Monitoring of Herbicide Effects and Water Stress on Plants Using the Mobile Picosecond-Fluorimeter

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ABSTRACT

Information on the actual photosynthetic activity of plants can be obtained from the analysis of the chlorophyll fluorescence decay. In a new study on vegetation stress experiments were also carried out *in situ* using the mobile picosecond-fluorimeter. The effects of a commercial liquid herbicide, typically and commonly sprayed in grain-field protection, were investigated in weeds and agricultural plants. This commercial product was a defined mixture of two individual herbicide components. The product was applied as recommended to the farmer. The measurements confirmed a selective operation of the herbicide, but also demonstrated that recommended concentrations are too high. Further laboratory experiments with water stress on plants showed that chlorophyll fluorescence decay is different according to the different types of vegetation stress. The average fluorescence decay time cannot be the only parameter to characterize different stress effects. However, using the mobile picosecond-fluorimeter in defined stress situations, plants can be thoroughly studied in their physiological behaviour.

INTRODUCTION

The absorption of light by the antenna pigments, the transfer of the absorbed photoenergy to the reaction centres, and the charge separation at the reaction centres of the photosystems I and II (PS I, PS II) are the first steps in the photosynthesis of plants. The photosystems act in combination via a chain of electron transfer molecules.

Following light absorption chlorophyll molecules at different functional sites and in different energetic situations contribute to the emission of photons. So chlorophyll fluorescence has become a valuable tool for investigations on photosynthetic species (for a review see Krause and Weis, 1991). Especially the analysis of the chlorophyll fluorescence decay after excitation with ultrafast light pulses yields detailed information on the actual state of photosynthetic activity (for a review see Holzwarth, 1991).

Effects of vegetation stress or pollution bring about variations of the chlorophyll fluorescence kinetics. This can be interpreted within the so-called exciton-radical pair model (Schatz et al., 1987, 1988) which includes the electron transfer from the photoexcited reaction centres II via a pheophytin molecule and the two quinone electron acceptors Q_A and Q_B to the plastoquinone-pool (van Gorkom, 1985, Holzwarth, 1991) to define the excited state relaxations. According to this model the rate constants for charge separation, charge stabilization and charge recombination strongly depend on the redox state of the first quinone electron acceptor Q_A

Application of the herbicide diuron (DCMU) blocks the electron transport chain after the first quinone electron acceptor Q_A of PS II (Velthuys, 1981, Trebst, 1991).

The rate of charge separation decreases and the rate of charge recombination increases, while no charge stabilization occurs in this case. Thus the fluorescence decay parameters are varied, and longer fluorescence decay times and higher fluorescence quantum yields of the PS II chlorophylls are observed (Holzwarth, 1991, Schatz et al. 1987, 1988).

Using chlorophyll fluorescence two methods for time-resolved investigations on the photosynthetic activity have gained importance. The first one makes use of the variable fluorescence yield, i.e. of the chlorophyll fluorescence induction (Schreiber, 1986, for a review see Briantais et al., 1986). However, following very recent publications (Trissl et al., 1993, Holzwarth, 1993) the interpretation of fluorescence induction curves generally has to be clarified.

The other method is based on the direct measurement of the chlorophyll fluorescence decay (Holzwarth, 1991). In earlier papers it has been demonstrated that picosecond fluorescence measurements are well suited for investigations on the actual state of photosynthesis of green plants and on the effects of vegetation stress (Moya et al., 1986, Maier-Schwartz et al., 1992).

Using an improved version of our mobile picosecond-fluorimeter we have shown very recently that marine photosynthetic organisms can be thoroughly studied in their polluted environmental conditions (Maier-Schwartz et al., 1994); tolerance of and adaptation to sulfide stress have been

characterized for different species of cyanobacteria from the Baltic Sea. Here we report on our latest investigations on the influence of important types of vegetation stress on plants, that is of herbicide application and of water deficiency.

MATERIALS AND METHODS

Up to now the size of our mobile picosecond-fluorimeter is comparable with a refrigerator. However, *in situ* investigations can be carried out easily as shown in an outdoor experiment, see Fig. 1. For electric power supply a portable generator (Honda EX 1000) was used.

