

## **pH DEPENDENCE OF THE EFFECTS OF DIURON, ATRAZINE AND DINOSEB ON THE LUMINESCENT PROPERTIES OF THYLAKOID MEMBRANES**

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**Summary.** The effect of the external pH in the range of 5.5 to 8.0 on the activity of three PSII herbicides (diuron, atrazine and dinoseb) was studied in isolated thylakoid membranes by means of prompt and delayed fluorescence induction kinetics. Well-expressed pH dependence was established for prompt fluorescence parameters ( $F_v/F_m$ ,  $F_i/F_m$ ) as well as for delayed fluorescence parameters ( $I_1$ ,  $I_2$ ). Two types of pH dependence of the herbicide effect were observed. The apparent resistance to herbicides of the urea/triazine type (diuron and atrazine) was found to be maximal at pH 6–6.5, although the amplitude of the herbicide effect was different between diuron and atrazine. The pH dependences for thylakoids treated with the phenolic herbicide, dinoseb, showed a minimum in the herbicide effect at pH 7.5.

The mechanism by which pH influences the herbicide sensitivity of thylakoid membranes is supposed to involve pH-induced conformational changes of the  $Q_B$ -binding site on the D1 protein of PSII, resulting in alteration of the affinity to the herbicide molecule. The conformation of the  $Q_B$ -site at a certain pH is characterized by maximal herbicide resistance. The optimal value of pH depends on the type of herbicide, specifically the location of the H-bond between the herbicide molecule and D1. The abrupt distinction in the behaviour of the different types of herbicides with respect to pH could serve as a basis to develop a test method to discriminate between different families of herbicides and to classify a given herbicide as belonging to one or another family.

**Keywords:** D1 protein, delayed fluorescence, electron transport, herbicides, pH, Photosystem 2, *Pisum sativum*, L., variable chlorophyll fluorescence

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**Abbreviations:** DF – delayed fluorescence; DCMU – diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES – N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); MES – 2-(N-Morpholino)ethanesulfonic acid; PF – prompt fluorescence; PQ – plastoquinone; PSI – Photosystem 1; PSII – Photosystem 2;  $F_o$  – constant fluorescence;  $F_i$  – fluorescence level at 150 ms;  $F_v$  – variable fluorescence;  $F_m$  – maximal fluorescence

## Introduction

A cardinal site of action of the most commonly used photosynthetic herbicides is the PSII acceptor side. The mechanism of inhibition stands on a competitive displacement of the PQ molecule from the  $Q_B$ -site on the D1-protein in the PSII reaction centre (Velthuys, 1981; Lavergne, 1982; Vermaas et al., 1984; Trebst, 1987). The bound herbicide thus prevents oxidizing of  $Q_A$  by  $Q_B$ . The quinone molecule binds to two amino acid residues of D1 – Ser-264 and His-215. Herbicides are divided into two superfamilies in respect of both their corresponding binding amino acid (Ser-264 or His-215) and their structure – the urea/triazine type and the phenol type superfamilies (Trebst, 1987; Oettmeier, 1992). The former includes the herbicides diuron and atrazine which bind to Ser-264. Dinoseb belongs to the latter, phenol type.

The herbicide binding requires a steric equivalence between the herbicide molecule and the  $Q_B$ -site (Mets and Thiel, 1989; Fuerst and Norman, 1991). By changing the conformation of the binding site one would expect to alter its affinity to the herbicides and hence the herbicide activity. Such alterations of PSII sensitivity to herbicides at different temperatures were found (Lambrev and Goltsev, 1999). One possible means for modifying the membrane proteins conformation could be pH of the surrounding medium.

An informative method for monitoring the state of PSII and the photo-induced electron transport is measuring the prompt and delayed chlorophyll fluorescence (Duysens and Sweers, 1963; Havaux and Lannoye, 1983). Induction curves of PF and DF are assumed to be good indicators for the photosynthetic quantum yield (Buttler, 1977), the electron transport capacity, redox state of the electron carriers (Krause and Weis, 1984; Schreiber and Schliwa, 1987) and the proton gradient across the thylakoid membranes (Havaux and Lannoye, 1983; Fork et al., 1985; Bilger and Schreiber, 1990).

The main purpose of this study was to analyse the effects of two herbicide types on the PSII activity in dependence of pH as monitored by prompt and delayed fluorescence.

## Materials and Methods

Pea plants (*Pisum sativum*, L.) were grown hydroponically in Knop's solution under natural sunlight (700 lx average daytime illumination).

Thylakoid membranes (broken chloroplasts) were isolated from leaves of 14-days plants by the method of Whatley and Arnon (1963) with modifications. The isolation medium contained 67 mM phosphate buffer, 5 mM MgCl<sub>2</sub>, 330 mM Sorbitol (pH 7.8). The chlorophyll concentration was 3 mg.ml<sup>-1</sup>, estimated according to Lichtenthaler (1987). The thylakoids were frozen and stored in liquid nitrogen with addition of glycerol (Gol'dfel'd et al., 1980).

