SO_2 Injury in Intact Leaves, as Detected by Chlorophyll Fluorescence

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The effects of short-time fumigation (0–60 min) of intact spinach leaves with SO_2 (2 ppm) on the photosynthetic apparatus were investigated. A rather high SO_2 concentration was applied to monitor immediate effects on the fluorescence behaviour with the influence of repair processes or secondary types of damage being minimized. Three different types of *in vivo* chlorophyll fluorescence measurements were used: Rapid induction kinetics (Kautsky effect), slow induction kinetics with repetitive application of saturation pulses (saturation pulse method), and decay kinetics following a single turnover saturating flash.

The slow induction kinetics with repetitive application of saturation pulses reacts in the most sensitive way indicating a primary damage at the level of the enzymatic reactions of the Calvin cycle.

It is suggested that stromal acidification upon SO_2 uptake interferes with light activation of Calvin cycle enzymes. With longer fumigation times also damage at the level of photosystem II becomes apparent: A decrease in variable fluorescence yield reflects a lowering of photosystem II quantum yield, and the slowing down of fluorescence relaxation kinetics reveals an effect on the secondary electron transport from Q_A to Q_B. The detrimental effects of SO_2 depend to a great extent on the application of light during fumigation. Besides a light requirement for SO_2 uptake by stomata opening also the possibility of photoinhibitory damage is discussed.

The susceptibility of leaves to photoinhibition may increase with a lowering of Calvin cycle activity by SO_2.

Introduction

During the course of their lives, plants are faced with natural as well as with artificial (*i.e.* man-made) stress factors, for example extreme temperatures, water and mineral deficiency, as well as herbicides and air pollutants, respectively [1]. SO_2 is a serious air pollutant, which is well known to cause visible damage (chlorosis and necrosis) and, at lower concentrations, a great number of physiological disturbances [2].

Chlorophyll fluorescence measurements have been successfully applied for assessment of the effects of physiological stress factors on plants [3, 4]. Advantages of fluorescence measurements are the immediateness and sensitivity of the recordings. Various types of fluorescence measurements can give specific information on possible sites of stress-induced damage, which is difficult to obtain by gas exchange photosynthesis measurements. The interpretation of the fluorescence data, however, is often complicated by the existence of several quenching mechanisms [5–7]. Recently, a measuring system was developed [8], which allows for continuous determination of photochemical (q_Q) and non-photochemical (q_E) quenching of fluorescence. This system is based on the selective recording of modulated fluorescence, which is not disturbed by so-called "saturation pulses", which are applied in order to determine the maximal fluorescence yield upon full reduction of photosystem (PS) II acceptors. Photochemical quenching, q_Q, is a measure for the rate of energy conversion at PS II reaction centers, with the primary plastoquinone acceptor Q_A in its oxidized form. Non-photochemical quenching, q_E, is primarily caused by energization of the thylakoid membrane [9, 10].

This paper deals with the question, in which way the chlorophyll fluorescence characteristics of green leaves are modified, when an increasing stress dosis is given by fumigation with SO_2. In the present study, on purpose a rather high concentration of SO_2 was applied, such that strong effects on the fluorescence behaviour were induced within one hour to minimize

Abbreviations: PS II, photosystem II; Q_A, primary plastoquinone electron acceptor of photosystem II; Q_B, secondary plastoquinone electron acceptor; q_Q, photochemical quenching; q_E, non-photochemical quenching; F_m, maximal fluorescence yield.

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possible interference of repair mechanisms and of secondary types of damage.

Materials and Methods

Plant material

Spinach plants (*Spinacia oleracea* cv. Yates hybrid) were grown in a greenhouse at a day/night cycle of 11/13 h, with 20/10 °C temperature, and a relative humidity of 20–30%.