The experimental setup is based on the approved standard technique of time-correlated single photon counting (O'Connor and Philips, 1984). The mobility of the measuring system was obtained by using a red laser diode and optical fibres for light guidance. Light excitation of the leaves was performed by a commercial laser diode at 655nm (Toshiba TOLD 9410) which was operated in pulsed mode with 80ps FWHM and a repetition rate of 500kHz. The laser excitation pulses were guided by an optical fibre of 4m length to a daylight blocking detector head in front of the leaves. To avoid nonlinear effects and partial closure of the photosystems the intensity was kept lower than 10^8 photons cm^{-2} $pulse^{-1}$. Fluorescence light was transmitted via a second optical fibre and an optical filter set (695nm, 10nm FWHM) to a fast photomultiplier (Hamamatsu, R1894). This selected wavelength is close to the emission maximum of the PS II chlorophyll fluorescence.

Decay curves were obtained by time to amplitude conversion of the observed photons and by counting in pulse-height-analysis mode within a 486-based computer with integrated DMA-card (Nucleus PCA-II). The apparatus function of the experimental setup was obtained by measuring the ultrafast fluorescence decay of the cyanine dye DQTCI in ethanol (10^{-5} molar, 40 ps fluorescence decay time). For this purpose a closed cuvette with the dye solution was mounted in front of the detector head.

Fig. 1 - In situ operation of the mobile picosecond-fluorimeter.

The decay curves were analysed using standard deconvolution techniques (Marquardt algorithm (Marquardt, 1963)) and assuming a multiexponential decay. The quality of the fits was tested by the chi-square criterium and by the random distribution of the weighted residuals. In all cases the recorded decay curves could be fitted by three exponential functions with very good quality ($1.0 \leq \chi^2_{RED} \leq 1.1$). For an exemplary result the chlorophyll fluorescence decay of a leaf of a dead nettle (*Lamium*) in healthy state is shown in Fig. 2.

It has been demonstrated previously that the average decay time τ_m is a suitable parameter (Moya et al., 1986, Maier-Schwartz et al., 1992, Schmuck et al., 1992) to characterize the actual state of photosynthetic activity of plants. This average decay time is calculated from the parameters of the fit analysis by

$$\tau_m = \frac{\int dt\, t\, F(t)}{\int dt\, F(t)} = \frac{\sum_{i=1}^{n} \alpha_i \tau_i^2}{\sum_{i=1}^{n} \alpha_i \tau_i}$$

In a rather good approximation this allows an easy evaluation. Since many chlorophyll molecules in different functional situations contribute to the observed emission, there is a wide variation of the individual parameters of the multiexponential fits. In more complicated situations this multiparameter set has to be discussed for physiological interpretation.

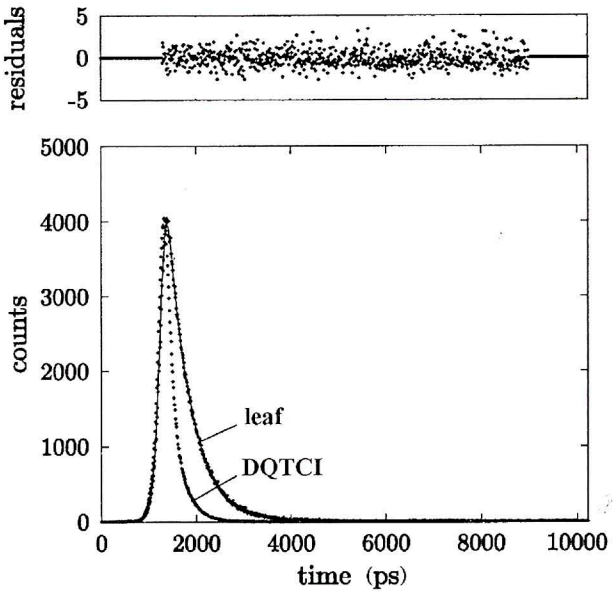

Fig. 2 - Linear plot of the fluorescence decay of the fluorescence scatterer DQTCI for obtaining the apparatus function and of the chlorophyll fluorescence decay of a dead nettle in healthy state. The full curve represents the best fit ($\alpha_1 = 0.62$, $\alpha_2 = 0.34$, $\alpha_3 = 0.04$, $\tau_1 = 100\,ps$, $\tau_2 = 387\,ps$, $\tau_3 = 789\,ps$, $\tau_m = 364\,ps$, $\chi^2_{RED} = 1.03$). *The randomly distributed weighted residuals confirm the good quality of the fit.*

In our experiments leaves of the grains triticale (*Triticale Alamo*) and barley (*Hordeum Vulgare*), of peas (*Pisum Sativum*), and of the weed dead nettle (*Lamium*) were investigated.