The effects of the following herbicides were studied: diuron – 3-(3,4-dichlorophenyl)-1,1-dimethylurea (SIGMA); atrazine – 2-ethylamino-4-chloro-6-isopropylamino-1,3,5-triazine (SERVA); dinoseb – 6-(sec-butyl)-2,4-dinitrophenol (SERVA).

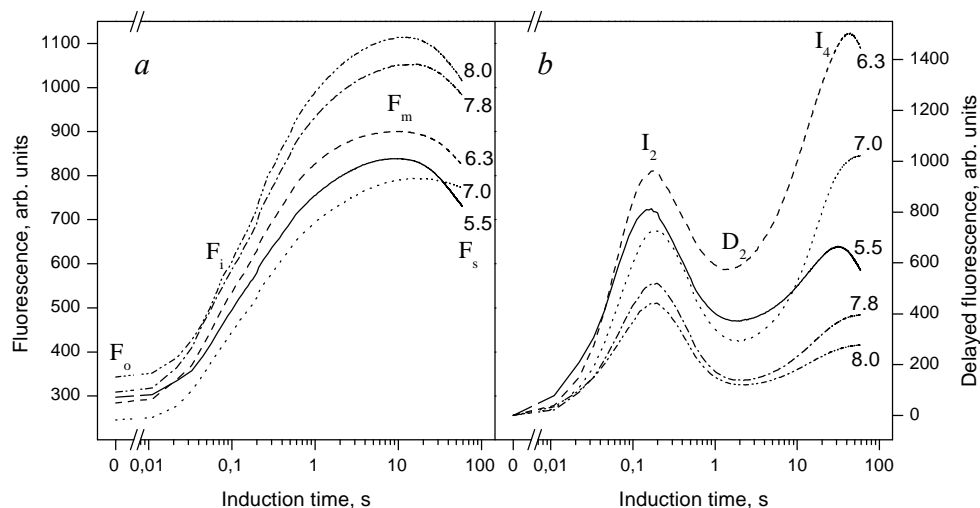
Just before the measurements, the thylakoid suspension was diluted in a buffered medium with defined pH, containing 25 mM MES (pH 5.5–6.3) or HEPES (pH 7.0–8.0) and 5 mM MgCl<sub>2</sub>, to chlorophyll concentration of 30 µg.ml<sup>-1</sup>. Herbicide solution was added to a final concentration of 10<sup>-6</sup>M for diuron and atrazine and 10<sup>-5</sup>M for dinoseb. Since dinoseb has a weaker herbicide activity (Dolchinkova et al., 1997), it was applied in 10-fold higher concentrations to achieve a comparable effect. The samples were kept in the measuring chamber in the dark for 3 min. Control thylakoids were diluted in the same solutions without herbicides.

Prompt and delayed fluorescence induction kinetics measurements were conducted using a phosphoroscope-based fluorometer FL2006 (*Test*, Russia) as described earlier (Goltsev and Yordanov, 1997). Induction kinetics of prompt and delayed fluorescence were registered simultaneously for a period of 1 min. The millisecond decay kinetics of delayed fluorescence dark relaxation were observed also for the same induction period. Prompt and delayed fluorescence parameters are presented as mean values and standard errors calculated from at least 3 repetitions.

## Results

The induction kinetics of prompt and delayed fluorescence of pea thylakoids, taken with FL-2006, have a characteristic shape, as shown in Fig. 1. The initial level of prompt fluorescence  $F_0$ , detected in the first period of 0.5 ms is referred to as constant fluorescence. It reflects the fluorescence from open reaction centres (Krause and Weis, 1991) as well as, to some extent, fluorescence from  $Q_B$ -nonreducing centres, which are closed during the first 2 ms after illumination start (Strasser et al., 2000). Closing of the  $Q_B$ -nonreducing centres corresponds to the so-called J peak in the induction curves, registered with 10 µs time resolution (Strasser et al., 1995). Taking into account that, for the light intensities used, the levels of fluorescence emission at J are expected to be low, we assume that the estimated values for the initial fluorescence are close to the real  $F_0$ .

