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PHOTOSYNTHESIS AND HIGH LIGHT STRESS*

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Summary. Exposure of plants to irradiances far above the light saturation point of photosynthesis, known as high-light stress, induces various responses including light adaptation of the photosynthetic apparatus and chloroplast ultrastructure by formation of sun-type chloroplasts. The latter possess a lower cross section for light absorption (less light-harvesting chlorophyll proteins) and higher rates of photosynthetic quantum conversion than shade-type chloroplasts. De-epoxidation of violaxanthin to zeaxanthin, increase in heat emission, rise of non-photochemical de-excitation of absorbed light quanta (qN) and photoinhibition of the photosynthetic pigment apparatus are further high-light stress responses.

The degree of photoinhibition can clearly be determined via measurements of the chlorophyll fluorescence relaxation kinetics and depends on the photon flux density of the high-light stress as is shown here with soybean leaves. A decrease in photochemical quenching qP and variable chlorophyll fluorescence ratios F_v'/F_m' or $\Delta F/F_m'$, and an increase of non-photochemical quenching qN are not yet an indication of a photoinhibition. One always has to determine the photoinhibitory quenching coefficient qI which is one of three qN components. However, in soybean leaves a photoinhibition (increase in qI) of the chloroplasts, as measured at the adaxial light exposed upper leaf side, did not affect the photosynthetic CO_2 assimilation rates (P_N) of the whole leaf. Thus, chlorophyll fluorescence measurements, usually carried out with low irradiance light, taken at the upper leaf-side alone are not representative for the physiological situation of the whole leaf. Chlorophyll fluorescence signatures have to be determined as well from the abaxial lower leaf side in order to correctly judge the physiological state of the leaf.

* This paper is dedicated to the 70-th anniversary of Prof. Dr. Sci. Ivan Yordanov.

Key words: Chlorophyll fluorescence, light adaptation, non-photochemical quenching, photoinhibition, sun-type chloroplast

Abbreviations: Chl – chlorophyll; F_v and F_m – variable and maximum Chl fluorescence of PSII in the dark adapted state; F'_v and F'_m – variable and maximum Chl fluorescence in the light adapted state; F_d – Chl fluorescence decrease from F_m to F_s at continuous illumination, F_o – ground value of Chl fluorescence; F_s – steady state Chl fluorescence 5 min after onset of illumination, P_N – photosynthetic net CO_2-assimilation; PPFD – photosynthetic photon flux density; PSII – photosystem II; qN – non-photochemical quenching coefficient of chlorophyll fluorescence; qP – photochemical quenching coefficient of Chl fluorescence; qI – non-photochemical quenching due to photoinhibition of photosystem 2; qT – non photochemical quenching due to state transitions; qE – non-photochemical quenching due to the build-up of a pH-gradient.

Introduction

The stress concept

Terrestrial plants are exposed to many kinds of natural stressors, such as water stress, heat and high-light stress or anthropogenic stresses, such as air pollution (exposure to SO_2, NO_X, O_3), acid rain, and acid morning dew (as reviewed by Lichtenthaler, 1996). Plants respond to these day-to-day or long-term stress exposures by particular stress-induced responses and stress coping mechanisms as well as non-specific stress responses which efficiently help plants to survive and which have been joined in a unifying stress concept for plants (Lichtenthaler, 1996, 1998). Within this concept four stages of plants in response to stress can be differentiated. During stress exposure they are: 1) the *response phase* (alarm reaction), 2) the *stage of resistance* and 3) the *stage of exhaustion* mostly followed by 4) the *regeneration phase*, in which the physiological function is gradually restored once the stressor is removed. The length (hours, days) of the regeneration phase to take the plant back to a normal physiological function depends on the strength, duration and type of stress.