Exciting leaves with light causes a fluorescence behaviour which reflects the previous state of illumination (Kautsky effect, see for example, Briantais et al., 1986). The influence of this has to be avoided to collect reproducible and reliable data. Fig. 3 exhibits the fluorescence intensity of a leaf with different preillumination. Though the laser diode had only very weak intensity, dark adapted leaves yielded a pronounced chlorophyll fluorescence induction, whereas sunlight adapted ones revealed hardly any variation of fluorescence intensity with time. But, when the decay curves were monitored at later times after the onset of excitation pulses they were very similar, see the corresponding average decay times τ_m of 513 ps and 473 ps, respectively. So the data acquisition was generally started later than fifteen minutes after the beginning of laser excitation.

A biochemical calibration for a total loss of photosynthetic activity was performed by treating the leaves with an aqueous solution of the herbicide DCMU (Velthuys, 1981, Trebst, 1991).

The concentration was chosen high enough for a total blocking of the electron transport chain from photosystems II.

As we have demonstrated previously (Maier-Schwartz et al., 1992), the average decay time τ_m then continuously in-

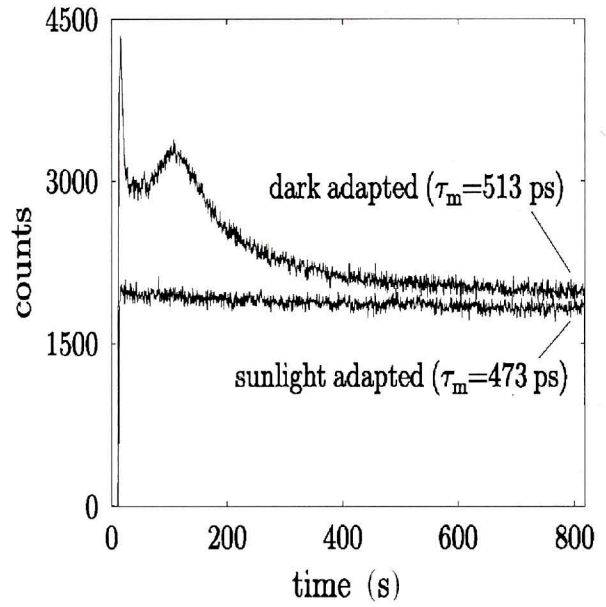

Fig. 3 - Induction curves of the chlorophyll fluorescence of a dead nettle measured with the low laser excitation intensity. The dead nettle was preilluminated by sunlight or dark adapted for 30 minutes, respectively. The fluorescence decays were measured fifteen minutes after the onset of laser excitation. The average decay times differ only slightly.

creases from a minimum to a maximum value within a short time.

The influence on various plants of a commercial liquid herbicide was investigated in outdoor experiments. This product is widely used in grain-field protection against weeds. Its active substances were 333g isoproturon and 166g bifenox per litre of the aqueous standard solution, which work optimally together according to a statement of the producer.

The commercial herbicide was sprayed with common agricultural methods at defined concentrations. For that purpose 3.5 to 4.5 litres of the standard solution were diluted in 300 litres of water for application to a field of one hectare.

Isoproturon has the same point of attack as DCMU (Fedtke, 1982). Bifenox is a diphenyl ether and induces light-dependent chlorophyll bleaching and lipid peroxidation. The light activation requires functional photosynthetic electron flow. However, the site of herbicide activation remains unclear. Some diphenyl ethers can inhibit electron flow at the reducing or oxidizing site of the plastoquinone pool. Since our standard solution contained a DCMU-type as well as a diphenyl ther herbicide, competing effects could occur (Gilham et al., 1987). Water stress experiments were carried out with detached leaves. They were wilted for up to five days in a climatic chamber at 22°C at a constant relative humidity of 60% and kept in darkness between the measurements.

