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Fluorescence kinetics of chloroplasts as indicators of disorders in the photosynthetic system
I. Comparative studies with greening leaves of *Vitis* and *Hordeum*

by

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Die Fluoreszenzkinetik von Chloroplasten als Indikator von Störungen des Photosynthesesystems
I. Vergleichende Untersuchungen an ergrünenden Blättern von *Vitis* und *Hordeum*

Zusammenfassung: Die Kinetik sowohl der verzögerten Fluoreszenz (Lumineszenz) als auch der prompten Fluoreszenz von Chloroplasten wird benutzt, um die photosynthetische Aktivität von Blattgeweben zu charakterisieren. Zunächst wurde der Ergrünungsvorgang von etiolierten *Vitis*-Stecklingen und *Hordeum*-Sämlingen untersucht. Die Datenerfassung und -auswertung wurde mittels eines Mikrocomputers durchgeführt.

Parallel zu der lichtinduzierten Entwicklung der Photosyntheseaktivität wurde der charakteristische Verlauf der Kinetik (Abnahme der Lumineszenz bzw. Anstieg und Abnahme der Fluoreszenz) deutlicher. Bei beiden Pflanzen war die Abfolge der Veränderungen gleich, es traten lediglich zeitliche Unterschiede auf. An Hand der Interpretationen aus der Literatur werden die Kinetiken diskutiert und die Methode als Verfahren vorgeschlagen, das eine schnelle Charakterisierung mancher Schäden des Photosynthesesystems in verschiedenen Pflanzen erlaubt.

Key words: leaf, photosynthesis, chlorophyll, light.

Introduction

Light energy absorbed by plant pigments is mainly used for photosynthesis, but part of it is always lost in the deexcitation processes of chlorophyll molecules which comprise luminescence (= delayed fluorescence), (prompt) fluorescence and heat emission (Buschmann *et al.* 1984). For further information see the reviews on luminescence by Lavorel (1975) and Malkin (1977), on fluorescence by Papageorgiou (1975) and Krause and Weis (1984).

When leaves which were dark-adapted for more than 15 min are transferred into the light, photosynthesis reaches its full rate only after more than 3 min. It is well established that the induction kinetic of the fluorescence is antiparallel to the rate of oxygen evolution (Strasser 1973; Walker *et al.* 1983) and CO_2 fixation (Ireland *et al.* 1985).

Damage of the photosynthetic apparatus, in particular of the electron transport system, leads to characteristic changes of these phenomena, the analysis of which might thus be a valuable tool for the study of plant diseases and disorders affecting the photosynthetic system, like chlorosis, fungal infections, or effects of chemicals (herbicides).

A detailed knowledge of the behaviour of luminescence and fluorescence under natural conditions is, however, a prerequisite for such analyses. In a first step comparative studies of the changes during light-induced greening of etiolated shoots of *Vitis vinifera* were carried out. To support the results the tests were repeated with *Hordeum* as a rather different plant. The greening process could be considered an 'inverse' model for chlorotic phenomena. It comprises the accumulation of chlorophylls and carotenoids and the formation of other substances, such as redox carriers and proteins that allow the gradual appearance of photosynthetic activity parallel to the formation of thylakoids (review: BRADBEER 1981).

In 1982 BLAICH *et al.* established an apparatus which could be used to localize and measure inhibition of photosynthesis on an intact leaf by means of luminescence analysis. In our studies presented here we try to correlate the induction kinetics obtained with this luminoscope and a fluorescence apparatus.

A detailed discussion of the underlying phenomena is to give a base for further results.

Material and methods

Plant material

Wooden canes of outdoor plants of *Vitis vinifera* cv. Müller-Thurgau were cut into pieces of 3—4 buds which were then cultivated for 30 d in water. Seedlings of *Hordeum vulgare* cv. Villa were grown for 5 d on tap water. Both were kept in total darkness. Shooted *Vitis* cuttings with 3—5 leaves and *Hordeum* seedlings with their primary leaf were then transferred into a climate chamber (22 °C, 60 % relative humidity) and illuminated with continuous white light (400 W OSRAM HQI-E, 47 W · m^{-2}). Before measuring the plants were dark-adapted for 2 h (for ½ h when measured after less than 24 h greening).