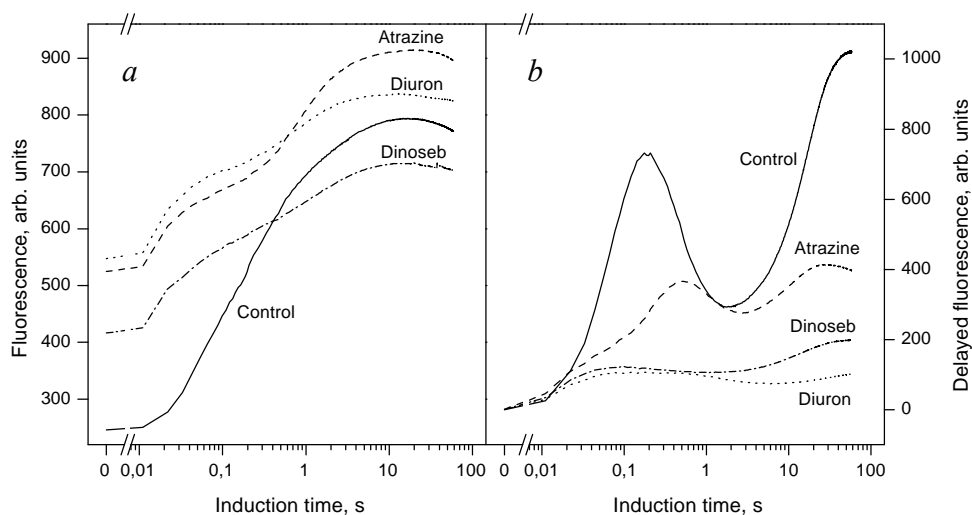
The rise from  $F_0$  to  $F_m$  may be monophasic (in control thylakoids – see Fig. 1a) or with an intermediate shoulder (I) at approximately 100–150 ms (in herbicides-treated objects – see Fig. 2a). The level of fluorescence at time 150 ms is denoted as



**Fig. 1.** Induction kinetics of prompt (a) and delayed (b) fluorescence of control thylakoids, suspended in media with different pH. Just before measuring the thylakoid suspensions were diluted in 25 mM MES/HEPES buffer containing 5 mM  $MgCl_2$  to a chlorophyll concentration of  $30 \text{ mg}\cdot\text{ml}^{-1}$  and kept in the dark for 3 min. Actinic light intensity was  $1200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

$F_i$ . This level probably reflects the relative share of  $Q_B$ -nonreducing centres (which also increases apparent  $F_0$ ) and the process of reaching steady-state levels of  $Q_A^-$ .

The excitation light applied in the FL-2006 fluorometer is not strong enough to completely reduce the PQ pool and close all reaction centres. The maximal level of fluorescence is reached after 10 seconds.



**Fig. 2.** Induction kinetics of prompt (a) and delayed (b) fluorescence of thylakoids incubated at pH 7 in the presence of  $10^{-6}$  M atrazine,  $10^{-6}$  M diuron or  $10^{-5}$  M dinoseb.

The induction curves of delayed fluorescence have two distinctive maximums – around 0.1–0.3 s (fast phase of the induction) and around or after 5 s (slow phase of the induction). The fast phase maximum is formed by two indistinguishable components, denoted as  $I_1$  and  $I_2$ . This maximum reflects the rate of electron transport in the PS II acceptor side. The slow phase maximum,  $I_4$ , is related to the enhancement effect of the transmembrane potential on the delayed light emission. The dip between  $I_2$  and  $I_4$  is referred to as  $D_2$ . Its depth is an indicator of the extent of the PQ pool reduction.

In order to quantitatively assess the photosynthetic characteristics, we calculated some parameters (absolute or relative) derived from the peak values. The parameters of prompt fluorescence are: the variable fluorescence ( $F_v$ ) calculated as the difference between maximal ( $F_m$ ) and constant fluorescence ( $F_o$ ); the ratio  $F_v/F_m$ , which is proportional to the maximal quantum efficiency of the PS II photochemical reaction (Krause and Weis, 1991); and the ratio  $F_i/F_m$ , an indicator of the relative share of  $Q_B$  non-reducing centres. The relative parameter of DF,  $(I_4 - D_2)/D_2$ , that is proportional to transmembrane proton gradient value was evaluated.

### Effects of pH on the prompt and delayed fluorescence kinetics

The prompt and delayed fluorescence induction kinetics of control thylakoids at some selected values of pH are presented in Fig. 1. The influence of pH on both types of luminescence is clearly demonstrated. The initial level ( $F_o$ ) and the maximal intensity ( $F_m$ ) of prompt fluorescence (Fig. 1a) are non-monotonously affected by pH. The changes in  $F_o$  and  $F_m$  are proportional and so, the relative variable fluorescence ( $F_v/F_m$ ) remains unchanged.

The deviations of the delayed fluorescence intensity at different values of pH (Fig. 1b) are even more pronounced than those of the prompt fluorescence. The overall intensity of delayed fluorescence appears to be maximal at pH 6.3 and decreases at more acidic or alkaline pH – pH 5.5 induces a 2.3-fold decrease, whereas pH 8.0 induces a 6-fold decrease compared to pH 6.3. Similar pH dependence of the intensity of DF was obtained by Haveman and Lavorel (1975).

The delayed fluorescence intensity is not uniformly affected by pH in the whole induction period – the strongest influence is observed on the slow maximum ( $I_4$ ).  $I_4$  has 50% higher intensity than  $I_2$  at pH 6.3, and it is lower than  $I_2$  at pH below 6 or above 7.5. The decrease in  $I_4$  is accompanied by a corresponding decrease in the rate of DF growth to  $I_4$  (the first derivative of the induction curve at the inflexion before  $I_4$ ).