RESULTS AND DISCUSSION

Calibration

Our experimental setup was optimized for observations of the chlorophyll fluorescence caused by photosystem II. So changes of the kinetics at PS II could be detected with special sensitivity.

Application of the herbicide diuron (DCMU) to the leaves caused an irreversible blocking of the photochemical relaxation rates of the light excited photosystems II and thus longest decay times of the chlorophyll fluorescence. We made use of this effect and define this procedure to be an adequate reproducible biochemical calibration for the loss of photosynthetic activity.

The healthy state of a leaf brings about the optimum for the average decay time τ_m whereas the DCMU-treated state represents the worst case. In this connection Fig. 4 (logarithmic plot) characterizes the measurements of the extrema of photosynthetic activity, that is the lower and upper limits for the chlorophyll-fluorescence decay curves of a leaf. Again the data were acquired for the common weed dead nettle. The corresponding average decay times were 364ps and 2125ps, respectively. For other plants slightly different values were determined.

When vegetation stress on plants brings about a reduction of the electron flow from photosystems II the chlorophyll fluorescence decay varies between the extrema pointed out above as long as there is no structural damage of the photosystems.

Herbicide stress

Fig. 5 (top) shows the variations of the chlorophyll fluorescence decay curves obtained when the leaves of a dead nettle were affected by the commercial herbicide. The data were acquired at distinct times (see Fig.) after herbicide contamination at an amount of 3.5 litre standard solution per hectare (see Materials and Methods). The calculated average decay times τ_m are plotted below as a function of the corresponding affecting time.

For comparison results with a 4.5l/ha application as recommended by the producer are also given. As can be seen from the plots, there was a dramatic loss of photosynthetic activity within a short period of time depending on the herbicide concentration. Comparing these data with the biochemical calibration (DCMU) reveals that the electron transport chain was totally blocked within a few hours. Probably concentrations lower than recommended for this herbicide would be sufficient for poisoning this weed. Similar characteristic results were obtained for other weeds (data not shown).

In order to study how cereals are affected by this herbicide, barley and triticale were treated in the same manner. After one day the average decay time τ_m reached a maximum close to the value of the DCMU-treated state, see Fig. 6. But within one week the average decay time continuously decreased towards values as observed for a healthy state. Thus there was a very pronounced recovery of photosynthetic

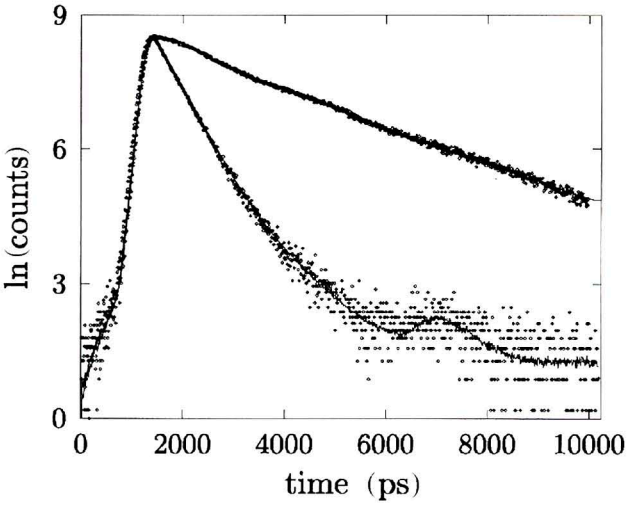

Fig. 4 - Logarithmic plot of the chlorophyll fluorescence decay and best fit curves of a leaf of a dead nettle in healthy state (bottom) and in the DCMU-treated state (top).