During the dark period the *Vitis* leaves are mainly expanding in area whereas the leaves of the *Hordeum* seedlings are growing in length. Because of their basal meristem *Hordeum* leaves show a gradation of different tissue age, the oldest parts being on the leaf tip. For our experiments we used the tissue 1.5 cm below the leaf tip which, according to previous studies, shows the fastest development (BUSCHMANN 1981).

Equipment

Induction kinetics of luminescence and fluorescence were detected with parallel samples. The fast and the slow kinetics of fluorescence were recorded simultaneously.

The luminescence measurement was carried out at room temperature with the luminoscope described by BLAICH *et al.* (1982). For excitation white light was used (Schott, Mainz; KL 1500, 2.1 W · m^{-2}) passing through a light pipe and illuminating a masked area of 2 × 2 cm. *Hordeum* leaves were cut into pieces of 2 cm (0.5—2.5 cm below the leaf tip) and then arranged to cover an area of 2 × 2 cm. Luminescence was detected with a photomultiplier (Proxitronic, FV 38800/2). During the measurement the mechanical chopper was set to 5 rotations/s. Time for illumination of the sample and for detection of luminescence was then 70 ms with 30 ms intervals. A microcomputer (Apple II+) was used to control the chopper and for data processing, including averaging of 3—10 repeated measurements as described by BLAICH (1985).

The fluorescence was detected at room temperature at 680 nm through one cut-off filter (Schott, Mainz; RG 665) and one interference filter (Schott, Mainz; DAL 680). For the excitation a He/Ne laser was used (Spectra Physics, type 134, 5 mW;

632.8 nm, 15 W · m^{-2}) passing its light through an electric shutter into one arm of a three-armed light pipe mounted to a 10 cm light-mixing glass rod. The illuminated area was a circle of about 4 mm diameter. *Vitis* leaves were measured beside the middle rip and the *Hordeum* leaves 1.5 cm below the leaf tip. The two arms of the light pipe, not used for excitation, were taken to detect fluorescence. For the fast fluorescence kinetics the signal detected by a photomultiplier (RCA, type 7265) was stored in a transient recorder (Datalab DL 901) and then plotted on a chart recorder. Simultaneously the signal of the slow kinetics was detected by a photodiode (Silicon Detector Corp., SD-444-41-11-261) and put directly on a chart recorder. The kinetic data were transferred into a microcomputer (Hewlett Packard 85) by means of a graphics tablet device. The average of the curves and the first derivative were calculated.

Chlorophylls were extracted from the leaves either by grinding the tissue with a mortar in 80 % (v/v) aqueous acetone or by a 16 h dark incubation, at room temperature, in dimethyl sulfoxide (DMSO) (modified method of HISCOX and ISRAELSTAM 1979). For both extracts the maxima position and the absorption coefficients of ZIEGLER and EGLE (1965) given for 80 % acetone were used.

Amount of chlorophyll in primary leaves of *Hordeum* seedlings transferred into continuous white light after 5 d of growth in the dark · The extracts were prepared either by a) grinding the tissue with a mortar in 80 % aquous acetone or by b) 16 h dark incubation in dimethyl sulfoxide (DMSO) · Each value represents the mean of 5 replicates · The numbers in brackets give the standard deviation

Chlorophyllmenge in Primärblättern von *Hordeum*-Sämlingen, die nach 5tägigem Wachstum im Dunkeln in helles Licht überführt worden waren · Die Extrakte wurden entweder a) durch Zerreiben des Gewebes in 80%igem wäßrigem Azeton oder b) durch 16stündige Dunkelinkubation von Blattscheiben in Dimethylsulfoxid (DMSO) hergestellt · Jeder Wert stellt das Mittel aus 5 Wiederholungen dar · Die Zahlen in Klammern geben die Standardabweichung an

Illumination time	Chlorophyll content (µg/g fresh weight)			
	a	b	a + b	a/b
a) 80 % Acetone extract				
3 h	82 (1.6)	16 (1.5)	98	5.1
6 h	132 (2.8)	33 (2.9)	165	4.0
12 h	300 (20.7)	86 (10.0)	386	3.5
24 h	405 (12.3)	119 (9.5)	524	3.4
48 h	528 (18.5)	130 (11.2)	658	4.0
72 h	572 (26.5)	161 (14.3)	733	3.5
b) DMSO extract				
3 h	72 (2.6)	13 (1.3)	85	5.5
6 h	114 (12.5)	33 (6.9)	147	3.5
12 h	265 (10.3)	78 (9.7)	342	3.4
24 h	408 (18.6)	109 (5.0)	517	3.7
48 h	515 (13.7)	127 (9.5)	624	4.1
72 h	561 (20.2)	149 (11.5)	710	3.8