Fumigation with SO_2

For fumigation, detached leaves were used. The leaf-stalks were cut again under water to maintain transpiration and were kept in little flasks filled with water. These flasks were sealed with terostate to make sure that SO_2 could only be taken up by the stomates and could not be solved in the water. Then the leaves were fumigated with 2 ppm SO_2 in a cuvette with a volume of 0.4×10^{-3} m^3 at room temperature (18–22 °C) and a light intensity of 90 W m^{-2}.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured with whole leaves, dark-adapted for 30 min before measurements. Measurements were carried out with a modulation fluorometer (PAM Chlorophyll Fluorometer, Walz, Effeltrich). Three different types of operation were used:

Rapid induction kinetics

For recording fluorescence induction curves (Kautsky effect) the dark-fluorescence level, F_0, was monitored at low modulation frequency (1.6 kHz); then the actinic light (20 W m^{-2}; 650 nm) was switched on for 3 sec, for which time the modulation frequency was automatically increased to 100 kHz (PAM 102, Walz). The induction curves were recorded on a storage oscilloscope (Nicolet 527), from where they were plotted by chart recorder (Philips PM 8252 A).

Slow induction kinetics with repetitive application of saturation pulses

The actinic light had an intensity of 20 W m^{-2}. Every 30 sec, a saturating light pulse (1.15×10^3 W m^{-2}) of 700 ms duration was applied. The resulting curves were recorded with a chart recorder; from these traces q_Q and q_E were calculated as described previously [8].

Decay kinetics following a single turnover saturating flash

Single turnover saturating flashes were applied with a miniature flash-lamp (XST 103, Walz). Special trigger and gating circuitry, provided with the fluorometer (PAM 103, Walz), caused automatic switching to 100 kHz modulation frequency for recording of rapid decay kinetics and protected the amplifier system from oversaturation during the flash. Curves were recorded on a storage oscilloscope, from where they were plotted by chart recorder. Half-decay times were determined for that part of the decay which reached a *quasi*-steady state within 3 ms.

Results

Rapid induction kinetics (Kautsky effect)

Fig. 1 shows the effect of SO_2 fumigation on the rapid induction kinetics of chlorophyll fluorescence in spinach leaves upon illumination with continuous light. The characteristic fluorescence transients are denoted in the control curve (0 min): When the measuring beam is switched on, the dark-fluorescence level, F_0, is monitored; at this state, all primary acceptors of PS II are oxidized. Upon onset of actinic light, fluorescence rises from F_0 through an intermediate step, I-D, to its peak level, P, from where it declines towards a steady state level (not shown).

With continuous fumigation of the leaves with SO_2, the following characteristic changes of the fluorescence curves are observed: The I-level rises, the I-D-decline becomes somewhat more pronounced, the D-P-rise is slowed down, and the decline from P is retarded. After 60 min SO_2 there is also some increase of the F_0-level. Similar results were previously reported by Shimazaki *et al.* [11].

Slow induction kinetics with repetitive application of saturation pulses

Fig. 2 shows original traces of the slow induction kinetics with saturation pulses given every 30 sec. In Fig. 3 the resulting kinetics of the quenching coefficients q_Q and q_E are depicted. The typical behaviour

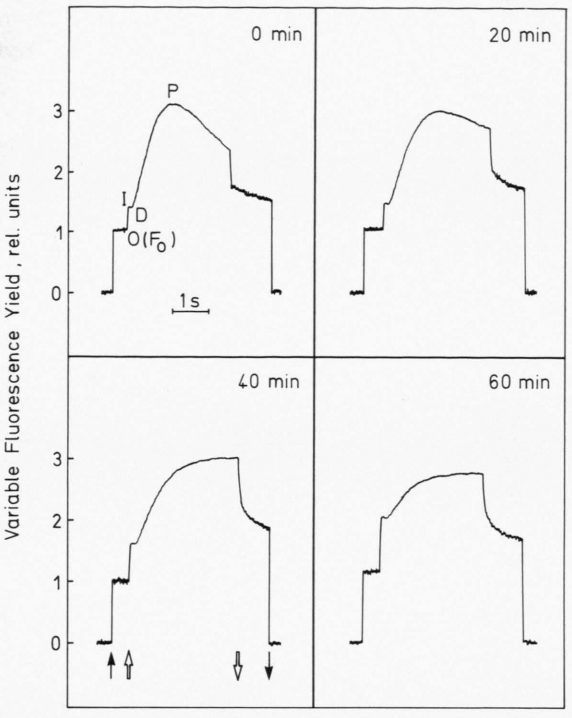

Fig. 1. Fluorescence induction curves of spinach leaves after SO_2 fumigation. The leaves were fumigated with 2 ppm SO_2 for 0, 20, 40, and 60 min in the light (90 W m^{-2}) and were dark-adapted for 30 min before measurements. Characteristic fluorescence levels: O (F_o): Dark-level fluorescence; I: Intermediate level; D: Dip; P: Peak level.
↑: Measuring beam on; ↓: Measuring beam off.
⇑: Actinic light on; ⇓: Actinic light off.