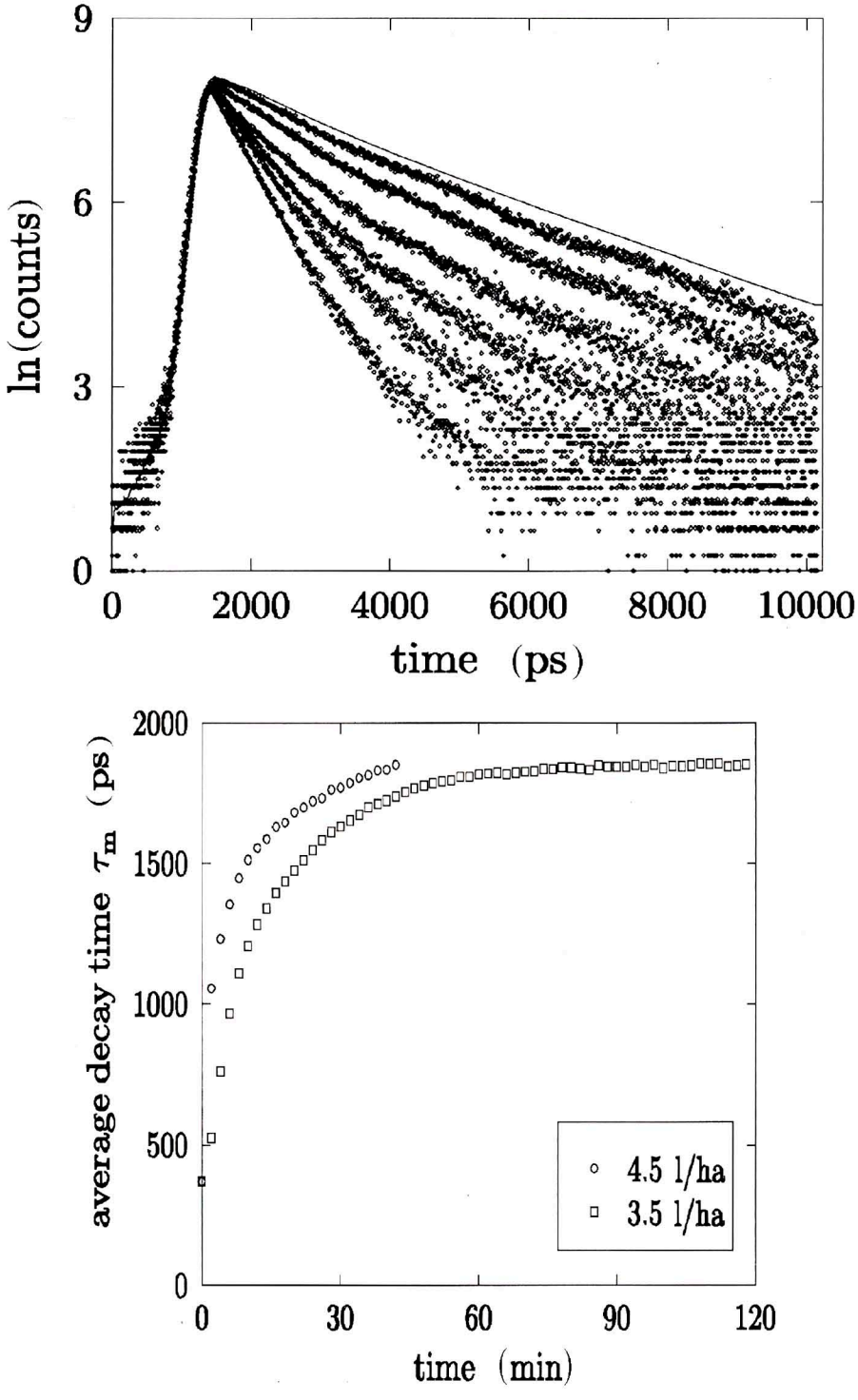

Fig. 5 -(top) Logarithmic plot of the chlorophyll fluorescence decay of the dead nettle monitored at defined times after contamination with the commercial herbicide (0, 2, 4, 8, 14, 112 minutes, from bottom to top). The concentration was 3.5 l per ha. The full curve represents the DCMU-treated state. (bottom) Plot of the calculated average decay times as a function of the affecting time of the commercial herbicide. Furthermore, data for herbicide application of 4.5 l per ha are given for comparison.