Results

Chlorophyll analysis

The comparison between the two methods of extraction demonstrates that a 16 h dark incubation with DMSO leads to approximately the same results as the time-consuming homogenization with 80 % aqueous acetone. The slightly lower values cannot be explained by an uncomplete extraction, as the tissue was always colourless after the incubation time and showed no chlorophylls when extracted by homogenization. Thus a small correction of the absorption coefficients should be sufficient to get the same results as by acetone homogenization. DMSO incubation is less tedious and losses during manipulation of the extracts are diminished.

The amount of chlorophylls accumulated in the primary leaves of *Hordeum* seedlings during growth in the light is shown in the table. During the first 12 h of illumination the chlorophyll *a/b* ratio decreased. After 24 h of light the rate of chlorophyll accumulation diminished.

Kinetic characteristics

During the induction phase luminescence reaches a kinetic maximum after approx. 10 s. Within about 3 min it declines to a low steady state value (Fig. 1 and 4). The decline was much slower in the leaves of *Hordeum*. The decrease from the kinetic maximum to the final steady state divided by the luminescence at that stage was taken as a value to characterize luminescence kinetics. It is referred to as '(relative) luminescence decline'.

The induction kinetics of fluorescence (Fig. 2 and 5) show a first sharp rise to the O level followed by a slower rise to the maximum P which is reached after about 1 s. Within 3 min fluorescence is at a constant low steady state value. Both rise and decline were faster for *Hordeum* leaves. Fluorescence reached its maximum always

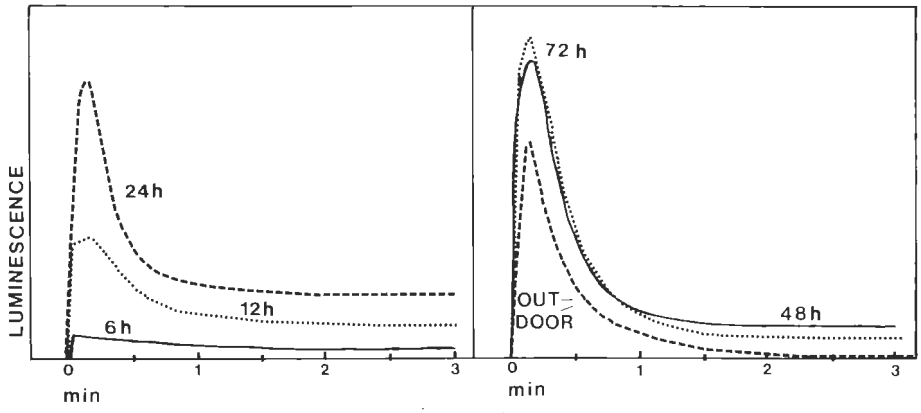

Fig. 1: Induction kinetics of the luminescence (= delayed fluorescence) of *Vitis* leaves taken from outdoor plants (10th leaf, July 12) and from cuttings transferred into continuous white light after 30 d of growth in the dark. Kinetics of the cuttings are shown from 6, 12, 24, 48, and 72 h of illumination. Means calculated from 8 replicates.

Induktionskinetik der Lumineszenz (= verzögerte Fluoreszenz) von *Vitis*-Blättern (10. Blatt von Freilandpflanzen, Entnahme: 12. Juli) und von angetriebenen *Vitis*-Stecklingen, die nach 30 d im Dunkeln ins Helle überführt wurden. Die Kinetik wurde nach 6, 12, 24, 48 und 72 h Beleuchtung gemessen. Mittelwerte aus 8 Wiederholungen.