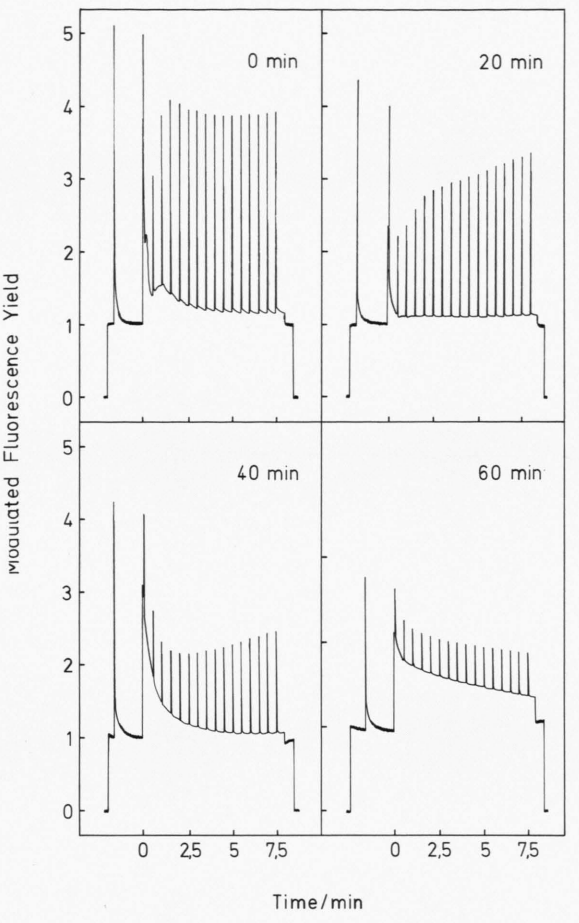

Fig. 2. Slow induction kinetics with repetitive application of saturation pulses after SO_2 fumigation (2 ppm). For explanation of experimental conditions see Materials and Methods.

of the control leaves is apparent from the 0 min curves of Fig. 2 and 3: When the light is switched on (actinic light and saturation pulses), fluorescence rises to its maximum level and then decays (through some intermediate levels) to a steady state value. The corresponding kinetics of q_Q show that there is first a fast reduction of the electron transport chain (q_Q decreases from 1 to 0.35) followed by a slower reoxidation (q_Q eventually increases to 0.93). Non-photochemical quenching, q_E, first increases from 0 to 0.56 (energization of the membrane) and then decreases again to a steady state value of 0.3 (relaxation of energization by consumption of energy in the Calvin cycle) (see also ref. [8]).

This pattern is strongly modified by SO_2 fumigation. Already after 20 min of SO_2 fumigation, two effects can be detected: On the one hand there is a decrease of the maximal fluorescence intensity and on the other hand relaxation of membrane energization is delayed, such that a steady state is not reached within the measuring time. Hence, the onset of Calvin cycle activity is already affected. However, this does not yet cause a lowering of electron transport rate, as q_Q behaves much like in the control.

After 40 min fumigation, electron transport rate is lowered. Reoxidation of Q_A^-, following initial reduction, is distinctly slowed down, and q_Q increases very slowly to 0.88 only.