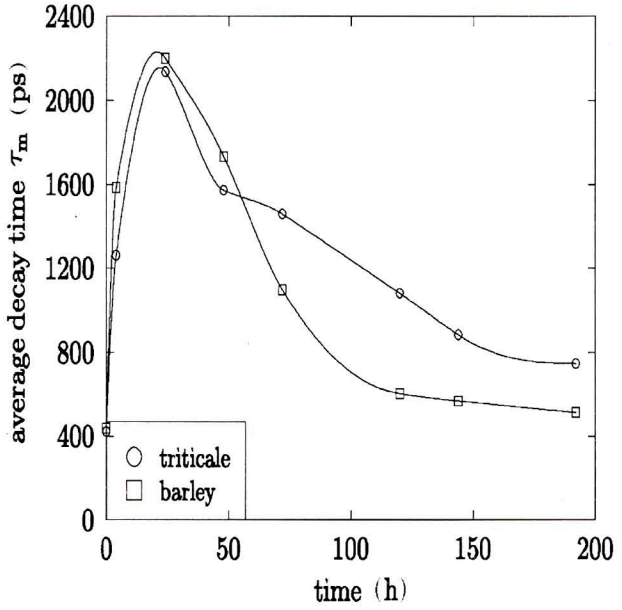

Fig. 6 - Average decay times of the chlorophyll fluorescence of barley and triticale plotted as a function of time after contamination by the commercial herbicide. The applied concentration was 3.5 l per ha.

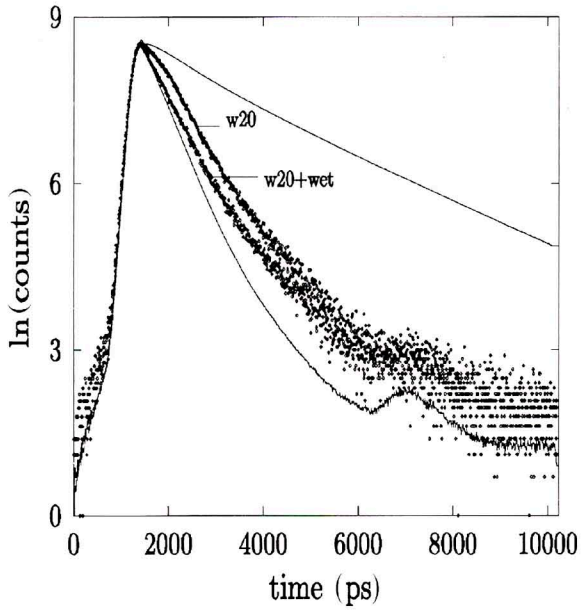

Fig. 7 - Logarithmic plot of the chlorophyll fluorescence decay of a dead nettle monitored in healthy state (lower full curve), after wilting for 20 hours (w20), and after subsequent wetting (w20 + wet), respectively. The DCMU-treated state is represented by the upper full curve.

activity after a period of less productivity. However, a recovery of the electron transport was never observed in weeds (data not shown).

Comparison of the observed chlorophyll fluorescence decay curves with the behaviour of DCMU-treated leaves reveals a strong similarity. This must be due to an analogue modification of the relaxation rates, and confirms that the blockade of the electron transport chain caused by this herbicide takes place behind the primary quinone electron acceptor Q_A. Obviously the DCMU-type herbicide component isoproturon is predominantly affecting the chlorophyll fluorescence.

In case of the weeds the blockade of electron transport is irreversible, whereas it appears to be reversible in case of barley and triticale. This could be due to a different response of the plants to the additional herbicide component bifenox. For further understanding investigations on the vegetation stress of the individual herbicide components would be necessary, which is outside the scope of this paper.

Water stress

There are several communications on effects of water stress on plants in the literature (see for example Sharkey, 1990 and Havaux, 1992). Recently a shortening of the average fluorescence decay time (Schmuck et al., 1992) has been reported, which cannot be understood in analogy to the effects of herbicide stress as given above. In earlier observations we found that slight reduction of the atmospheric humidity reversibly decreased the photosynthetic activity and thus prolonged the chlorophyll fluorescence decay (Maier-Schwartz et al., 1992). But, when the humidity was kept saturated, for example with varied temperature, the photosynthetic activity remained unaltered.