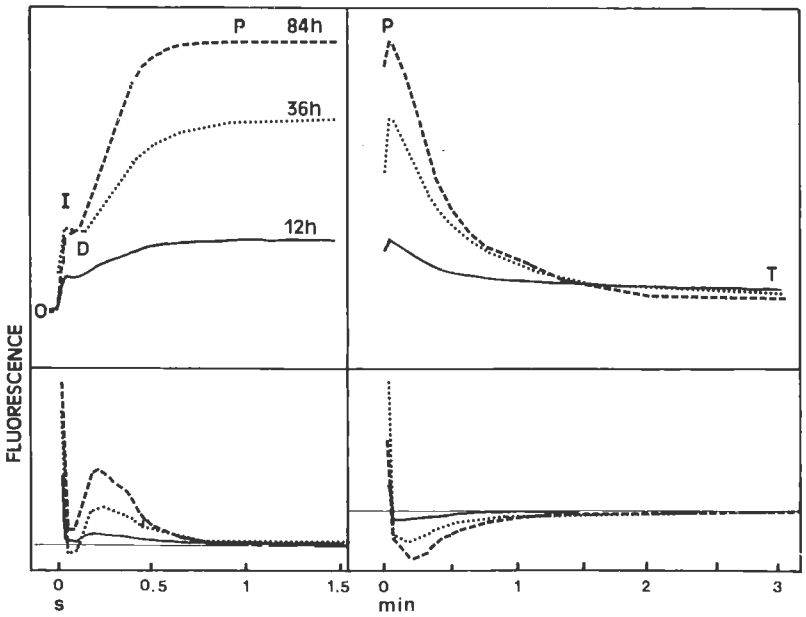

Fig. 2: Induction kinetics of the (prompt) chlorophyll fluorescence of *Vitis* leaves according to Fig. 1. Fast (left part) and slow kinetics (right part) were measured simultaneously after 12, 36 und 84 h of illumination. Means calculated from 8 replicates. For explanation of the capital letters see 'Discussion'.

Induktionskinetik der (prompten) Chlorophyllfluoreszenz von *Vitis*-Blättern entsprechend Abb. 1. Schnelle (links) und langsame Kinetik (rechts) wurden gleichzeitig nach 12, 36 und 84 h Beleuchtung erfaßt. Mittelwerte aus 8 Wiederholungen. Erläuterung der Großbuchstaben s. „Discussion".

earlier than luminescence and in most cases showed an earlier decline to the final state (compare Fig. 1 with Fig. 2 and Fig. 4 with Fig. 5). The relative rate of fluorescence rise from the level O to the kinetic maximum P, divided by the fluorescence at the O level, was calculated as a characteristic value.

Vitis cuttings

During greening in the light the luminescence curves of the leaves of *Vitis* cuttings showed an increase of the kinetic maximum (Fig. 1), the following decline became faster and the relative luminescence decrease was more pronounced (Fig. 3).

The fluorescence kinetics of the *Vitis* cuttings showed a rise in their kinetic maximum and in the rate of fast increase and slow decline (Fig. 2). The time for reaching the kinetic maximum and the steady state remained nearly constant (derivatives in Fig. 2). The values for both relative fluorescence rise and decline increased during the greening process (Fig. 3).

Hordeum seedlings

The primary leaves of etiolated *Hordeum* seedlings transferred into the light showed a rise in the kinetic maximum of luminescence throughout the first 72 h of illumination (Fig. 4). The decline rate increased with the illumination time and the value for the decrease (Fig. 6) became higher during the greening period. The maximum fluorescence (Fig. 5) decreased during growth in light.