### Effects of herbicides on the prompt and delayed fluorescence kinetics

Incubation of thylakoids in the presence of atrazine, diuron or dinoseb brings on considerable alterations on the induction curves of prompt and delayed fluorescence, as can be seen in Fig. 2. The initial fluorescence level ( $F_o$ ) is approximately doubled compared to the control while the maximal fluorescence level ( $F_m$ ) is less affected

(Fig. 2a). Two effects arise from this: 1) the variable fluorescence, being the difference between  $F_m$  and  $F_o$ , is decreased, and 2) the increase from  $F_o$  to  $F_m$  is decelerated. The prompt fluorescence of control thylakoids in the time range from 30 ms to 300 ms shows a monophasic logarithmic increase (Hsu et al., 1989). The induction curves of herbicide-treated thylakoids express an additional shoulder in this region with an inflexion at about 150 ms ( $F_i$ ). Thus, the increase to  $F_m$  is at least biphasic. The magnitude of the herbicide-induced effects on the prompt fluorescence depends on the type of herbicide used.  $F_o$  and  $F_i$  are highest for the diuron-treated objects, slightly lower for atrazine and lowest for dinoseb.

The induction curves of the delayed fluorescence (Fig. 2b) show that incubation with herbicides causes an overall suppression of the delayed fluorescence intensity. Diuron-treated thylakoids appear to be the most influenced – DF intensity decreases by one order, as compared to the control. Dinoseb induces just about the same level of inhibition as diuron, when applied at 10-fold higher concentrations. Atrazine is less effective compared to diuron at the same concentration.

The shape of the induction curves also undergoes substantial changes when herbicides are added. The most sensitive component is the slow maximum –  $I_4$ , which is completely suppressed at some values of pH. The fast phase maximum of the induction kinetics is not only diminished, but also its position on the time scale is changed. As it was mentioned above, in control thylakoids the first maximum is formed of two undistinguished components –  $I_1$  and  $I_2$ . In herbicide-treated objects the slower component  $I_2$  is suppressed and  $I_1$  becomes predominant.

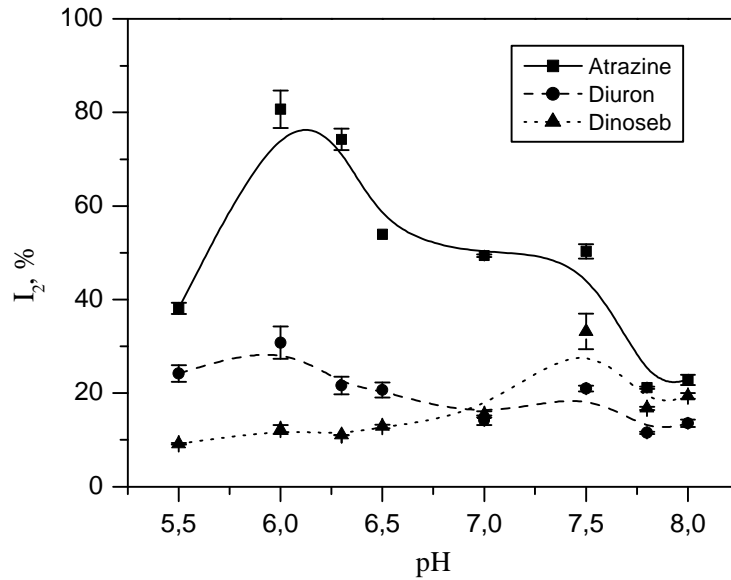
### **pH dependence of the parameters of PF and DF of herbicide-treated thylakoid membranes**

Figures 3–5 show the dependence of some parameters of the prompt and delayed fluorescence induction curves on pH. In order to exclude the effect of pH itself on the induction kinetics and to focus solely on the influence of pH over the herbicide activity, the data are normalized to the control.

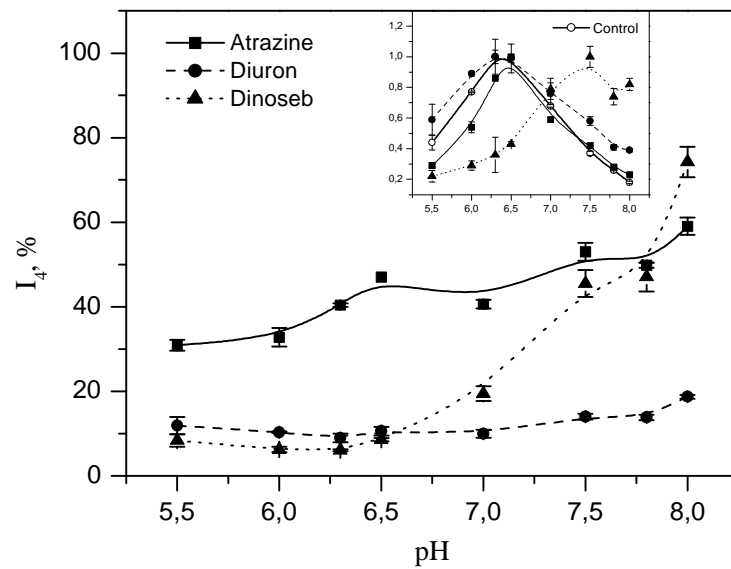
The pH-dependence of the delayed fluorescence induction curve maximum ( $I_2$ ) is shown in Fig. 3. For atrazine and diuron-treated thylakoids, the effect on  $I_2$  is greatest at the highest pH – 8.0 and decreases at lower pH. The minimal effect is found at pH 6.3 and 6.0 for atrazine and diuron, respectively. The shape of the pH-dependence is similar for atrazine and diuron, although the maximums are slightly shifted.

