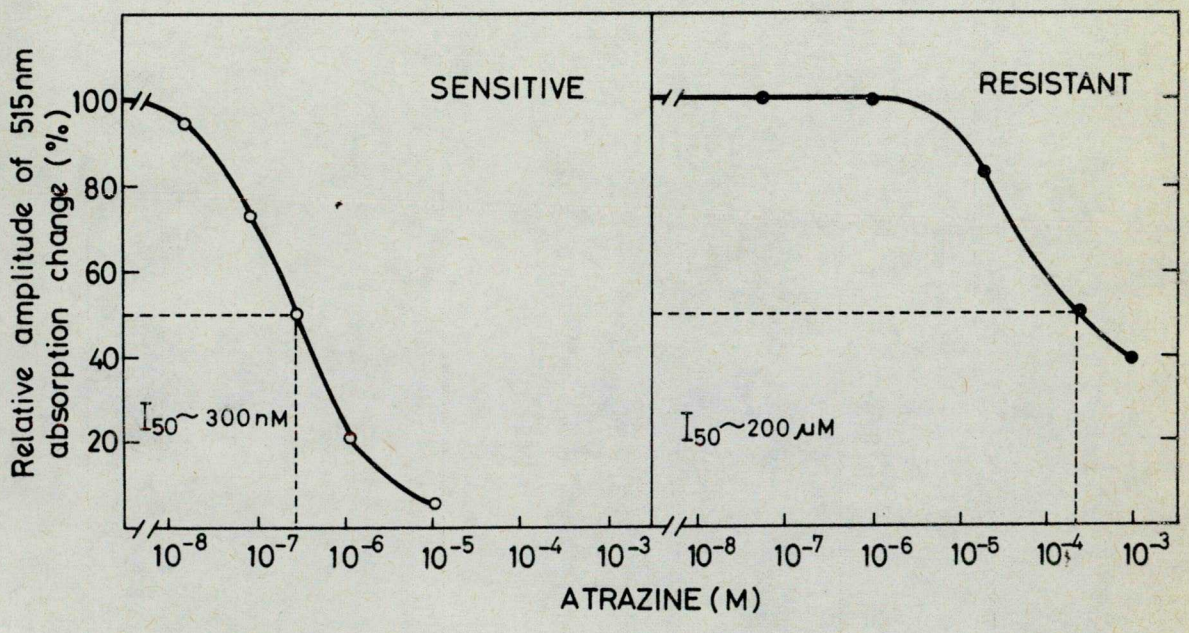


Fig. 14 Determination of $I_{50}$ concentration in isolated susceptible and resistant chloroplasts of E. canadensis L. by plotting the amplitude of the signals of flash-induced 515 nm absorption change as a function of increasing atrazine concentration

## 4.3 Chlorophyll fluorescence induction transient analysis in isolated chloroplasts of triazine-susceptible and triazine-resistant biotypes of E. canadensis L.

Fluorescence rise depends on the redox state of Q, the primary acceptor of PS II, also known as the quencher of fluorescence. If Q is reduced, fluorescence rise is faster than if it is oxidised. In the following experiment the relative intensity of the fluorescence induction signals were analysed by using different concentrations of PS II herbicides (DCMU, DNOC and atrazine) in isolated triazine-susceptible and triazine resistant E. canadensis L. chloroplasts. The intensity of the signals was found to rise faster with increased concentrations of DCMU and DNOC for both biotypes (Fig. 15 and 16). DCMU at a concentration of 1 μM has completely saturated the fluorescence induction curve which is indicated by the fast fluorescence rise in both biotypes. DNOC also acted similarly at concentrations lower than 10 μM but with further increases in concentrations (100 μM and 300 μM) the signals decreased greatly in amplitude (Fig. 16).

On the other hand, both biotypes showed differential sensitivity towards atrazine. 10 μM atrazine almost completely saturated the fluorescence induction curve in the case of susceptible biotype but a similar concentration did not change the signal at all in the

case of resistant one (Fig. 17). 2 mM atrazine was needed for resistant biotype to get complete saturation curve like the susceptible one.

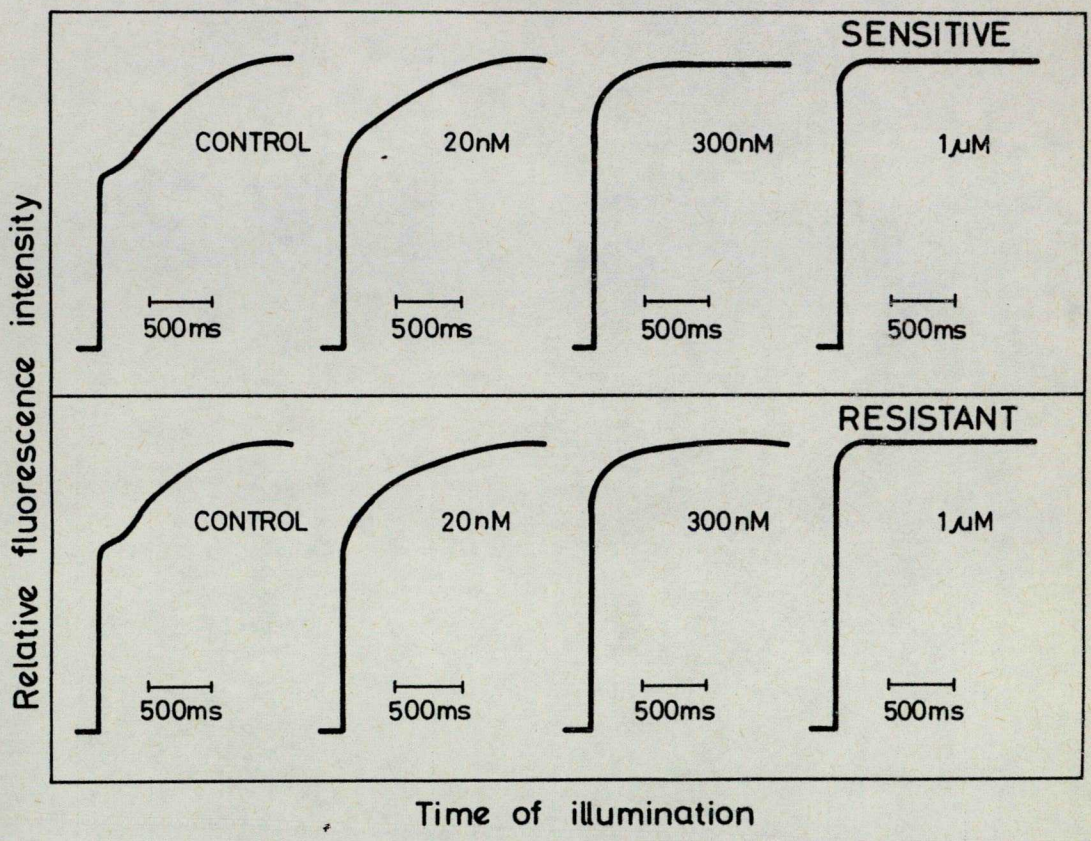


Fig. 15 Fluorescence induction curves of isolated susceptible and resistant chloroplasts of E. canadensis L. in the presence of DCMU