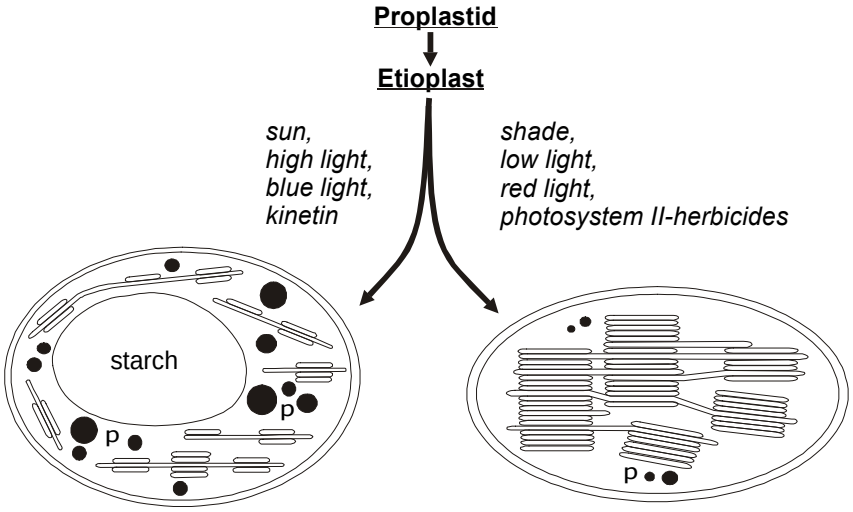
Adaptation of chloroplasts

All plant stressors will either directly or indirectly affect the photosynthetic apparatus and its function. There exist long-term adaptations of the Chl and carotenoid content and chloroplast structure (within a few days), e. g. the formation of high-light adapted sun chloroplasts (Lichtenthaler, 1981; Lichtenthaler et al., 1981, 1982a,b, 1984; Meier and Lichtenthaler, 1981; Wild et al., 1981, 1986). Moreover, there are

short-term declines in photosynthetic function (within minutes or hours), e. g. induced by closure of stomata due to temperature and water stress (e. g. Schindler and Lichtenthaler, 1996), or by high irradiance-induced impediment of photosynthetic quantum conversion and electron transport, e. g. photoinhibition (see below) as seen in the non-invasive method of Chl fluorescence measurements (Lichtenthaler et al., 1986; Lichtenthaler and Rinderle, 1988; Lichtenthaler, 1988, 1992; Krause and Weis, 1991). Long-term high-light stress, often associated with water and temperature stress, leads to a decline of the chlorophyll content and thylakoid frequency of resulting in the formation of sun-type chloroplasts that possess much less LHC-II (LHCPs) and a much higher capacity for photosynthetic quantum conversion, electron transport, and CO_2 assimilation (Fig. 1) than shade-type chloroplasts (Meier and Lichtenthaler, 1981; Lichtenthaler et al., 1981, 1982, 1984). These sun-type chloroplasts with lower amounts of high-harvesting pigments are better suited for high quanta fluence rates, and their high rates of photosynthetic quantum conversion will help the plant to avoid damage or photoinhibition by high-light stress. Yet, at dry and sunny periods the high-light adaptation response of chloroplast structure and function is often not sufficient enough to protect the photosynthetic apparatus during mid-day hours against partial and temporal photoinhibition of the chloroplasts, especially in the upper layer of the sun-exposed palisade parenchyma cells (e. g. Barber and Anderson, 1992; Lichtenthaler et al., 1992; Schindler and Lichtenthaler, 1996).

Photoinhibition of the photosynthetic apparatus

A decline in photosynthetic quantum conversion is usually measured by either a decline in the values of the Chl fluorescence ratio F_v/F_m measured in the non-functional, dark-adapted state 1 of the photosynthetic apparatus which represents the maximal photochemical yield of PSII when all reaction centres are open. An equivalent ratio can also be determined in the illuminated state of the photosynthetic apparatus as ratio F_v'/F_m'. A related Chl fluorescence ratio is the actual quantum efficiency of PS II in the illuminated state $\Delta F/F_m'$ (Genty et al., 1989). A decline in photosynthetic function can also be checked via the variable Chl fluorescence decrease ratio (Rfd-ratio = F_d/F_s) which is a more useful and precise value as it also includes the functional, light-adapted state 2 of the photosynthetic apparatus (cf. Lichtenthaler and Rinderle, 1988; Lichtenthaler and Miehé, 1997). A decline in the ratios F_v/F_m, F_v'/F_m', $\Delta F/F_m'$ or F_d/F_s (Rfd-values) does not necessarily mean that the photosynthetic apparatus is photoinhibited as, besides photoinhibition, there exist several other forms of non-photochemical de-excitation of absorbed light quanta and particular Chl fluorescence quenching mechanisms (e. g. heat emission, state 1/state 2 transitions, formation of a pH-gradient) causing a decline in F_v/F_m, Rfd-values and other Chl fluorescence ratios. Photoinhibition causes a breakdown of the D1-protein of PS II and requires a regeneration of several hours or more (Krause and Weis, 1991; Barber and Anderson, 1992).