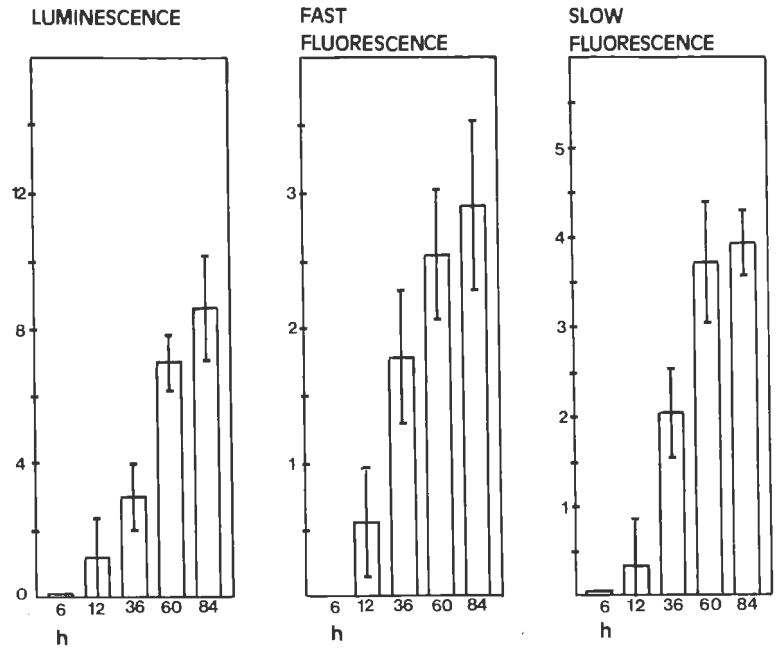

Fig. 3: Values for relative luminescence decrease (left), relative fluorescence rise (middle) and decline (right) of *Vitis* leaves. For explanation see Fig. 1.

Werte der relativen Abnahme der Lumineszenz (links) und der relativen Zunahme (Mitte) bzw. Abnahme (rechts) der Fluoreszenz von *Vitis*-Blättern. Erläuterung s. Fig. 1.

A shoulder at the beginning of the fast fluorescence rise occurring after 6 h of illumination disappeared during the further greening process (see the first sharp dip of the derivative in Fig. 5). During the first 24 h of greening the slow kinetic curve passed a minimum before reaching the steady state. Later on a secondary peak appeared in the slow decline which is visible especially in the first derivative (Fig. 5) as a shoulder (24 h of illumination) or as a second maximum (72 h). The values for both relative fluorescence rise and decline became higher during the illumination period (Fig. 6). A constant maximum of the rise value was reached after 24 h of illumination and after 48 h for the decline value. The values for *Vitis* cuttings were higher than that of *Hordeum* seedlings, especially during the first 24 h of illumination.

Discussion

In order to discuss the induction kinetics two phases have to be distinguished: the fast rise and the subsequent slow decline.

Fast rise: After the onset of excitation both luminescence and fluorescence rise to a kinetic maximum. The kinetic characteristics of fluorescence (compare Fig. 2) are termed O, I, D, P, T (MOHANTY and GOVINDJEE 1974).

The O level reached immediately after the onset of excitation reflects the amount of antenna chlorophylls (MATHIS and PAILLOTIN 1981).

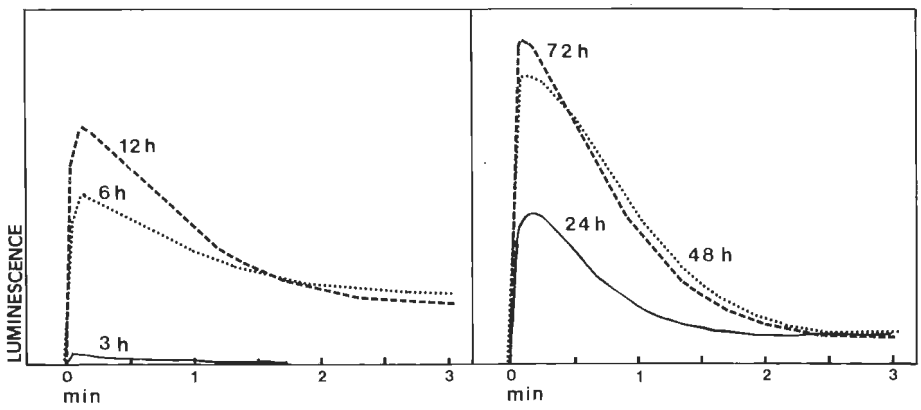

Fig. 4: Induction kinetics of luminescence (= delayed fluorescence) of primary leaves taken from 5-day-old etiolated *Hordeum* seedlings transferred into continuous white light. For explanation see Fig. 1.

Induktionskinetik der Lumineszenz (= verzögerte Fluoreszenz) von Primärblättern 5 d alter etiolierter *Hordeum*-Sämlinge. Erläuterung s. Fig. 1.