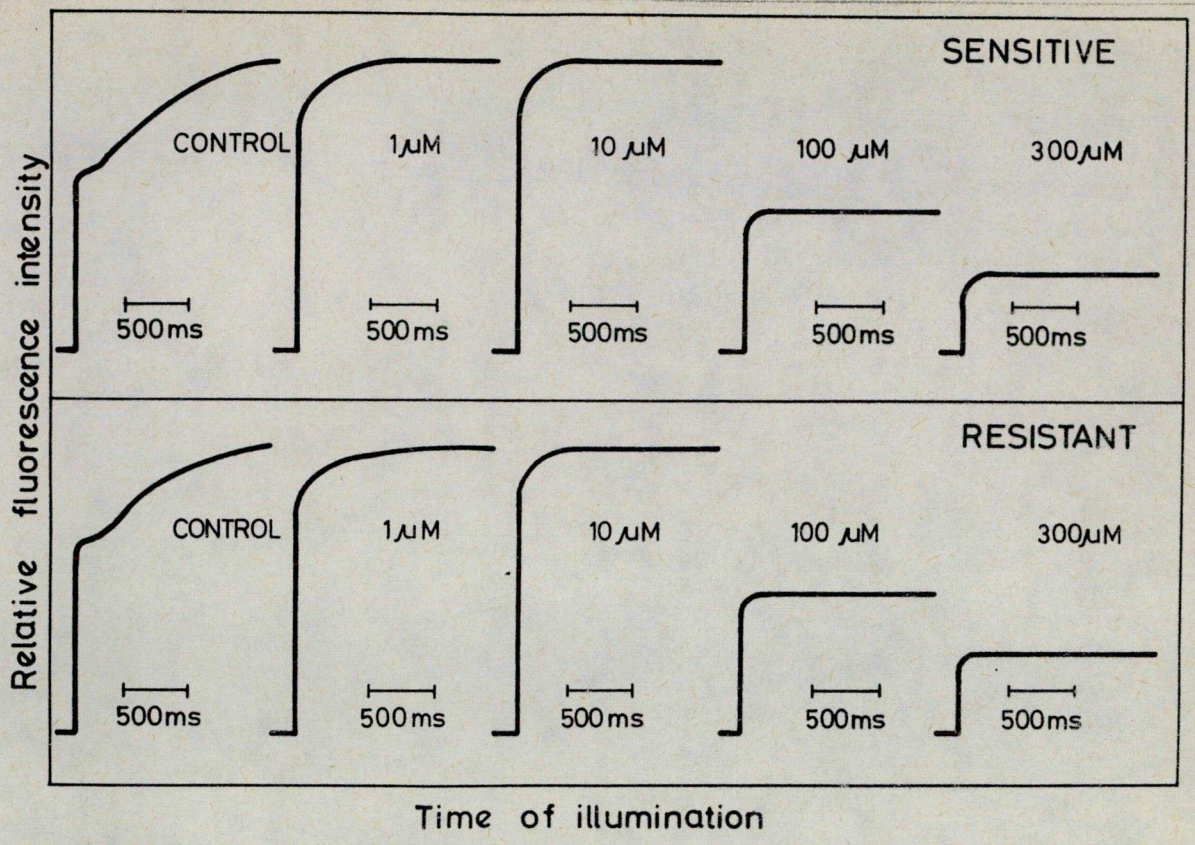


Fig. 16 Fluorescence induction curves of isolated susceptible and resistant chloroplasts of E. canadensis L. in the presence of DNOC

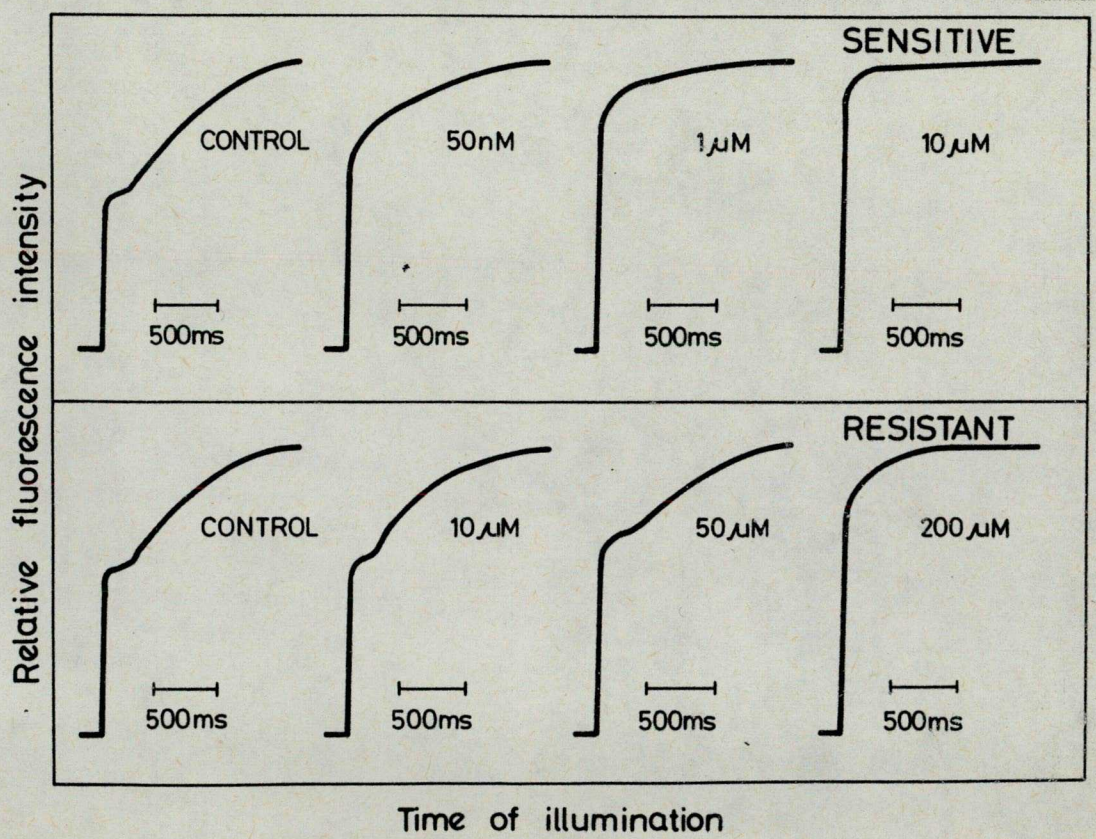


Fig. 17 Fluorescence induction curves of isolated susceptible and resistant chloroplasts of E. canadensis L. in the presence of atrazine

One of the striking observations in the measurement of room temperature fluorescence induction in the isolated chloroplasts of susceptible and resistant biotypes was that during illumination of dark adapted chloroplasts, the fluorescence increase from the "original level" observed immediately after the onset of illumination (the $F_o$ level) to the slightly higher intermediate value ($F_I$) was dramatically higher in the resistant biotype in comparison to the susceptible one (Fig. 18).

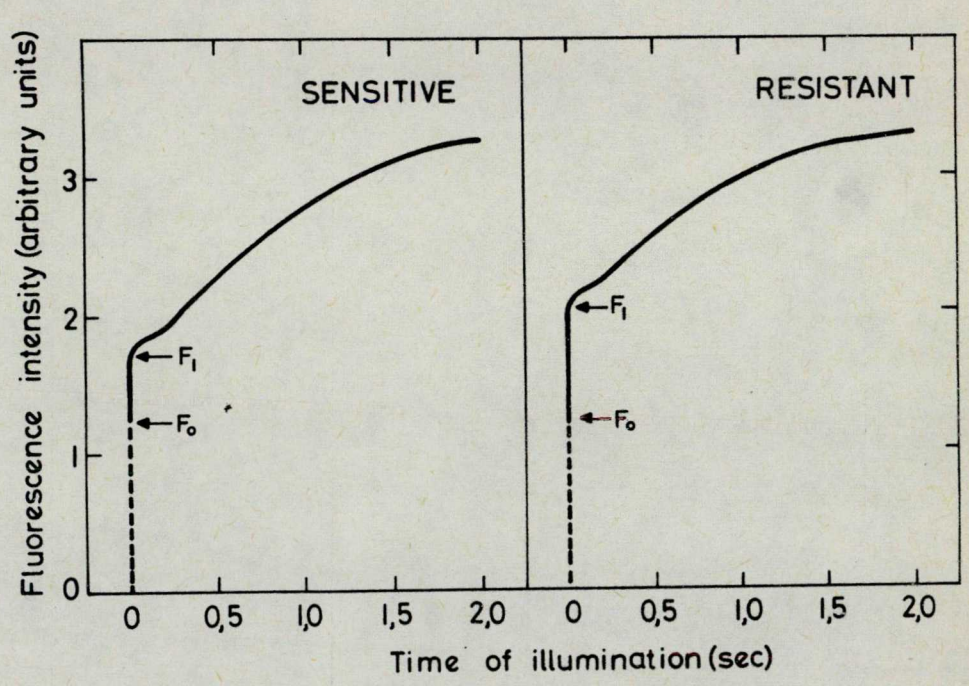


Fig. 18 Chlorophyll fluorescence transient changes observed upon illumination of dark adapted chloroplasts isolated from susceptible and resistant biotypes of E. canadensis L. $F_o$ levels of fluorescence were determined within 5 ms after full shutter opening. The $F_I$ level of fluorescence in untreated samples was calculated from the fluorescence intensity after 50 ms of sample illumination

A quantitative evaluation of fluorescence rise curve is possible by calculating the ratio of intensity of fluorescence at $F_I$ to that at $F_M$ (where $F_M$ is the maximum level of fluorescence). The value generated by the expression:

$$\Delta F_I/\Delta F_M = (F_I - F_o)/(F_M - F_o)$$

represents the proportion of the total variable fluorescence yield which occurs during the rapid (intermediate) phase of the fluorescence rise. $F_I$ in this equation is taken as the fluorescence intensity at the begining of the linear intermediate portion of the transient. The $\Delta F_I/\Delta F_M$ value was found to be almost double (0.38) in the case of resistant chloroplasts in comparison to the susceptible ones(0.20).