Sun-type Chloroplast
(high-light chloroplast)

1. low amounts of thylakoids per chloroplast section
2. narrow grana stacks
3. few thylakoids per granum
4. low stacking degree
5. more exposed chloroplast membranes
6. higher proportion of Chl *a*-proteins CPI and CPIa
7. higher Chl *a/b* ratios: 2.9 - 3.4
8. large starch grains
9. many and large plastoglobuli
10. lower chlorophyll content per chloroplast
11. high rates of net CO_2 assimilation P_N
12. light saturation of P_N at a high photon flux density

Shade-type Chloroplast
(low-light chloroplast)

1. high thylakoid frequency per chloroplast section
2. broad grana stacks
3. very high grana stacks
4. high stacking degree
5. more appressed thylakoids
6. high amounts of light-harvesting Chl *a/b*-proteins LHCII (LHCPs)
7. low Chl *a/b* ratios: 2.4 - 2.7
8. no starch
9. few small plastoglobuli
10. more total chlorophyll per chloroplast
11. low rates of net CO_2 assimilation P_N
12. early light saturation of P_N

Fig. 1. Development of chloroplasts from proplastids via etioplasts to either sun-type or shade-type chloroplasts depending on light quality and light quantity. The general adaptation response of chloroplasts to high-light and low-light growth conditions can also be induced at medium irradiances by endogenous compounds such as cytokinins (kinetin) or by photosystem II herbicides (diuron, bentazon, atrazin). The sun-type chloroplasts possess an ultrastructure, pigment composition and photosynthetic rates which are quite different from those of shade-type chloroplasts. The chloroplast adaptation scheme presented here is based on the results of Lichtenthaler (1981 and 1984), Lichtenthaler et al. (1981, 1982a,b, 1984), Meier and Lichtenthaler (1981).

It can be differentiated from other non-photochemical Chl fluorescence-quenching processes by measuring the relaxation kinetics of Chl fluorescence in a dark period following high-light treatment (Demmig and Winter, 1988; Quick and Stitt, 1989; Walters and Horton, 1991; Burkart, 1994). Here we compare the influence of the photon flux density of a high-light stress exposure on the degree of photoinhibition in green fully developed leaves of soybean as measured via Chl fluorescence and by net CO_2-fixation rates.

Material and Methods

Plants

Soybean plants (*Glycine max* L.) were grown in the greenhouse of the Botanical Garden for 2–3 weeks at a PPFD of 300–400 $\mu mol.m^{-2}.s^{-1}$ at 22 °C and a relative humidity of 60 ± 10%. For the experiments fully differentiated green leaves were taken.

Pigment determination

Chlorophylls *a* and *b* and total carotenoids (x+c) were determined in the same leaf pigment extract in 100% acetone using the redetermined extinction coefficients and equations established by Lichtenthaler (1987).