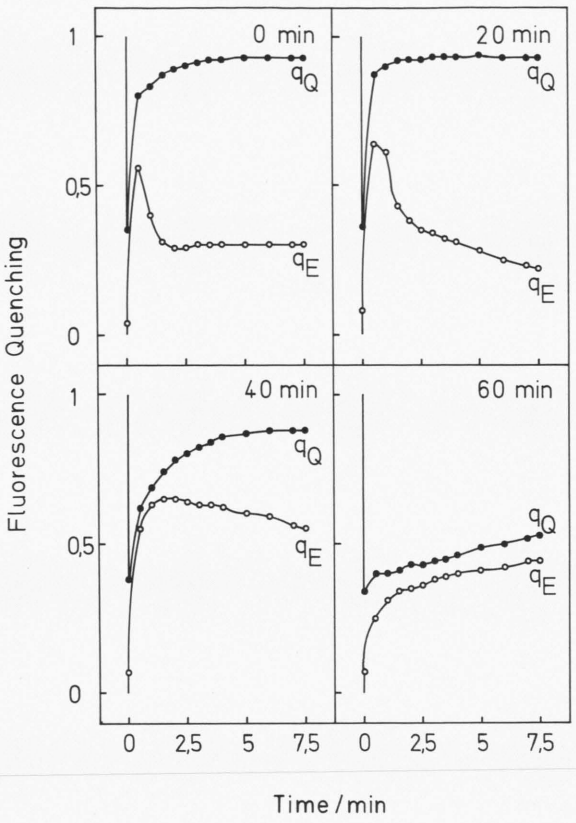

Fig. 3. Induction kinetics of q_Q and q_E (derived from the curves in Fig. 2). For experimental conditions see Materials and Methods.

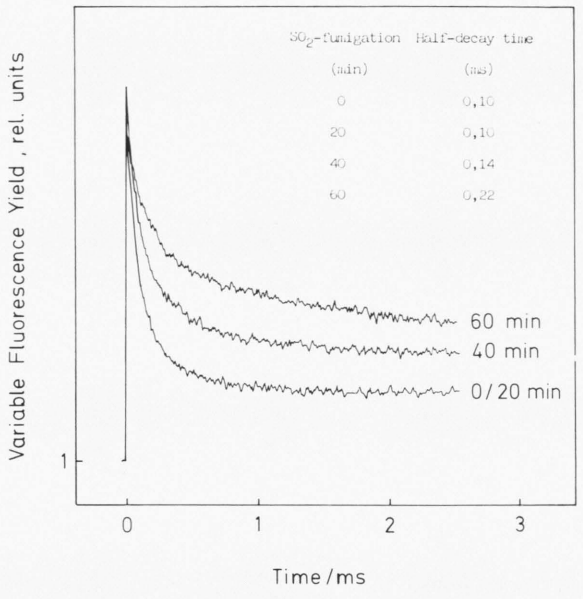

Fig. 4. Fluorescence decay kinetics following a single turnover saturating flash. Modification of the kinetics by SO_2 fumigation (2 ppm) for 0, 20, 40, and 60 min. Time 0 denotes the moment when photodetector-gating is inactivated, which occurs about 100 μsec following the flash. The half-decay time corresponds to the time required for fluorescence to decline 50% between its maximum at time 0 and its minimum at 3 ms. For other experimental conditions see Materials and Methods.

After 60 min there is only weak reoxidation of Q_A, reduced during the first seconds of illumination (q_Q increases from 0.34 to 0.53), and relaxation of membrane energization does not occur anymore. Actually, this behaviour reflects strong inhibition of Calvin cycle and electron transport activity similar to the behaviour observed after application of heat and water stress or of the ATPase inhibitor tentoxin [12].

Decay kinetics following single turnover saturating flash

Upon application of a single turnover saturating flash, a single charge separation is induced at every PS II reaction center. With the rapid modulation fluorometer it is possible to monitor the re-oxidation kinetics of Q_A^-, from where the electron is transfered to the two-electron gate Q_B [13].

Fig. 4 shows original recordings of fluorescence decay kinetics following a single turnover flash in spinach leaves, which were fumigated with 2 ppm SO_2 for up to 60 min. The half-decay times are shown in the inset of the figure: After 20 min, the half-decay time is not yet affected. It increases from 0.1 to 0.14 ms with 40 min fumigation and to 0.22 ms with 60 min fumigation.

Hence, it may be concluded that the fumigation with SO_2 has caused also inhibition of the electron transfer step between Q_A and Q_B.

Discussion

In this study three different types of *in vivo* chlorophyll fluorescence measurements were used to detect effects of a short-time fumigation of intact spinach leaves with a relatively high level of SO_2. With this approach one may expect to monitor the immediate effect of this toxic gas on the photosynthetic apparatus, with the overlapping influences

of repair processes and secondary types of damage, which certainly play a role under natural conditions, being minimized. The different fluorescence methods give information on different parts of the photosynthetic apparatus. The decay kinetics following a single turnover saturating flash (Fig. 4) very specifically reflect the primary electron transfer step at PS II reaction centers, while the induction kinetics in continuous light (Fig. 1) more generally are effected by the intersystem electron transport. The saturation pulse method (Figs. 2 and 3) gives information on both, electron flow rate and energy status of the thylakoid membrane, as controlled by the performance of the Calvin cycle.