## 4.4 Investigation of thermoluminescence in isolated chloroplasts of triazine-susceptible and triazine-resistant biotypes of *E. canadensis* L.

Thermoluminescence (TL) originates from PS II and the bands of the glow curve can be related to charge recombination between positively charged donors and negatively charged acceptors (Inoue and Shibata, 1979; Demeter et al. 1979). The main band of TL has been found to appear at + 30°C during continuous heating from - 60°C to + 80°C in the case of susceptible chlo-

roplasts. This band at + 30°C has been designated as B-band by Inoue and Shibata (1978, 1979).

It has been established that the glow curve of TL in isolated chloroplasts is strongly influenced by the addition of PS II herbicides (Sane et al. 1977; Ichikawa et al. 1975). In the following experiment, the relative intensity of the main band of the glow curve of TL was measured by using different concentrations of PS II herbicides (DCMU, DNOC and atrazine) in isolated triazine-susceptible and triazine-resistant chloroplasts of E. canadensis L.. The B-band at + 30°C has been found to be replaced gradually by a new band at + 10°C due to mixing of different concentrations of DCMU with the susceptible chloroplasts (Fig. 19). Atrazine and DNOC also replaced the B-band by new bands appearing at + 8°C and - 10°C respectively in susceptible chloroplasts (Fig. 20). The new bands appearing at + 10°C, + 8°C and - 10°C in the TL due to the treatment of susceptible chloroplasts by DCMU, atrazine and DNOC respectively can be related to the primary electron acceptor Q, and called Q-band (Demeter et al. 1982). Plotting the amplitude of the B-band as a function of DCMU concentrations, the $I_{50}$ value was found to be around 80 nM (Fig. 21).

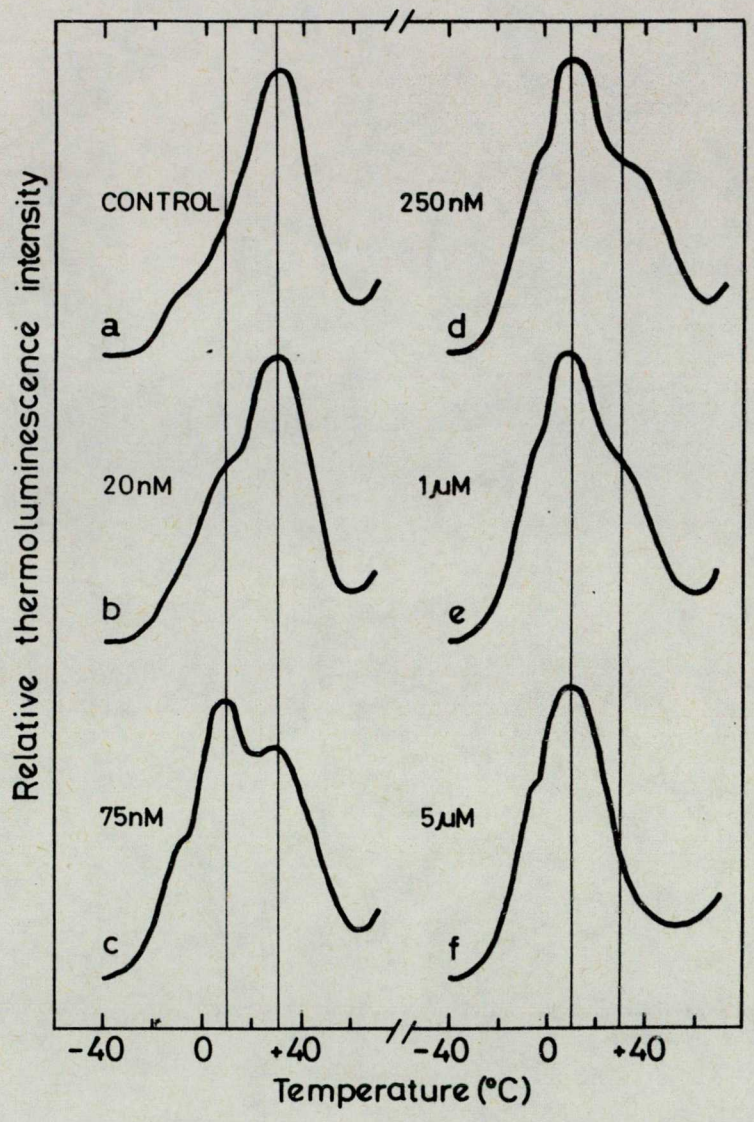


Fig. 19 Gradual disappearance of the B-band at + 30°C and concomitant appearance of the Q-band at + 10°C in the glow curve of TL due to the treatment of susceptible chloroplasts with DCMU

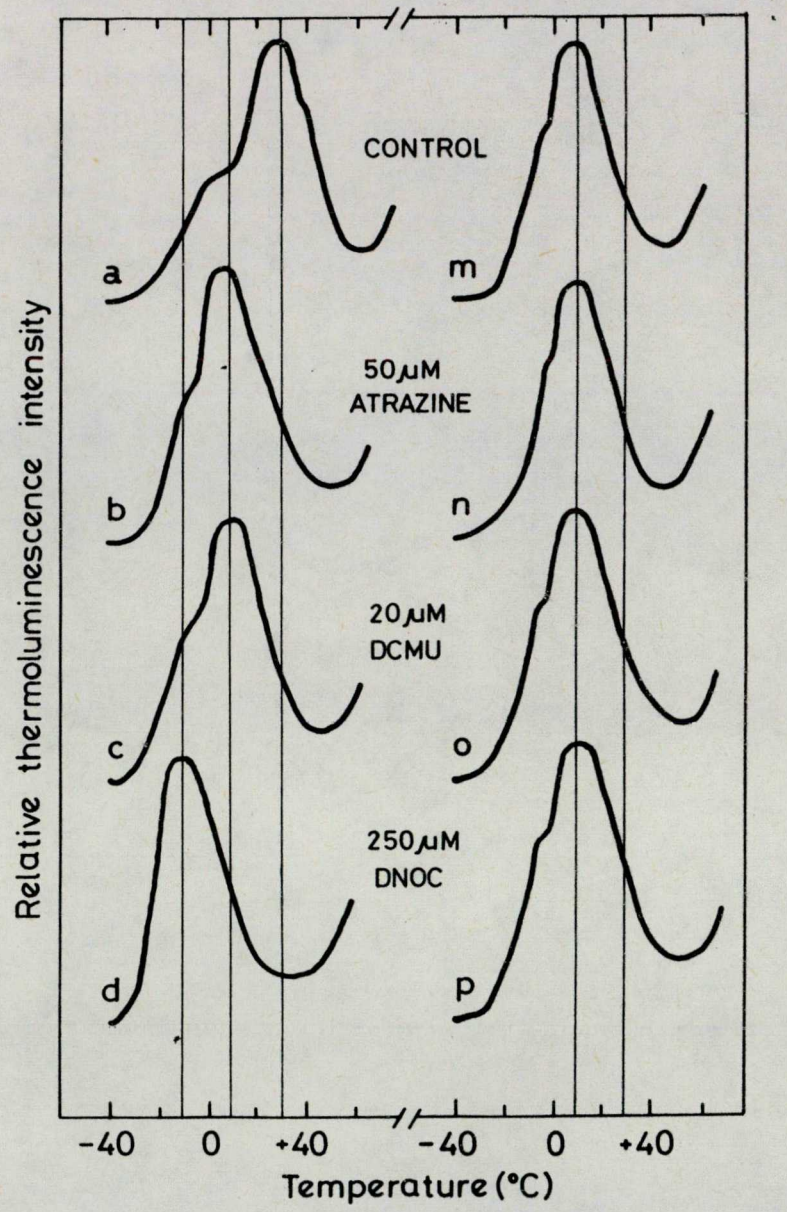


Fig. 20 Comparison of the effects of atrazine, DCMU and DNOC on the peak positions of the glow bands of TL in susceptible (a, b, c and d) and resistant (m, n, o and p) chloroplasts