The curve representing dinoseb-treated thylakoids is entirely dissimilar by shape. The lowest pH media induce the strongest suppression of  $I_2$ . With increasing pH, the amplitude of  $I_2$  is increased to a maximum at pH 7.5. The figure indicates a stronger inhibitory effect of diuron and dinoseb than atrazine. The maximal effect of dinoseb is 9% of the control, of diuron – 12% and of atrazine – 22%.

Generation of proton gradient across the membrane enhances the delayed fluorescence and results in the  $I_4$  maximum of the DF induction curve (Fig. 4). The pH depen-

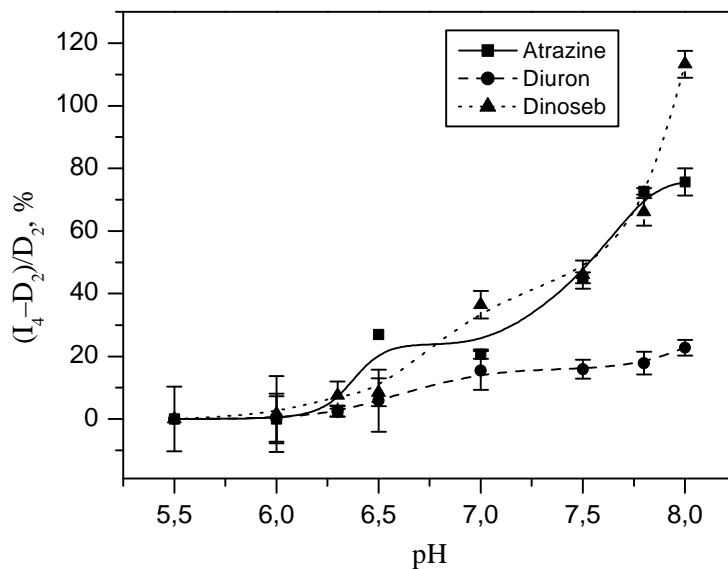


**Fig. 3.** pH dependence of the herbicide effect on the delayed fluorescence peak  $I_2$ . For each point the  $I_2$  values are normalized to the corresponding control.

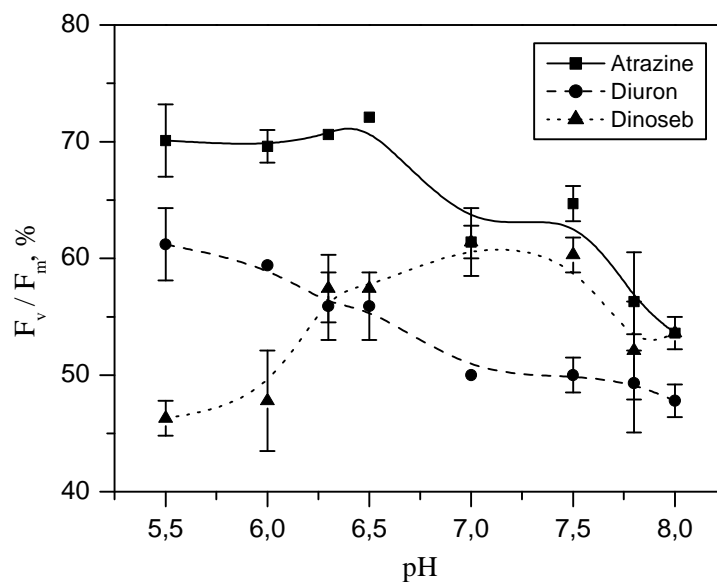


**Fig. 4.** pH dependence of the herbicide effect on the delayed fluorescence peak  $I_4$ . For each point the  $I_4$  values are normalized to the corresponding control.

dence of the herbicide effect on this peak is similar to that of the ratio  $(I_4 - D_2)/D_2$ , used to assess the level of the transmembrane gradient (Fig. 5). All examined



**Fig. 5.** pH dependence of the herbicide effect on the delayed fluorescence parameter  $(I_4 - D_2)/D_2$ . For each point the  $(I_4 - D_2)/D_2$  values are normalized to the corresponding control.