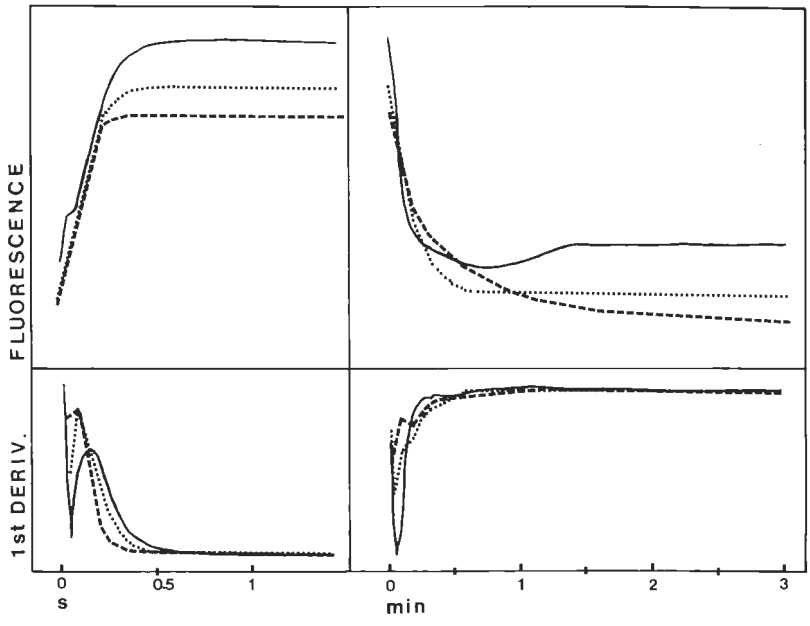

Fig. 5: Induction kinetics of the (prompt) chlorophyll fluorescence of primary leaves taken from *Hordeum* seedlings. For explanation see Fig. 2.

Induktionskinetik der (prompten) Chlorophyllfluoreszenz von Primärblättern etiolierter *Hordeum*-Sämlinge. Erläuterung s. Fig. 2.

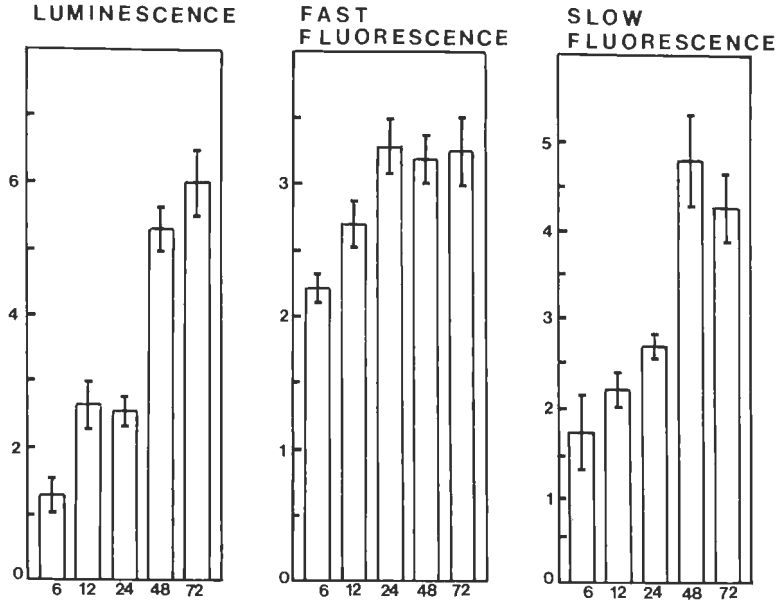

Fig. 6: Values for luminescence and fluorescence kinetics of *Hordeum* seedlings. For explanations see Fig. 1.

Werte der Lumineszenz- und Fluoreszenzkinetik von *Hordeum*-Sämlingen. Erläuterung s. Fig. 1.

The fast rise (< 1 s) from the O level to the kinetic maximum P is considered an indicator for the amount of reduced quencher molecules (DUYSENS and SWEERS 1963), which accumulate because the electron transport is not fully functionate.

Between the O and P level sometimes a small 'inflection' I and a subsequent 'dip' D is visible showing up as a sharp minimum in the first derivative.

The rise from O to I was related to the onset of the 'oxygen gush', the first short outburst of oxygen evolution (PAPAGEORGIOU 1975).

The decline between I and D was explained by an early PS I mediated reoxidation of reduced quencher molecules, due to the onset of the electron transport at the rate-limiting step between the plastoquinone-pool and the cytochrom b6/f-complex (SATOH and KATOH 1981). Later a PS I reaction, possibly the oxidation of NADPH in the Calvin cycle, is supposed to become rate-limiting for the induction and thus leads to the slower rise of the signal to the kinetic maximum P (MUNDAY and GOVINDJEE 1969), which is correlated to a decrease of the oxygen evolution at the end of the 'oxygen gush' (PAPAGEORGIOU 1975). The value for the relative fluorescence rise, sometimes termed 'variable fluorescence' (definition see 'Results') reflects the size of light-harvesting chlorophylls surrounding the reaction center of PS II. This can be taken as an indicator for the capacity for quencher reduction of PS II reactions (KITAJIMA and BUTLER 1975).