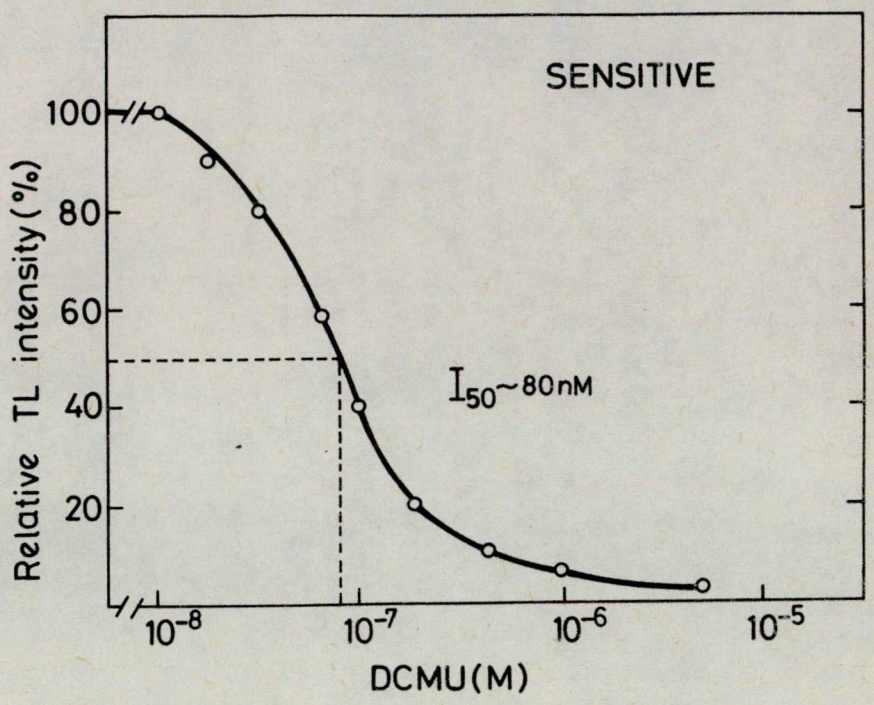


Fig. 21 Estimation of $I_{50}$ concentration of DCMU in the susceptible chloroplasts by plotting the amplitude of the B-band as a function of increasing concentrations of DCMU

Interestingly, no B-band at + 30°C was observed in the TL of resistant chloroplasts even in untreated control sample. A band at + 10°C (Q-band) was found only in this case. The band remained unchanged (Fig. 20) at high concentrations of atrazine, DCMU and DNOC (50 μM, 20 μM and 250 μM respectively).

Since DCMU and DNOC induced TL bands are well separated from each other, therefore, we tried to follow the displacement of these herbicides in the chloroplast membrane. Fig. 22 shows the concentration dependence of the displacement of DNOC by DCMU.

DNOC treatment gave a TL band at - 10°C in susceptible chloroplasts. The addition of increasing concentrations of DCMU to the DNOC treated samples caused a gradual disappearance of the band at - 10°C. Simultaneously a TL band at + 10°C emerged and increased in intensity. The appearance of the TL band at + 10°C is a characteristic of DCMU treatment.

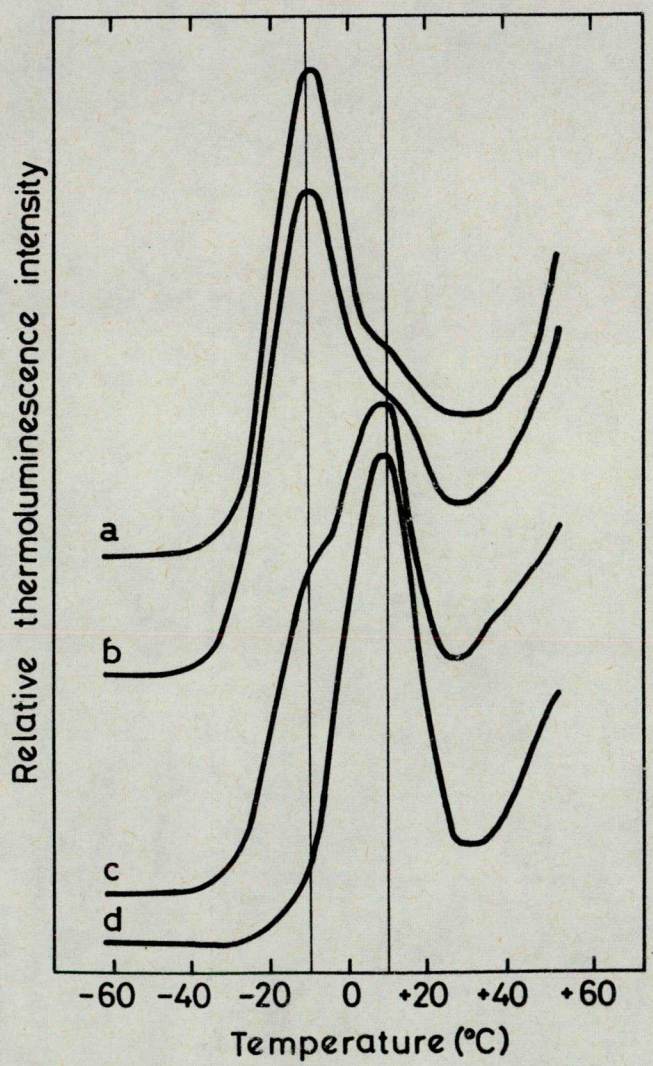


Fig. 22 Displacement of bound DNOC from the susceptible chloroplast thylakoids by DCMU as monitored by TL. After 5 min incubation of the samples with 200 μM DNOC, different concentrations of DCMU were added and TL was recorded after 2 min. a/ no DCMU added, b/ 75 nM DCMU, c/ 200 nM DCMU, d/ 1 μM DCMU

In order to obtain detailed knowledge about the participation of either the primary acceptor, Q or the secondary acceptor, B in the generation of TL in both susceptible and resistant chloroplasts, flash experiments were carried out. The results obtained from flash experiments revealed that in the susceptible chloroplasts after 5 min and 6 hr dark adaptation respectively, the amplitude of the B-band at + 30°C oscillates with a periodicity of 4 showing maxima at 2nd, 6th and 10th flashes (Fig. 23a and b).