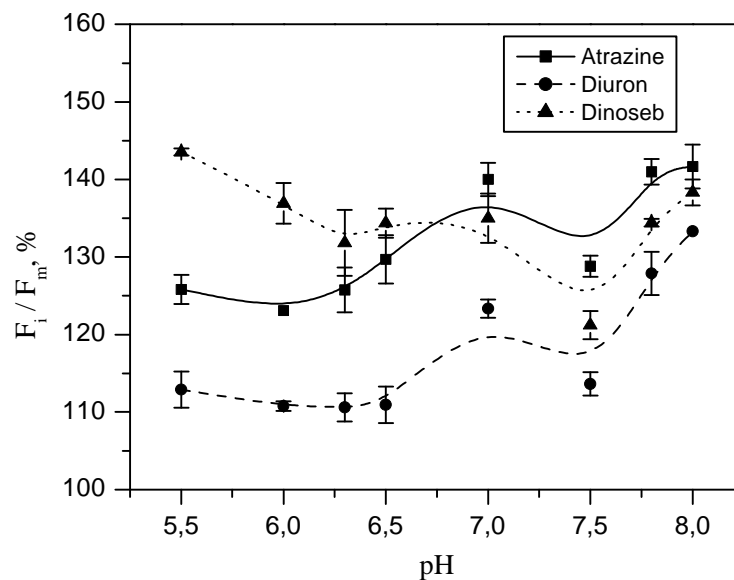
**Fig. 6.** pH dependence of the herbicide effect on the prompt fluorescence parameter  $F_v/F_m$ . For each point the  $F_v/F_m$  values are normalized to the corresponding control.



herbicides inhibit this parameter to a comparable extent. At pH 5.5 no proton gradient is generated (the parameter is completely suppressed). The three herbicides are distinguished by their effect above pH 7.5. Diuron is the strongest inhibitor and dinoseb, in contrast to the others, appears to stimulate the proton gradient since the values for  $(I_4-D_2)/D_2$  exceed the control.

The influence of pH on the relative variable fluorescence,  $F_v/F_m$ , for herbicide-treated thylakoids is shown in Fig. 6 normalized to the control. Diuron is again stronger than atrazine but the pH dependences for both herbicides follow the same pattern. The differences with the control are least pronounced in low pH media and extend with increasing pH. The minimal inhibition of the variable fluorescence is observed in a pH 8 medium.  $F_v/F_m$  is 48% of the control for diuron-treated thylakoids and 53% for atrazine. The maximum in the pH dependences is shifted for diuron compared to atrazine with 0.5–1 unit toward acid pH. The dependence of  $F_v/F_m$  in dinoseb-treated objects in the pH interval of 5.5–7.0 is reciprocal to the curves representing atrazine and diuron-treated ones.  $F_v/F_m$  has the lowest values at pH 5.5 (48% of the control).

The herbicide effect on the induction kinetics of prompt fluorescence may be evaluated by the magnitude of the herbicide-induced maximum in the time range 100–150 ms. The amplitude of prompt fluorescence at 150 ms –  $F_i$ , normalized to  $F_m$ , is shown in Fig. 7 in percents of the control. The similarities between this parameter and  $F_v/F_m$  regarding their pH dependences are evident, taking into consideration that  $F_i/F_m$  changes reciprocally with the herbicide effect. The effect of diuron and atrazine

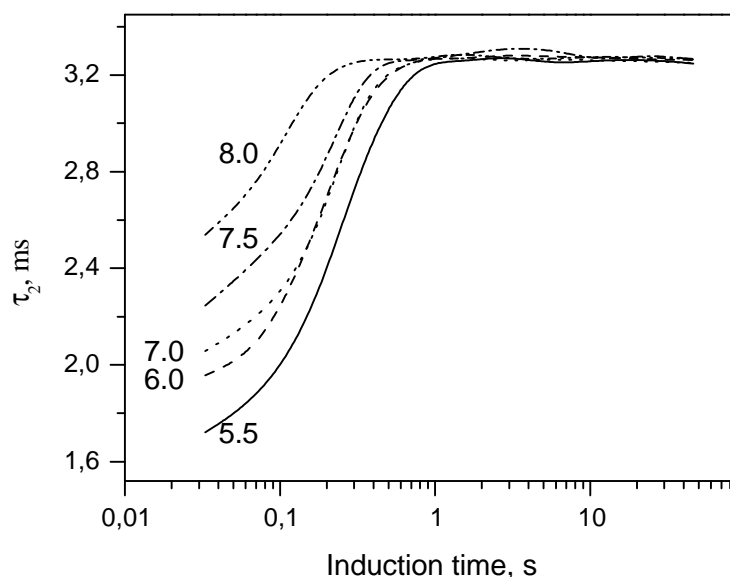


**Fig. 7.** pH dependence of the herbicide effect on the prompt fluorescence parameter  $F_i/F_m$ . For each point the  $F_i/F_m$  values are normalized to the corresponding control.

is maximal at pH 8 and decreases at more acidic media. In contrast, dinoseb is most effective at pH 5.5.