Chlorophyll fluorescence parameters

The Chl fluorescence decrease ratio $Rfd = F_d/F_s$ were measured of dark-adapted leaves using the LITWAF fluorometer (Lichtenthaler and Rinderle, 1988; Lichtenthaler and Miehé, 1997). The Chl fluorescence measurements were performed using a PAM-fluorometer (Walz, Effeltrich, Germany) as described by Schreiber et al. (1986), Schindler and Lichtenthaler (1996) and Burkart (1994). Modulated red light (0.1 $\mu mol.m^{-2}.s^{-1}$) was applied to determine F_o. The continuous red actinic light amounted to 300 $\mu mol.m^{-2}.s^{-1}$ and the white saturating light pulsed (1s) to 3000 $\mu mol.m^{-2}.s^{-1}$. These kinetics allowed to determine the maximal photochemical yield of PS II in the illuminated state (F'_v/F'_m), the actual quantum efficiency of PS II ($\Delta F/F'_m$) as well as the photochemical (qP) and non-photochemical (qN) quenching coefficients. These Chl fluorescence parameters were determined according to Schreiber et al. (1986) and van Kooten and Snel (1990). Assuming that qN consists of the components qE + qT + qI, the individual components of the non-photochemical quenching qN were calculated from Chl fluorescence relaxation kinetics at room temperature in the dark phase (subsequent to the high light exposure) using saturating light pulses (cf. Walters and Horton, 1991) and are presented as percentages of total qN.

These three components of non-photochemical Chl fluorescence quenching can be differentiated as they possess differential relaxation times. The fastest relaxing component qE (2–4 min) is related to the development of the pH-gradient ΔpH in the thylakoid lumen. As it shows up at high irradiance conditions above the light saturation point of photosynthetic CO_2 assimilation, it has also been termed "high energy quenching" coefficient qE. qT as the medium fast relaxing component (ca. 10–20 min) describes the "state transitions" of the two photosystems, whereas the slow relaxing component qI (> 40 min) indicates the degree of photoinhibition of PS II.

The Chl fluorescence kinetics were measured simultaneously with the CO_2-assimilation rates by placing the glass fiber fluorescence detection arm of the PAM fluorometer on top of the gas exchange cuvette containing the attached trifoliate leaflets.

Gas exchange measurements

Net CO_2 and transpiration measurements were performed with a CO_2/H_2O-porometer (Walz, Effeltrich, Germany) and used for the calculation of stomatal conductance (gH_2O values), see also Schindler and Lichtenthaler (1996) and Ball (1987). White light of different PPFDs was provided by a slide projector.

High light treatment

The leaves of soybean plants (*Glycine max*) were illuminated for 25 min at different PPFDs up to 2000 µmol.m^{-2}.s^{-1} with continuous white light from a slide projector. An infra-red filter and a ventilator were used to exclude infra-red radiation and heating up of the leaf. The measurement of one relaxation kinetic took at least half a day.

Repetition of experiments

The values shown in the figures represent the values from one kinetic measurement. Repetition with different leaves resulted in slightly different absolute values but showed the same trends and results.

Results and Discussion

Fully developed green leaves of three-week-old soybean plants grown in the green house at a PPFD of 200 µmol.m^{-2}.s^{-1} showed a Chl *a*+*b* content of 34 ± 3 µg.cm^{-2} leaf area and a total carotenoid (x+c) level of 8.3 ± 0.6 µg.cm^{-2}. The pigment ratio for Chl *a/b* amounted to 3.1 (± 0.1), and the ratio Chls/carotenoids (*a*+*b*)/(x+c) to 4.9 (± 0.2). The leaves thus possessed the regular pigment composition and ratios as found in photosynthetically active leaves of other plants (Lichtenthaler et al., 1992; Babani and Lichtenthaler, 1996; Schindler and Lichtenthaler, 1996).

The leaves' photosynthetic apparatus of the well-watered soybean plants was functionally fully intact. This was seen in normal values of the different Chl fluorescence parameters, such as photochemical quenching qP of 0.85 to 9, the maximum photochemical yield of PS II in the illuminated state F_v'/F_m' of 0.76 to 0.78, and also in the dark F_v/F_m of 0.80 to 0.85, the actual quantum efficiency of PS II $\Delta F/F_m'$ of 0.72 to 0.76, as well as in the variable Chl fluorescence decrease ratio with Rfd-values of 2.6 to 3.1. The non-photochemical Chl fluorescence quenching coefficient qN exhibited fairly low values of 0.2 to 0.3 which are found at this rate in fully functional, non-stressed leaves of green plants under these measuring conditions. Also, the photosynthetic CO_2 assimilation rates P_N measured at a PPFD of 1000 $\mu mol.m^{-2}.s^{-1}$ showed good values of 12 to 14 $\mu mol.m^{-2}.s^{-1}$.