The effect of water stress to detached leaves of the dead nettle is demonstrated in Fig. 7. Wilting for 20 hours in the climatic chamber caused a slower chlorophyll fluorescence decay indicating a partial blockade of electron transport. When the stressed leaves were immersed in water for 2 hours a partial recovery of photosynthesis occurred and the decay became faster again (see Fig. 7).

When the leaves continued to wilt, the reversibility was lost to some extent and the chlorophyll fluorescence decay showed a different behaviour (not shown in Fig. 7). On the one hand, there was a higher amount of the fast decay component than in the case of a herbicide stressed state and on the other hand, there was a prominently slower decay component. The calculated average decay times were remarkably shorter than in severely herbicide stressed situations.

These observations were confirmed in a laboratory experiment with peas. The detached leaves were investigated in healthy, DCMU-treated and wilted state (after 5 days). The results for the calculated average decay times as a function of emission wavelength are given in Fig. 8. There is only a small variation of τ_m with wavelength. At 715nm, in the range of higher PS I contribution the fluorescence decayed slightly faster. However, the decay times differ considerably

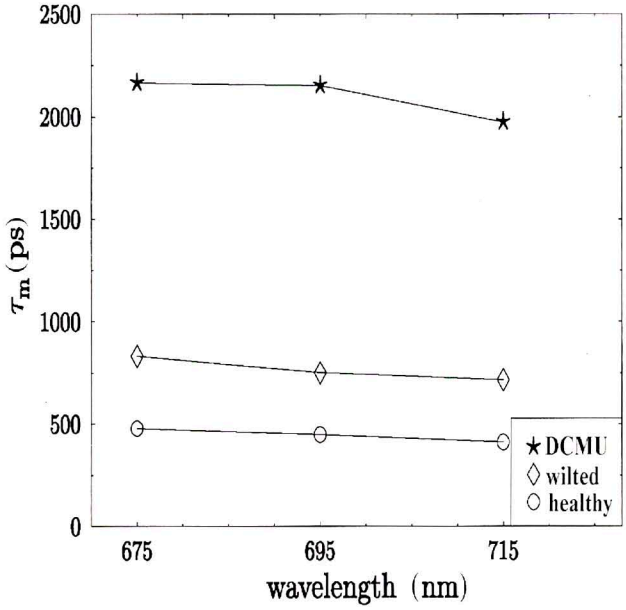

Fig. 8 - Average decay times of the chlorophyll fluorescence of leaves of peas as a function of the emission wavelength. Comparison of the data for the healthy, the wilted (5 days, see text) and for the DCMU-treated state.

for the different types of vegetation stress. Obviously, herbicide stress and water stress produce distinct types of reactions compared with the situation of healthy plants.
Thus the average decay time cannot be the only parameter to characterize the type and the level of stress on a leaf. Additionally the variation of the individual exponential decay parameters and their dependency on wavelength have to be discussed. For clarifying more details the development of the chlorophyll fluorescence decay with wilting and drying leaves is investigated in subsequent experiments.

CONCLUSIONS

In this paper we presented the results of our latest investigations on monitoring vegetation stress on plants using a mobile picosecond-fluorimeter. The performance and the calibration of our experimental setup for analyzing the chlorophyll fluorescence decay was optimized to ensure reliable and easy operation in the field. This was proved in an *in situ* study of the effects caused by the application of a commercial herbicide to a grain-field. The different effects on cereals and weeds were monitored temporally, thus affirming the selective response of the plants to this herbicide.
Experiments with water stress on plants showed a definitely different chlorophyll fluorescence decay. Trying to understand the different mechanisms on a molecular scale, for example in the so-called Z-scheme of photosynthesis, one has to keep in mind that herbicides often affect the electron transfer chain between photosystem II and photosystem I. On the other side, water deficiency affects the structural part as well as the water splitting complex, which supplies the electrons for the reduction of oxidized reaction centres II. Hence water stress produces somewhat more complicated situations.
Further investigations on the chlorophyll fluorescence decay of plants are necessary to extend the use of this tool in the analysis of vegetation stress. However, in a situation of defined stress this method is already well suited for monitoring.

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