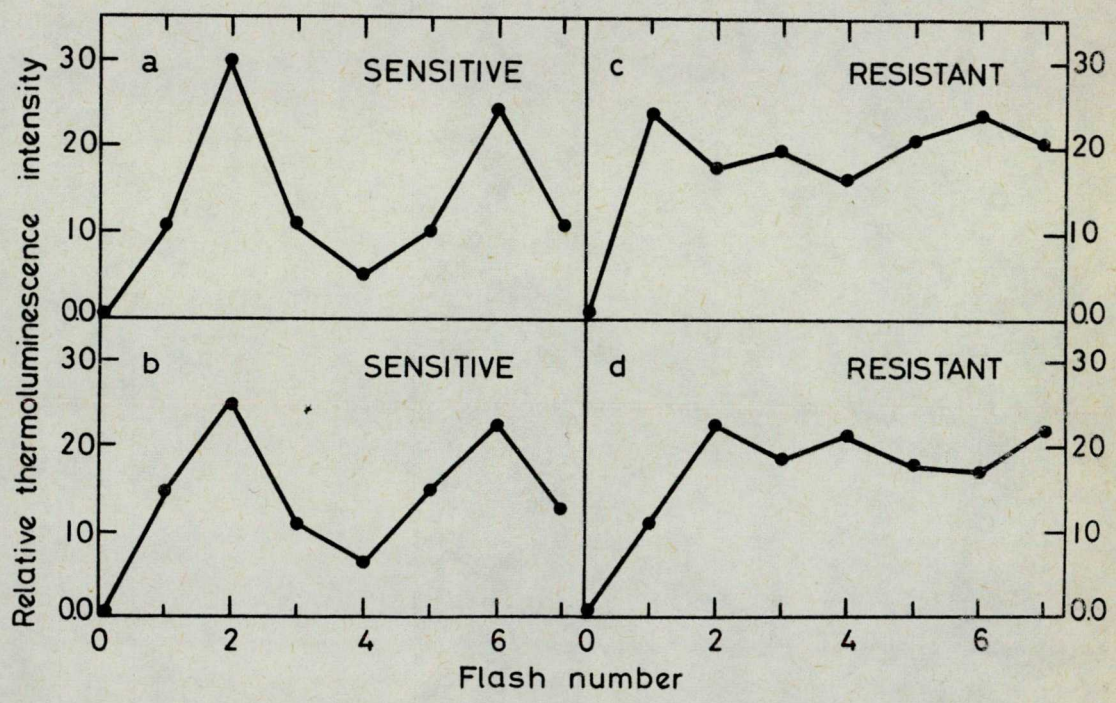


Fig. 23 Oscillatory patterns of TL bands as a function of excitation flash members in the susceptible and resistant chloroplasts.
a and b: period-4 oscillation of the B-band height in susceptible chloroplasts after 5 min and 6 hr dark adaptation respectively.
c and d: period-2 oscillation of the Q-band height in the resistant chloroplasts after 5 min and 6 hr dark adaptation respectively

This means that after 5 min or 6 hr dark adaptation of susceptible chloroplasts, the B pool is in the steady-state distribution, that is $B/B^-$ is $\sim 1$ (Lavergne and Etienne, 1980; Velthuys, 1980). After excitation of the sample either by even or uneven number of flashes 50% of the B pool always is in the half-reduced semiquinone state, $B^-$ and consequently the oscillation of the TL emission is determined by the 4 successive charge accumulation states of the water-splitting system. This can be concluded from the large period-4 oscillation of the TL yield in the case of susceptible biotype.

On the other hand, oscillatory pattern is quite different in the case of resistant biotype. After short dark incubation (5 min) of chloroplasts, the amplitude of the Q-band at + 10°C was found to oscillate with a periodicity of 2 exhibiting maxima at uneven flash numbers (1, 3, 5, 7 etc.). The situation is quite reverse if the resistant chloroplasts are allowed to stay in dark for 6 hr. In this case, the Q-band at + 10°C also oscillates with a periodicity of 2 but shows maxima at even number of flashes (2, 4, 6, 8 etc.) as can be seen in Fig 23c and d.

# 5 DISCUSSION

Photosynthetic oxygen evolution measurement from $H_2O$ to different electron acceptors is one of the most widely used techniques in the field of herbicide research. Many authors applied this method in the determination of site of action and $I_{50}$ concentration of various groups of inhibitors (Tischer and Strotmann, 1979; Arntzen et al. 1979; Codd and Schimid, 1980).

We also applied this technique in the investigation of triazine resistance of Erigeron canadensis L.. In our experiment DCMU and DNOC normally inhibited the oxygen evolution in both susceptible and resistant biotypes. On the other hand, both biotypes showed differential sensitivity towards atrazine. The R/S value was found to be $7.5 \times 10^2$ in the case of E. canadensis L., which is comparable with $10^3$ in the case of Amaranthus retroflexus L.. Our results are in agreement with results obtained previously by Pfister and Arntzen (1979). They reported that the degree of resistance in A. retroflexus L. was very large for all symmetrical triazines (R/S value = $10^3$). In their experiment with DCPIP (dichlorophenol-indophenol) photoreduction ($H_2O \longrightarrow$ DCPIP), the R/S value of atrazine was found to be equal to $10^3$. Arntzen et al. (1979) observed very small differences in the effectiveness of diuron

in inhibiting electron transport in susceptible versus resistant biotypes of A. retroflexus L. but the difference was very large in the case of atrazine. Pfister et al. (1979) demonstrated that in some cases 1000-fold higher concentrations of various s-triazines were needed to inhibit electron transport to a similar extent in the case of resistant chloroplasts as compared to the susceptible ones of Senecio vulgaris L.. In their observations diuron was only slightly less effective in the triazine-resistant than in the susceptible chloroplasts. Droppa et al. (1981) found that in the susceptible plants of A. retroflexus L. the oxygen evolution was completely inhibited by low concentrations of atrazine whereas in resistant plants even a 100-fold concentration of atrazine inhibited oxygen evolution only 40%. Oettmeier et al. (1982) observed that to achieve equal inhibition of photosynthetic electron transport in resistant and susceptible chloroplasts of A. retroflexus L. a 251-fold concentration of atrazine was necessary for the resistant chloroplasts as compared to the susceptible ones.

In our experiments triazine-susceptible and triazine-resistant biotypes responded equally towards

DNOC treatment. This result is at variance with the earlier observations of Pfister and Arntzen (1979) and Oettmeier et al. (1982) who reported that chloroplasts which are highly resistant to triazines are actually more susceptible to certain phenols and nitrophenols. The results obtained by the oxygen evolution measurement were supported by fluorescence induction experiments. It is well documented that the fluorescence rise depends on the redox state of Q, the primary acceptor of PS II. If Q is reduced fluorescence rise is faster than if it is oxidized (Duysens and Sweers, 1963; Papageorgious, 1975). In our experiment DCMU dramatically stimulated the rate of fluorescence rise observed upon illumination of dark adapted susceptible and resistant chloroplasts - an observation similar to Pfister and Arntzen, (1979). In lower concentrations DNOC affected fluorescence transients, a pattern identical to that of DCMU in both biotypes. However, at higher concentrations (100 μM and 300 μM) DNOC greatly decreased the amplitude of the fluorescence induction signals. Picrate and BNT (2-bromo-4-nitrothymol) also decreased the fluorescence rise in a similar manner like DNOC (not shown in the figures). As it has been reported previously (Ghanotakis et al. 1982) that picrate and DNP (2-4-dinitrophenol) are ADRY-agents (Accelerating the deactivation reactions

of water splitting enzyme system Y), therefore, we concluded that the diminishing effect of DNOC on fluorescence rise of susceptible and resistant chloroplasts at higher concentrations might be due to its ADRY effect on oxygen evolving side of PS II.

The fluorescence rise in chloroplasts of both biotypes caused by these herbicide treatments is due to the inhibition of re-oxidation of the primary acceptor Q by the secondary acceptor B ($Q^-B \rightleftharpoons QB^-$) in the acceptor side of PS II (Zankel and Kok, 1971). Atrazine behaved similarly like DCMU in the case of susceptible chloroplasts. Ten μM atrazine completely inhibited the electron transfer from Q to B in the case of susceptible biotype but a similar concentration could not block the electron transfer at all in the case of resistant ones. About 200-fold concentration was needed in the resistant chloroplasts to get a similar induction curve as in the susceptible one. Similar results have been demonstrated by Arntzen et al. (1979) in A. retroflexus L..

Photosynthetic oxygen evolution and fluorescence induction measurements are commonly used methods in the field of herbicidal effects on photosynthesis. It has been observed that the light-induced 515 nm absorbtion change is also greatly influenced by herbicide (PS II herbicides) treatment of chloroplasts

(Roux and Faludi-Dániel, 1977; Van Assche, 1979; Garab et al. 1983). Bearing these findings in mind, we assumed that the method of 515 nm absorption change can be applied in the approximate determination of $I_{50}$ concentrations of herbicides in the treated chloroplasts and in subsequent evaluation of R/S ratios of susceptible and resistant biotypes.