The rise to the maximum of the luminescence kinetic takes about 10 s and is much slower than that of fluorescence. According to MALKIN (1977) the kinetics of luminescence and fluorescence are parallel when measured simultaneously. In our case the

luminescence kinetics is probably slower because of the intermittent dark phase, which is absent in the case of the fluorescence measurement.

The luminescence signal is supposed to be proportional to the amount of activated reaction centers of PS II as it is considered to originate from the recombination of charge separation in the reaction centers of PS II (LAVOREL 1975). The fast rise to the kinetic maximum indicates the low rate of charge separation during the first seconds of induction. As our system detects within the range of 70 ms, the luminescence signal is determined by the redox reactions of the plastoquinone pool and by the decay of the pH gradient and the membrane potential correlated with photophosphorylation (LAVOREL 1975). Our kinetics show that activated reaction center chlorophylls are still accumulating when the fluorescence is already declining. Obviously Q oxidation (partially reflected as a fluorescence decline, see below) is functioning earlier than ATP consumption in the Calvin cycle which leaves the plant in a 'high energy state' with strong luminescence (ITOH *et al.* 1971). This high energy state contributes also to the decline of fluorescence (energy quenching, see below).

Slow kinetic decline: Subsequent to the rise both luminescence and fluorescence reach a steady state (T) within about 3 min. This decline has been explained mainly by three mechanisms:

a) The oxidation of the quencher Q, which proceeds faster when the following redox reactions of the electron transport chain and the CO_2 fixation become fully functional (DUYSENS and SWEERS 1963) or when oxygen acts as an electron acceptor (VIDAVER *et al.* 1981);

b) the 'energy quenching', which is described as energy consumption related to the chemiosmotic pH gradient (BRIANTAIS *et al.* 1979);

c) by the enhanced energy transfer to PS I which emits less fluorescence than PS II and which is termed state 1 — state 2 transition (CHOW *et al.* 1981).

The decline in the luminescence kinetics measured in the range of more than 1 ms is explained by an enhanced functioning of the electron transport chain on both sides of PS II (the plastoquinone-pool and the S-states of the water splitting enzyme), and by the concomittant onset of ATP consumption which decreases the high energy state (see above).

The luminescence declines somewhat later than fluorescence. This, too, may be due to the intermittent dark phases which lead to a slower kinetic rise (see above). The relative decline of luminescence (definition see 'Results') is much stronger than that of fluorescence indicating that the factors determining luminescence are much more out of balance than those controlling fluorescence.

During greening in the light the decline of the chlorophyll *a/b* ratio shows that the biosynthesis of *b* starts later than that of *a* (HENNINGSON and BOARDMAN 1973). The *a/b* ratio remains on a relatively high level presumably because of the further growth of the primary leaf, which then contains a high proportion of young leaf tissue.

At the very first second of illumination the accumulated protochlorophyllide is phototransformed into chlorophyllide, a process which can be followed directly due to changes in the fluorescence emission spectrum (BUSCHMANN and SIRONVAL 1984). When the pigment biosynthesis has accumulated sufficient chlorophylls, chlorophyll-protein complexes are formed as an integrated part of the thylakoid membrane. This is accompanied by changes in the fluorescence emission and excitation spectrum (BUSCHMANN 1981). PS I activity is established earlier than PS II activity (OELZE-KAROW and BUTLER 1971).

In the beginning of chlorophyll synthesis the reaction centers operate with small antenna units (PLESNICAR and BENDALL 1973). After about 1 d of growth in the light it contributes mainly to the enlarging of these units. Both luminescence and fluorescence

are indicators for the functioning of PS II (see above). During the greening process PS II activity accompanied by the oxygen evolution is increased. This is shown by the specific changes of relative fluorescence and luminscence.