### pH effects on the decay kinetics of the delayed fluorescence dark relaxation

The FL-2006 apparatus permits registration of decay kinetics of dark relaxation with resolution of  $5 \mu\text{s}$ . Such decay kinetics can be recorded at different times during the induction period. The curves are then fitted as a 2-exponential decay with half-times  $\tau_1 \approx 0.5 \text{ ms}$  and  $\tau_2 \approx 1.5 \div 3.5 \text{ ms}$  (Zaharieva et al., 1998). The changes in  $\tau_2$  during the induction period are represented in Fig. 8 for control thylakoids in media with different pH. In all cases, a common trend is observed:  $\tau_2$  is initially low (1.7 to 2.5 ms) and a sigmoidal rise occurs during the first second of the induction until reaching



**Fig. 8.** Effect of pH on the lifetime of the millisecond component of the DF dark relaxation kinetics,  $\tau_2$ .

steady-state values (3.2 ms). It is supposed (Zaharieva et al., 1998) that  $\tau_2$  is inversely proportional to the overall rate constant of the  $Q_B^{2-}$  oxidation. These are 1) electron transfer towards the PQ pool and 2) back reaction of recombination through the reaction centre. At times around 1 s after the actinic light onset, the PQ pool becomes reduced and  $\tau_2$  is formed mainly by the back reaction. Therefore, the sigmoidal rise of  $\tau_2$  corresponds to the process of the PQ pool reduction.

The effect of pH on the induction course of  $\tau_2$  takes place just in the initial phase: with decreasing pH of the medium, the initial values of  $\tau_2$  monotonously decrease, while the steady-state times remain unchanged.

## Discussion

The fluorescence properties of thylakoid membranes are sensitive to a multitude of features of the photosynthetic machinery. The pH of the external medium could impact the prompt and delayed fluorescence directly or indirectly by several ways. One possible line of influence is a direct modification of the fluorescent molecules. It is known that chlorophylls are converted to their corresponding phaeophytins at low pH (van Gorkom et al., 1976; Lichtenthaler, 1987). However, this effect takes place below pH 2 (van Gorkom et al., 1976). Protonation/deprotonation of thylakoid membranes may change the accessibility of chlorophylls to fluorescence quenchers (as zeaxanthin) or the concentration of quenching molecules due to activation of the xanthophyll cycle (Ruban and Horton, 1999).

The surface-electrical properties of thylakoid membranes control a number of photosynthetic phenomena including chlorophyll fluorescence, thylakoid stacking, membrane-conformational changes and electron transport (Barber, 1980). pH influences these properties by neutralizing the surface charge (Scoufflaire et al., 1982). At low pH values (5.1–5.4) thylakoid membranes aggregate and form grana.

The membranes approach each other and stack, but homogeneous distribution of PSI, PS II and light-harvesting complexes remains (Barber, 1980; Karukstis and Sauer, 1985). Furthermore, charge neutralization does not induce so substantial variation in chlorophyll *a* fluorescence intensity as does cation screening, presumably because energy transfer from PS II to PSI is maintained in the absence of domain formation (Barber, 1980).

Delayed fluorescence is determined by the quantum yield of fluorescence as well as the probability of radiative recombination (Lavorel, 1975), which also could be dependent on pH. The pH value may affect the rate of electron transport and hence, the induction kinetics of prompt and delayed fluorescence. It is established that the efficiency of the primary photochemical reaction does not depend on pH in the range of 2.5–8, i.e. the number of active reaction centres remains constant (van Gorkom et al., 1976; Schatz and Witt, 1984). The rate of electron transport is maximal at pH 6–7 (Ben-Hayyim et al., 1976; Barr and Crane, 1980). It is obvious that these steps of the electron transfer chain, which require participation of hydrogen ions (protonation of plastoquinol), are dependent on pH. Apart of this, the oxygen-evolving complex is inhibited at low pH (Haveman and Lavorel, 1975; van Gorkom et al., 1976).

Dependence on pH may be found also for components of the electron-transport chain that do not involve protons directly. Protonation/deprotonation of the protein groups may induce conformational changes altering the redox potentials of the electron carriers (Demeter and Sallai, 1986). This would affect not only the rate of the forward reactions but mostly the back reactions. The pH-induced acceleration of the back reaction (radiative recombination of the separated charges in the PSII reaction

centre) probably determines the observed stimulation of delayed fluorescence by medium acidification (Fig. 1).