In our measurements, DCMU and DNOC equally decreased the 515 nm absorption change signals in both susceptible and resistant chloroplasts of E. canadensis L.. Atrazine, on the other hand, showed differential diminishing tendency of 515 nm absorption change signals in resistant biotype in comparison to the susceptible one. Five μM atrazine almost completely abolished the signal in the case of susceptible biotype whereas a similar concentration could not change the signal in the case of resistant one. The $I_{50}$ value was 300 nM for susceptible biotype and 200 μM for the resistant one. The R/S ratio was calculated out to be $5 \times 10^2$ which is nearly equivalent to $7.5 \times 10^2$ obtained in oxygen evolution measurement.

Oxygen evolution, 515 nm absorption change and fluorescence induction measurements could be applied in the determination of $I_{50}$ concentration of atrazine in E. canadensis L.. However, these techniques could not give any data about the changes occurring at the

acceptor side of PS II, accompanying with the appearance of herbicide resistance in E. canadensis L.. Therefore, we tried to introduce a new method, thermoluminescence (TL), for the investigation of the mechanism of herbicide resistance in plants. It has been stated that TL provides information about the redox states of the primary and secondary acceptors of PS II (Inoue and Shibata, 1979; Droppa et al. 1981). The main TL band at + 30°C is related to the secondary acceptor, B and another band appearing at + 10°C can be related to the primary acceptor, Q (Demeter et al. 1982). Any change in the redox state of these acceptors is reflected on the peak positions and the amplitude of the respective bands (Demeter, 1982). Thus TL seems to be a useful technique for studying the mechanisms and sites of action of herbicides that inhibit the electron transport of PS II at the level of the secondary acceptor, B.

In the current experiment, the B-band at + 30°C has been found to be replaced by the Q-band at + 10°C, + 8°C and - 10°C due to the treatment of susceptible chloroplasts by DCMU, atrazine and DNOC respectively. These herbicides blocked the electron transport from Q to B (Trebst and Draber, 1979) and as a result electrons accumulated on Q (Velthuys and Amesz, 1974). Charge recombination of the reduced primary acceptor,

$Q^-$ with the donor side of PS II generated the Q-band. Plotting the amplitude of the B-band as a function of DCMU concentration, the $I_{50}$ value was found to be around 80 nM. This finding is in good co-relation with our data obtained from oxygen evolution and 515 nm absorption change experiments.

The differences in the peak positions of the Q-bands may be explained in the following ways:
1/ The structural modifications of the proteinacious components of Q and B due to binding of DCMU, atrazine and DNOC changes the mutual orientation and separation of Q and P-680, so that probability of the reverse electron transport from Q to P-680 might also change and thus the position of the related TL band as well.
2/ TL originates from charge recombination occurring between positively charged donors and negatively charged acceptors (Lurie and Bertsch, 1974; Ichikawa et al. 1975; Arnold, 1966; Inoue and Shibata, 1979; Demeter et al. 1979). The peak position of a TL band is determined by the redox span between the donor and acceptor molecules participating in the recombination (Vass et al. 1981). The herbicide induced change in the redox state of Q is reflected in shifts in the peak positions of the Q-bands. The two different peak positions (at around + 10°C for DCMU and atrazine and at - 10°C for DNOC) obtained in our TL measurements

can be supported by a classification made by Trebst and Draber (1979), who classified urea-type and phenolic herbicides into two different groups on the basis of their chemical structures. We therefore, assume that herbicides belonging to two different groups have different effects on the state of Q which is evident from our results. Böger et al. (1981) proposed different binding sites for urea-type and phenolic herbicides. Our results can also be supported by the work of Pfister et al. (1981) who reported a 32-34 kD polypeptide for binding urea-type herbicides and Oettmeier et al. (1982) who reported a 41 kD polypeptide for binding phenolic herbicides (see Fig. 3) on the reducing site of PS II.

Since DCMU and DNOC induced TL bands are well separated from each other, therefore, we tried to follow the displacement of these herbicides in the susceptible chloroplast membrane. The result obtained from displacement experiment suggests that DCMU, atrazine and DNOC have a common site of action in the chloroplast membrane. Our displacement experiment can be supported by the work of Reimer et al. (1979) and Trebst, (1979) who demonstrated that BNT (Bromonitrothymol) a phenolic herbicide has replaced the bound radioactively labelled metribuzin ($C^{14}$-metribuzin) from the thylakoid membrane in the concentration range

of their $I_{50}$ value. Oettmeier and Masson (1980) showed that the ureas interfere noncompetitively but the phenolic herbicides interfere competitively with the specific binding site of the phenolic herbicide, dinoseb.

The apparent contradictory results can be resolved by explaining that there might have different but overlapping binding areas for DCMU, atrazine and DNOC. The binding of one herbicide may influence the binding of the other. Our interpretation can be supported by a model (see Fig. 2) proposed by Trebst, (1980).

In the above TL experiments it has been noticed that the B-band appeared at + 30°C in the case of susceptible biotype. Due to the treatment of DCMU, atrazine and DNOC, the electron transport is blocked between the primary and secondary acceptors and consequently the B-band has been found to be replaced by the Q-band at + 10°C, + 8°C and - 10°C in susceptible chloroplasts. Surprisingly, B-band did not appear at all at + 30°C even in untreated resistant-chloroplasts. A band at + 10°C (Q-band) has been found to appear in the glow curve of the TL. These observations suggest that following light excitation, in the resistant chloroplasts, the primary acceptor pool remains in reduced state for a long period while in the susceptible biotype Q is quickly reoxidized by the secondary acceptor B.

A mechanism which can explain our result would be that the semiquinone, $B^-$ is stabilized (mid-point potential of $B/B^-$ is raised) so that the centers would be predominantly in $B^-$ state after dark adaptation.

The more positive potential of $B^-$ explains why the singly reduced secondary acceptor $B^-$ does not take part in charge recombination reaction and consequently the lack of the B-band in the TL spectrum.

It has been shown by Bowes and Crofts (1981) that in susceptible chloroplasts the rate of the reaction $Q^-B \longrightarrow QB^-$ is faster than the reaction $Q^-B^- \longrightarrow QB^{2-}$. According to the redox theory of quinones (Clarke, 1960) a more positive redox potential of $B^-$ results in an equivalent lowering of the redox potential of the $B^{2-}$. Thus in resistant chloroplasts the $Q^-B^- \longrightarrow QB^{2-}$ reaction is even slower than in the susceptible ones. Since according to our assumption the majority of the centers are in $QB^-$ state in the resistant chloroplasts, after light excitation the slow $Q^-B^- \longrightarrow QB^{2-}$ reaction causes the appearance of Q-band.

Our TL experiment can be well supported by the results of fluorescence induction experiments. The comparison of the initial portion of the fluorescence increase in susceptible and resistant biotype chloroplasts showed a much higher "intermediate" ($F_I$) level

in the resistant chloroplasts than in susceptible ones. This implies that the rate of re-oxidation of the primary electron acceptor by the secondary electron acceptor pool is slower in the resistant biotype than in the susceptible one.

The conclusion drawn from fluorescence induction measurements is in agreement with our TL observation which shows that in resistant chloroplasts, after short dark adaptation (5 min) in a sequence of flashes the amplitude of the Q-band at + 10°C oscillates with a periodicity of 2 exhibiting maxima at uneven flash numbers (1, 3, 5, 7 etc.). In resistant chloroplasts after short dark adaptation the majority of the centers are in $QB^-$ state and after uneven flash numbers the slow $Q^-B^- \longrightarrow QB^{2-}$ reaction occurs. After even number of flashes the faster $Q^-B \longrightarrow QB^-$ reaction takes place. As a result a greater portion of the primary acceptor remains in reduced state for a along period after uneven than after even flash numbers consequently the amplitude of the Q-band shows a period two oscillation with maxima at uneven flash numbers.

Conversely, after long dark adaptation (6 hr) of resistant chloroplasts, when the secondary acceptor pool is in oxidized state, a reverse oscillation pattern was observed showing maxima at 2, 4, 6 and 8 flashes. Since after the first flash the majority of

the centers would be in $Q^-B$ state ($Q^-B \longrightarrow QB^-$ reaction) and after the second flash the centers would be in $Q^-B^-$ state ($Q^-B^- \longrightarrow QB^{2-}$ reaction), the oscillation pattern is opposite to that found after short dark adaptation.