During greening the values for the rate of fluorescence rise increase faster than those for fluorescence decline. This shows that the electron transport around the quencher Q is faster than the energy quenching processes and the state 1 — state 2 transition. According to POPOVIC *et al.* (1984) oxygen plays an important role as electron acceptor in the early stages of greening and by this mechanism photoinhibition should be prevented. During greening the relative luminscence decrease is further enhanced when fluorescence values have already reached their maxima. This indicates that the steady state balance of charge separation is established late. In fully green leaves the decrease of relative luminscence becomes much higher than that of relative fluorescence. This indicates that during the induction period the processes responsible for the luminescence decrease (charge separation controlled by the electron transport at the plastoquinon-pool, deactivation of the S-states of the water splitting enzyme and photophosphorylation) has to be enhanced much more than the process responsible for the fluorescence decrease. It can be concluded that the fluorescence decline is less influenced by the functioning of the electron transport than the fluorescence rise.

The comparison of the greening process of *Vitis* cuttings with that of *Hordeum* seedlings demonstrates that the succession of different developmental processes is essentially the same for both species. The very first steps of photosynthesis proceed faster in the leaves of *Hordeum* seedlings than in the leaves of *Vitis* cuttings. This is shown by the faster rise of both luminescence and fluorescence and by the only slightly visible intermediate I. The disappearing of the I-D dip during greening of *Hordeum* leaves may be explained by the decreasing proportion of PS I activity during leaf development (PLESNICAR and BENDALL 1973). Compared to *Vitis* cuttings the electron transport in *Hordeum* seedlings is diminished because the reaction centers are deactivated much slower which is indicated by the slower luminescence decrease. Fluorescence decrease, however, is faster for the *Hordeum* seedlings than for the *Vitis* cuttings. This leads to the assumption that state 1 — state 2 transitions and energy quenching are established earlier in *Hordeum* seedlings. The kinetic minimum found only in *Hordeum* seedlings illuminated for 6 and 24 h resembles the kinetic found during penetration of a PS II herbicide. At that time the electron transport seems not yet fully functioning at a site close to PS II. The shoulder following the fluorescence maximum P in *Hordeum* leaves illuminated for more than 24 h is an indicator of a transient block of Calvin cycle functions. Q is supposed to become more reduced because NADPH is less oxidized and the pH gradient should be decreased because of phosphorylation of ribulose monophosphate (WALKER *et al.* 1983).

During greening the kinetic maximum of the fluorescence may be increased — as in the case of *Vitis* — because of the higher capacity for quencher reactions. But — as in the case of *Hordeum* — it may also be decreased because of the accumulating chlorophyll. Possibly this enhances the reabsorption of chlorophyll fluorescence, decreases the proportion of the intensity of the excitation light per absorbing pigments and/or increases the oxidation rate of the quencher Q by increasing the amount of electron acceptors available, i. e. more electron transport chains and more CO_2 fixation capacity (KRAUSE and WEIS 1984). The O level shows the same tendency as the maximum. In the case of *Vitis* cuttings the O level increases during greening, which can be explained by the enlarging of the antenna. In the case of *Hordeum* seedlings the O level decreases during pigment accumulation because of the increased reabsorption of fluorescence. However, the excitation light could also be transferred more efficiently to the less fluorescing PS I.

Our study demonstrates that the interpretation of induction kinetics of luminescence and of fluorescence can be taken as a tool to characterize disorders mainly of the following photosynthetic activities:

a) The excitation and de-excitation of PS II reaction centers (luminescence),

b) the redox reaction of the quencher Q (fast fluorescence rise),

c) the energy state of the thylakoids, an expression of the pH and potential difference related to the photophosphorylation (luminescence and slow fluorescence decline).

d) the state 1 — state 2 transitions (slow fluorescence decline).

Summary

Kinetics of both delayed fluorescence (luminescence) and prompt fluorescence of chlorophyll are used to characterize photosynthetic activity in leaf tissues. In a first attempt the greening process in etiolated *Vitis* cuttings and *Hordeum* seedlings was studied. Data sampling and processing, including averaging of the kinetics, was carried out by means of a microcomputer. Parallel to the light-induced development of photosynthetic activity the kinetic changes (i. e. luminescence decrease, fluorescence rise and decrease) became more prominent and transitory secondary peaks appeared. For both plants the succession of the kinetic characteristics was the same; it only differed in the time of appearance. The kinetics are discussed according to interpretations given in literature. The method is proposed as a tool to quickly characterize certain damages of the photosynthetic system in different plants.

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