Similar considerations suggest that the conformation of the  $Q_B$ -site is modified by the stromal proton concentration. Consequently, a natural assumption would be that the affinity of the  $Q_B$ -site to plastoquinone and hence – the rate of electron transfer via  $Q_B$ , is also affected by pH. This could explain how low pH accelerates the dark relaxation decay of DF in the ms range (as seen in Fig. 8). Another possible explanation of the pH dependence of  $\tau_2$  regards the role of protons as a reagent in the protonation of  $Q_B^{2-}$ . At more alkaline pH the concentration of protons becomes rate limiting for this reaction. As a result, the lifetime of  $Q_B^{2-}$  is increased and so is  $\tau_2$ .

Prompt and delayed fluorescence are sensitive to  $\Delta$ pH, which depends on pH of the medium. The influence of the proton concentration on the rate of intersystem electron transport contributes also to this sensitivity since the electron transport is the energy source for proton gradient generation. Finally, the buffer capacity of the intrathylakoid medium depends on pH, too. These factors control the complex pH dependence of the delayed fluorescence parameter  $(I_4 - D_2)/D_2$  (data not presented).

To a certain extent the observed pH effects might be due to changes in the electrostatic properties of the thylakoid membrane as a whole or of some of its components, which could alter the structure of essential proteins such as the D1 protein of the PSII reaction centre. The three herbicides used allow one to quantify the possible pH-induced changes in the  $Q_B$  locus of the D1 protein. The herbicide effect on the electron-transport reactions in the acceptor-side of PS2 is highly sensitive to pH of the medium (Fig. 3, 6, 7).

The location on D1 which binds  $Q_B$  or herbicides respectively, consists of amino acid residues 211 to 275 (Bowyer et al., 1991; Trebst, 1987), exposed to the stromal surface of the thylakoid membrane. The structure of this site is highly sensitive to changes in pH of the medium. The new conformation of the inside  $Q_B$  niche would determine its affinity to herbicides. The ability of certain herbicides to bind is controlled by the structural analogy between the herbicide molecule and the  $Q_B$  site and the position of the herbicide in the site, fixed by H-bonds with certain amino acids.

We suppose that at a certain pH the  $Q_B$  site attains such a conformation that the resistance to a given herbicide is maximal. The pH dependence curve of parameters related to the electron transport through the site therefore would be bell-shaped. Such a shape is indeed found for a number of investigated parameters of PF and DF ( $I_2$ ,  $F_v/F_m$ ,  $F_i/F_m$  – see Figs. 3, 6, 7). Moreover, for herbicides forming H-bonds with different amino acids (Ser-264 or His-215), the optimal conformation would be different, i.e. occurring at different pH. This is evident from 1) the similar pH dependence curves of all parameters for diuron and atrazine (Ser-264 binding), and 2) the rather dissimilar curves for dinoseb (His-215 binding). The resistance to atrazine and diuron is maximal at more acidic pH (6–6.5) and to dinoseb – at pH 7.5 (Fig. 3). Since the parameters are not equally related to the electron transport through the  $Q_B$ -site, the

pH dependences of their resistance may slightly differ from each other, but the general trend remains the same ( $F_v/F_m$ ,  $F_i/F_m$ ). It should be emphasized that the differences in the pH curves are not only between superfamilies but also within one superfamily (between diuron and atrazine). This points out that the location of the H-bond is not the only factor to determine the herbicide activity of a particular molecule (Trebst, 1987).

The type of the pH dependence of  $I_4$  (Fig. 4) and  $(I_4-D_2)/D_2$  (Fig. 5) which are supposed to correlate with the transmembrane potential is unlike the rest of the parameters. In the presence of herbicides the electron transport as a whole is inhibited and the level of the photoinduced transmembrane potential ( $\Delta pH$ ) is low. The rate of electron transport and  $\Delta pH$  are not linearly related. Even if the electron transport through PSII is mostly suppressed, proton gradient can be generated by residual linear electron transport or by cyclic electron transport around PSI. In such conditions the energization is to a large extent dependent on pH of the medium. The ratio  $(I_4-D_2)/D_2$  increases nearly exponentially with increasing pH (Fig. 5). Thus, parameters which are not uniformly related to the electron transport through the  $Q_B$ -site do not have the typical bell-shaped pH curve with a maximum corresponding to the maximal resistance to a given herbicide.

In conclusion, it could be summarized that the inhibition efficiency of the herbicides over the electron transport reactions in PS II depends on the transient conformation of the  $Q_B$ -site. This conformation can be modified by changing the temperature (Lambrev and Goltsev, 1999) or the pH of the external side of the thylakoid membrane. The pH dependence of the herbicide effect is highly specific in respect of the location of the H-bond between the herbicide and the D1 protein. The analysis of the pH dependences of the prompt and delayed fluorescence parameters demonstrates that herbicides of the urea/triazine type have minimal activity at pH 6–6.5 while herbicides of the phenolic type are least effective at pH 7.5. Since the induction curves of PF and DF are highly sensitive to the electron transport chain activity in the  $Q_B$ -binding site, they could be used as a test method to classify newly synthesized substances as herbicides of the Ser-264 or the His-215 superfamily.

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