Our results are in accordance with Bowes et al. (1980) who got higher fluorescence transients during the $Q^-B^- \longrightarrow QB^{2-}$ electron transfer and lower in the course of $Q^-B \longrightarrow QB^-$ reaction. The oscillation pattern of our TL measurements in resistant chloroplasts comply with their oscillation of fluorescence level as a function of flash numbers.

In susceptible chloroplasts the Q-band is shifted upon herbicide addition indicating that not only the state of B but that of Q also is influenced by herbicide binding to the chloroplast membrane. Opposite to this in resistant chloroplasts the Q-band remained unchanged even at high concentration of DCMU, atrazine and DNOC. This observation suggests that in the resistant chloroplasts the Q-B complex has changed due to the mutation of chloroplast DNA (Pfister and Arntzen, 1979) and herbicide binding at the level of the secondary acceptor does not exert any effect on the redox state of the primary acceptor.

# 6 SUMMARY

Differential effects of PS II herbicides (DCMU, atrazine and DNOC) on the electron transport system of photosynthesis were investigated in triazine-susceptible and triazine-resistant biotypes of Erigeron canadensis L. and Amaranthus retroflexus L..

The data obtained from four different experiments can be summarized in the following manners:

1. The herbicides used in our experiments (DCMU, atrazine and DNOC) affect the reducing side of PS II, between the primary acceptor Q and the secondary acceptor B of the photosynthetic electron transport chain.

2. Both susceptible and resistant biotypes responded equally to DCMU and DNOC treatments. Both biotypes, on the other hand, showed differential sensitivity towards atrazine. About 750-fold concentration was needed to inhibit electron transport to a similar extent in the resistant biotype as compared to the susceptible one. The R/S ratio was found to be $7.5 \times 10^2$ for E. canadensis L. and $10^3$ for A. retroflexus L..

3. It was found that the methods of fluorescence induction, 515 nm absorption change and thermoluminescence can be applied in the approximate determination of $I_{50}$ value of PS II herbicides in the treated plants, in addition to the oxygen evolution measurement.

4. In the susceptible chloroplasts, herbicide binding influences the redox state of the primary acceptor which is reflected on the peak position of the main TL band, about + 10°C for urea type (DCMU and atrazine) and - 10°C for phenolic (DNOC) herbicides. These differences in the peak positions of the TL bands indicate that the effects of these herbicides on the primary acceptor of PS II are different.

5. TL investigation illustrated that the action sites of urea-type (DCMU and atrazine) and phenolic (DNOC) herbicides are in interaction with each other. DCMU and atrazine can replace DNOC from its binding site in the chloroplast membrane.

6. In the susceptible biotype the main TL band which is related to the secondary acceptor B, appears at + 30°C. This band is totally absent in the resistant biotype. To explain this result, it is sug-

gested that the mid-point potential of the secondary acceptor B is more positive in the resistant chloroplasts than in the susceptible ones. Thus the reduced secondary acceptor B, can not undergo back reaction with the donor side of PS II and hence the B-band is missing.

7. In the resistant chloroplasts a TL band at + 10°C can be observed which is assigned to the primary acceptor, Q (Q-band). This observation indicates that in the resistant biotype the reoxidation of the primary acceptor Q, by the secondary acceptor B is much slower than in the susceptible biotype. Consequently the primary acceptor is in reduced state in dark adapted resistant chloroplasts and can easily undergo back-reaction with the donor side of PS II.

8. In the resistant chloroplasts, dark adapted for a short period, the amplitude of the Q-band oscillated with a periodicity of two showing maxima at uneven flash numbers (1, 3, 5, 7 etc.). After long term dark adaptation of chloroplasts an inverse binary oscillation was observed with maxima after even number of flashes (2, 4, 6, 8 etc.). The interpretation of the oscillatory pattern given in the "Discussion section" suggests that

i. in resistant chloroplasts, dark adapted for a short period, a high percentage of the secondary acceptor pool exists in semi-reduced state $B^-$

ii. the rate of electron transfer $Q^-B \longrightarrow QB^-$ is faster than the rate of $Q^-B^- \longrightarrow QB^{2-}$ electron transfer reaction.

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# REFERENCES

Amesz, J. and Duysens, L.N.M. (1977) In Topics in Photosynthesis - Primary Processes in Photosynthesis (Barber, C.J., ed.) 149-185, New York, Elsevier, North Holland

Arnold, W. (1966) Science, 154, 1046-1049

Arntzen, C.J., Ditto, C.L. and Brewer, P.E. (1979) Proc. Natl. Acad. Sci., USA, 76, 278-282

Berversdorf, W.D., Lerman, W.J., Erickson, L.R. and Machado, S.V. (1980) Can. J. Genet. Cytol., 22, 167-172

Böger, P. and Kunert, K.J. (1979) Z. Naturforsch, 34c, 1015-1025

Böger, P., Sandmann, G. and Miller, R. (1981) Photosynthesis Res. 2, 61-74

Bogorad, L., Jolly, S.O., Kidd, G. and McIntosch, L. (1980) in Genome Organization and Expression in Plants (Leaver, C.J., ed.) 291-304, Plenum Press, London

Bouges-Boquet, B. (1973) Biochem. Biophys. Acta, 314, 250-256

Bowes, J.M. and Crofts, A.R. (1981) Archives of Biochemistry and Biophysics, 209(2), 682-686

Bowes, J.M., Crofts, A.R. and Arntzen, C.J. (1980) Archives of Biochemistry and Biophysics, 200(2), 303-308

Burke, J.J., Wilson, R.F. and Swafford, J.R. (1982) Plant Physiol. 70, 24-29

Clarke, W.M. (1960) Oxidation-reduction Potential of Organic Systems, Waverly Press, Baltimore, Md.

Codd, G.A. and Schimid, G.H. (1980) Z. Naturforsch, 35c, 649-655

Croze, E., Kelly, M. and Horton, P. (1979) FEBS Lett. 103, 22-26

Darr, S., Machado, S.V. and Arntzen, C.J. (1981) Biochem. Biophys. Acta, 634, 219-228

Delieu, T. and Walker, D.A. (1972) New Phytol. 71, 201-255

Demeter, S. (1982) FEBS Lett. 144(1), 97-100

Demeter, S., Droppa, M., Vass, I and Horváth, G. (1982) Photochemistry and Photobiophysics, 4, 163-168

Demeter, S., Herczeg, T., Droppa, M. and Horváth, G. (1979) FEBS Lett. 100, 321-324

Droppa, M., Horváth, G. and Demeter, S. (1981) Z. Pflanzenphysiol. Bd., 105, 75-79

Duysens, L.N.M. and Sweers, H.E. (1963) In Studies Microalgae and Photosynthetic Bacteria.(Jpn. Soc. Plant Physiol., eds.) 353-372, Univ. of Tokyo Press, Tokyo

French, C.S. (1960) Chlorophylls in vivo and in vitro. In Encyclopedia of Plant Physiology (Ruhland, W. ed.) Springer Verlag, Heidelberg, 5(1), 259-271

Gabbott, P.A. (1969) Prog. Photosynth. Res., 3, 1712

Garab, Gy.I., Burgos, A.A.S., Zimányi, L. and Faludi-Dániel, Á. (1983) FEBS Lett. 154(2), 323-327

Garab, Gy.I., Chernisheva, S., Kiss, J.G. and Faludi-Dániel, Á. (1976) FEBS Lett. 61(2), 140-143

Ghanotakis, D.E., Yerkes, C.T. and Babcock, G.T. (1982) Biochem. Biophys. Acta 682, 21-31

Govindjee and Van Rensen, J.J.S. (1978) Biochem. Biophys. Acta 505, 183-213

Gressel, J. (1982) Plant Science Letters, 25, 99-106

Haehnel, W. (1976) Biochem. Biophys. Acta, 440, 506-521

Hill, R. and Bendall, D.S. (1960) Nature, 186, 136-137

Ichikawa, T., Inoue, Y. and Shibata K. (1975) Biochem. Biophys. Acta, 408, 228-239