The presented data suggest that the parameter reacting in the most sensitive way to SO_2 fumigation is the fluorescence induction pattern of fluorescence with repetitive application of saturation pulses. This pattern was already markedly changed after 20 min fumigation (Fig. 2) when the rapid induction kinetics (Figs. 1 and 4) were hardly affected. Actually, the invariance of q_Q with 20 min fumigation (Fig. 3) also indicates that electron transport rate as such is not yet disturbed. As has been previously suggested for the case of other stress factors [12] it appears that the changed saturation pulse induction pattern indicates an effect of SO_2 on the activation of Calvin cycle (*i.e.* the carboxylation reaction) while electron flow to molecular oxygen (Mehler reaction or photorespiration) may account for the invaried rates of charge separation at PS II. In addition, however, it is also apparent that the maximal fluorescence yield, F_m, observed with a saturation pulse, is already somewhat lowered after 20 min fumigation. Hence, there seems to be some loss in PS II quantum yield which tends to favour q_Q [14]. This loss in PS II quantum yield becomes very pronounced with 60 min fumigation.

One mode of SO_2 action undoubtedly is related to its acidifying properties [15–18]: The main diffusion barrier for gases into a leaf is formed by the stomates (apart from the cuticle); once inside the leaf, SO_2 reacts immediately (because of its high solubility) with the aquaeous phase, and the resulting ions are distributed according to a pH-dependent equilibrium:

$$SO_2 + H_2O \rightleftharpoons (SO_2 \times H_2O) \rightleftharpoons HSO_3^- + H^+ \rightleftharpoons SO_3^{--} + 2H^+.$$

Hence, SO_2 leads to an acidification, which is the stronger the higher the pH value in the respective cell compartment. The most alkaline compartment in a plant cell is the stroma of an illuminated chloroplast; it will therefore be acidified most by SO_2. As chloroplast stroma enzymes are characterized by a sharp pH optimum, this acidification leads to an inhibition of enzyme function. In particular, acidification may prevent light activation of Calvin cycle enzymes, while the major thylakoid functions, primary reactions, electron and ion transport are still intact [19].

Sulfur dioxide acts not only as an acidic gas, but it can also cause conformational changes in proteins, *e.g.* membrane-bound or soluble enzymes (as a review see [20]). At present it is not possible to differentiate between these two modes of action.

An additional aspect is brought into the discussion by the fact that the effects of SO_2 fumigation, as described above, depended to a large degree on the simultaneous application of light. When the same treatment was carried out in darkness, the effects were much less pronounced (not shown). This finding may be in part accounted for by the light requirement for stomata opening. However, one has to consider the possibility of photoinhibition as well, although the applied light intensity of 90 W m^{-2} was relatively low. It is well known that a light intensity, which is without harm when other conditions are favorable, may become photoinhibitory, when the photosynthetic apparatus is affected by stress parameters. One may suspect that the primary damage by SO_2 at the level of Calvin cycle activation will induce an increased susceptibility to photoinhibition. Actually, the lowering of variable fluorescence yield (most pronounced after 60 min fumigation) and the slowing down of the Q_A reoxidation kinetics following a single turnover flash, would be in agreement with similar effects observed with photoinhibitory treatment alone [21, 22].

In conclusion, the presented results show that various types of chlorophyll fluorescence measurements, which can be carried out with a readily available, portable instrument, provide rapid and detailed information of the progressive damage of the photosynthetic apparatus by SO_2 fumigation in the light. A primary damage at the level of the dark enzymatic reactions of the Calvin cycle may be separated from later damage in the vicinity of PS II. At present, it is not clear to what extent this later damage is caused by photoinhibition. This point has to be clarified in

future work. Also, after the phenomenology of SO_2-induced changes of the fluorescence behaviour has been investigated by short-term fumigation at relatively high SO_2-concentration, it will be important to extend these measurements to longer incubation times at SO_2-levels which are more close to those observed in nature with man-made air pollution.

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