Chlorophyll fluorescence measurements

In order to determine the dependence of the non-photochemical quenching coefficient qN on the incident light, we determined its rise with increasing irradiance of white light (duration 25 min) and also checked by dark relaxation kinetics the individual components of qN, such as the high energy quench qE, the photoinhibitory quench qI, and the quench component qT related to state transitions of the photosynthetic apparatus. The irradiance tested ranged from 10 to 2000 $\mu mol.m^{-2}.s^{-1}$.

The non-photochemical Chl fluorescence quenching qN increased 8 times, from 10 to 2000 $\mu mol.m^{-2}.s^{-1}$, whereas the photochemical quenching qP declined by 61%, and the maximum photochemical yield of PS II in the illuminated state F_v'/F_m' decreased by 39% and the actual quantum efficiency $\Delta F/F_m'$ by 76% (Fig. 2A). The decrease in qP, F_v'/F_m' and $\Delta F/F_m'$ as well as the increase in qN are usually taken as indicators that the photosynthetic apparatus, its quantum conversion and electron transport are partially blocked (Lichtenthaler and Rinderle, 1988; Krause and Weis, 1991), and this should result in a decrease of the photosynthetic rates P_N.

When differentiating between the three components of qN, one recognizes that, at a low PPFD of only 10 $\mu mol.m^{-2}.s^{-1}$, the state transitional quenching component qT makes up 67% of the total qN, whereas the high energy quench qE amounts only to 6% of qN (Fig. 2B). It is amazing that under these low-light conditions the photoinhibitory component qI contributed already 27% to the overall non-photochemical quenching qN. It may be questioned whether the saturating light pulses given during the relaxation measurements prevent a full recovery of the signal (cf. Quick and Stitt, 1988; Burkart, 1997). With increasing irradiance the component qT declined to ca. 5% of qN at 2000 $\mu mol.m^{-2}.s^{-1}$ indicating that, at higher irradiances above the light saturation of photosynthesis, the state transitions only play a very minor role in non-photochemical quenching of Chl fluorescence as changes in excitation energy distribution between the photosystem already occur at lower irradiances. The components qI and qE, in turn, increased with increasing PPFD, and at 2000 $\mu mol.m^{-2}.s^{-1}$ qI amounted to 52% and qE to 43% of the overall qN, respectively (Fig. 2B).