Inoue, Y. and Shibata, K. (1978) FEBS Lett. 85, 193-197

Inoue, Y. and Shibata, K. (1979) Trends Biochem. Sci. 4, 182-184

Izawa, S. (1977) In Encyclopedia of Plant Physiology, New Series, Vol. 5 (Trebst, A. and Avron, M. eds.) 226-282, Berlin, Springer

Izawa, S. and Good, N.E. (1975) Biochem. Biophys. Acta, 102, 20-38

Kerr, M.M. and Wain, R.L. (1964) Ann. Appl. Biol. 54, 447-450

Khanna, R., Pfister, K., Keresztes, A., Van Rensen, J.J.S. and Govindjee (1981) Biochem. Biophys. Acta, 634, 105-116

Klimov, V.V., Dolan, E. and Ke, B. (1980) FEBS Lett. 112, 97-100

Knox, R.S. (1975) In Bioenergetics of Photosynthesis (Govindjee, ed.), 183-221, New York, Academic Press

Laasch, H., Pfister, K. and Urbach, W. (1981) Z. Naturforsch, 36c, 1041-1049

Laasch, H., Pfister, K. and Urbach, W. (1982) Z. Naturforsch 37c, 620-631

Laasch, H., Urbach, W. and Schrieber, U. (1983) FEBS Lett. 159(1,2), 275-279

Lavergne, J. (1982) Biochem. Biophys. Acta 682, 345-353

Lavergne, J. and Etienne, A.-L. (1980) Biochem. Biophys. Acta, 593, 136-148

LeBaron, H.M. and Gressel, J. (1982) The nature and characteristics of herbicide resistance. Herbicide Resistance in Plants (LeBaron, H.M. and Gressel, J. eds.), 349-362, Wiley, New York

Lurie, S. and Bertsch, W. (1974) Biochem. Biophys. Acta, 357, 420-428

Machado, S.V., Arntzen, C.J. and Stephenson, G.R. (1978) Weed Sci., 26, 318-322

Machado, S.V. and Bandeen, J.D. (1977) Res. Rep. Can. Weed Comm. East. Sect., 305

Machado, S.V., Bandeen, J.D. and Stephenson, G.R. (1978) Res. Rep. Expert Comm. Weeds East. Sect., 339

Mullet, J.E. and Arntzen, C.J. (1981) Biochem. Biophys. Acta, 635, 236-248

Oettmeier, W., Johanningmeier, U. and Neumann, E. (1983) J. Bioen. and Biomemb. 15(2), 43-65

Oettmeier, W. and Masson, K. (1980) Pest. Biochem. Physiol. 14, 86-97

Oettmeier, W., Masson, K., Fedtke, C., Konze, J. and Schmidt, R.R. (1982) Pest. Biochem. and Physiol. 18, 357-367

Oettmeier, W., Masson, K. and Johanningmeier, U. (1982) Biochem. Biophys. Acta, 679, 376-383

Papageorgious, G. (1975) Bioenergetics of Photosynthesis, (Govindjee, ed.) 319-371, Academic Press, New York

Pfister, K. and Arntzen, C.J. (1979) Z. Naturforsch. 34c, 996-1009

Pfister, K., Radosevich, S.R. and Arntzen, C.J. (1979) Plant Physiol. 64, 995-999

Pfister, K., Steinback, K.E., Gardner, G. and Arntzen, C.J. (1980) Plant Physiol, 65, S-10

Pfister, K., Steinback, K.E., Gardner, G. and Arntzen, C.J. (1981) Proc. Natl. Acad. Sci. USA, 78, 981-985

Pillai, P. and John, J.B. St. (1981) Plant Physiol. 68, 585-587

Radosevich, S.R. and Appleby, A.P. (1973) Weed Sci., 21, 497-500

Radosevich, S.R. and Devilliers, O.T. (1976) Weed Sci. 24, 229-232

Reeves, S.G. and Hall, D.O. (1973) Biochem. Biophys. Acta, 314, 66-78

Regitz, G. and Ohad, I. (1975) In Proc. IIIrd. Int. Congress on Photosynthesis (Avron, A., ed.) 1615-1625, Elsevier, Amsterdam, ISBN, 0-444-41312-X

Reimer, S., Link, K. and Trebst, A. (1979) Z. Naturforsch. 34c, 419-426

Renger, G. (1976) Biochem. Biophys. Acta, 440, 287-300

Renger, G., Hagemann, R. and Dohnt, G. (1981) Biochem. Biophys. Acta, 636, 17-26

Rosenberg, J.L., Sahu, S. and Bigat, T.K. (1972) Biophysics J., 12, 839-850

Roux, E. and Faludi-Dániel, Á. (1977) Biochem. Biophys. Acta, 461, 25-30

Ryan, G.E. (1970) Weed Sci. 18, 614-616

Sane, P.V., Desai, T.S., Tatake, U.G. and Govindjee (1977) Photochem. Photobiol. 26, 33-39

Shipman, L.L. (1980) Photochem. Photobiol., 31, 137-167

Shipman, L.L. (1981) J. theor. Biol. 90, 123-148

Solymosi, P. (1981) Novenytermeles, 30, 57

Steinback, K.E., Pfister, K. and Arntzen, C.J. (1981) Z. Naturforsch. 36c, 98-108

Tischer, W. and Strotmann, H. (1977) Biochem. Biophys. Acta, 460, 113-125

Tischer, W. and Strotmann, H. (1979) Z. Naturforsch. 34c, 992-995

Trebst, A. (1979) Z. Naturforsch, 34c, 986-991

Trebst, A. (1980) 5th International Congress on Photosynthesis, Greece

Trebst, A. and Draber, W. (1979) In Advances in Pesticide Science, Part 2 (Greissbühler, H. ed.) 223-234, Pergamon Press, Oxford

Trebst, A. and Harth, E. (1974) Z. Naturforsch. 29c, 232-235

Van Assche, C.J. (1979) Advances in Pesticide Science, Part 2 (Greissbühler, H. ed.) 494-498, Pergamon Press, Oxford

Van Rensen, J.J.S. (1971) Meded Landbouwhogesch, Wageningen 71(1), 1-80

Van Rensen, J.J.S. and Hobé, J.H. (1979) Z. Naturforsch. 34c, 1021-1023.

Van Rensen, J.J.S. and Kramer, H.J.M. (1979) Plant Sci. Lett. 17, 21-27

Van Rensen, J.J.S., Van der Vet, W. and Van Vliet, W.P.A. (1977) Photochem. Photobiol. 25, 579-583

Van Rensen, J.J.S., and Van Steekelenburg, P.A. (1965) Meded Landbouwhogesch Wageningen 65(13), 1-8

Van Rensen, J.J.S. and Vermaas, W.F.J. (1981) Physiol. Plant. 51, 106-110

Van Rensen, J.J.S., Wong, D. and Govindjee (1978) Z. Naturforsch. 33c, 413-420

Vass, I., Horváth, G., Herczeg, T. and Demeter, S. (1981) Biochem. Biophys. Acta 634, 140-152

Velthuys, B.R. (1982) In Quinones in Energy Conserving Systems (Trumpower, B.L., ed.) Academic Press, New York, in press

Velthuys, B.R. (1980) Annu. Rev. Plant. Physiol. 31, 545-567

Velthuys, B.R. and Amesz, J. (1974) Biochem. Biophys. Acta, 333, 85-94

Vermaas, W.F.J., Arntzen, C.J., Gu, L.-Q. and Yu, C.-A. (1983) Biochem. Biophys. Acta, 723, 266-275

Vermaas, W.F.J. and Van Rensen, J.J.S. (1981) Biochem. Biophys. Acta, 636, 168-174

Warwick, S.I. and Black, L. (1980) Can. J. Plant Sci. 60, 751

Wessels, J.S.C. and Van der Veen, R. (1956) Biochem. Biophys. Acta 19, 548-549

Witt, K. (1973) FEBS Lett. 38, 116-118

Zankel, K.L. and Kok, B. (1971) Methods in Enzymology, (Pietro, A.S. ed.), 218-238, Academic Press, New York