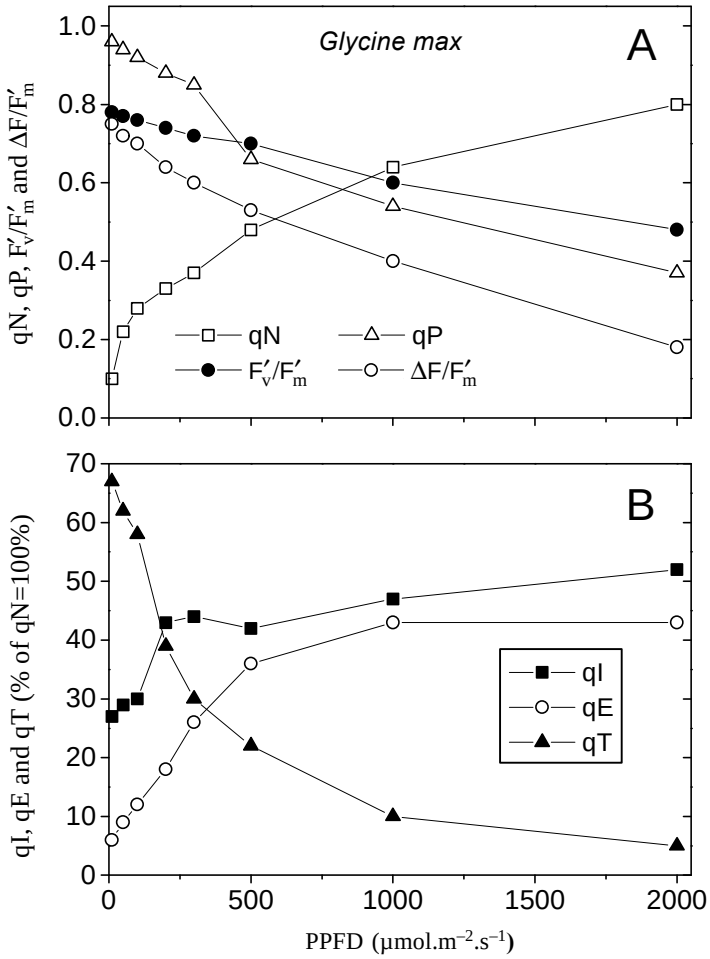


Fig. 2. Changes in chlorophyll fluorescence parameters and ratios during a 25 min illumination of soybean leaves at increasing irradiances (PPFD) up to 2000 µmol.m^{-2}.s^{-1}. **A)** Decline of photochemical Chl fluorescence quenching qP, the variable fluorescence of PS II in the illuminated state, F'_v/F'_m and the actual quantum efficiency of PSII, $\Delta F/F'_m$, as well as increase of non-photochemical Chl fluorescence quenching qN. **B)** Changes in the percentage of the individual components of the non-photochemical Chl fluorescence quenching qN with increasing irradiance: increase of photoinhibitory quenching (qI) and the quenching related to the build-up of a pH gradient (qE) and decline of the state transition quenching component qT.

The increase in qI indicating a progressing inhibition of the photosynthetic apparatus of soybean leaves was anticipated. The strong rise in the high energy quenching qE, indicating a progressing acidification of the thylakoid lumen, demonstrated that

in soybean also the latter process plays an essential role in the protection of the photosynthetic pigment and electron transport system against excessive high-light conditions. This acidification of the thylakoid lumen also activates the de-epoxidase as the essential enzyme of the high-light driven xanthophyll cycle which reduces violaxanthin via antheraxanthin to zeaxanthin. The progressing zeaxanthin accumulation in thylakoids with increasing PPFD is well known and had been documented in various plants (Demmig-Adams and Adams, 1992; Lichtenthaler et al., 1992; Schindler and Lichtenthaler, 1994, 1996). Zeaxanthin apparently separates the light-harvesting Chl-carotenoid proteins (LHC-II) from the reaction centre of PS II, thus reducing the excitation cross section of PS II, and in this way it acts in the photoprotection of the PSII against photoinhibition and photooxidation (cf. Ruban et al., 1992; Gilmore and Yamamoto, 1993; Walters and Horton, 1993; Lichtenthaler and Schindler, 1994). It is also possible that zeaxanthin reacts in a non-enzymatic reaction with highly reactive oxygen species and thus protects the photosynthetic apparatus against photooxidative damage, a hypothesis proposed by Schindler and Lichtenthaler (1997). Zeaxanthin accumulation at high-light conditions has not been studied in soybean but seems to play a major role in the photoprotection of PS II in soybean plants. The acidification of the thylakoid lumen, as the prerequisite for zeaxanthin formation, is well documented in the rising percentage of qE shown in figure 2B.

These results also demonstrate that a decline in F'_v/F'_m, $\Delta F/F'_m$ and qP, and the rise in qN with increasing PPFD is not only caused by a partial photoinhibition of the photosynthetic apparatus. In the case of soybean leaves it is also due to a strong increase in the high energy quenching component qE. The component qT at 2000 $\mu mol.m^{-2}.s^{-1}$ is relatively small, it may be an indication that state transitions of the photosystems also occur at a high PPFD. One should consider that these might be correlated to some extent to an adaptation response of the "medium-light" chloroplasts (medium-light growth conditions) to the high-light situation. Horton and Hague (1988) also showed that qI decreased with an increase in PPFD, whereas Quick and Stitt (1988) did not detect an increase of qT above a PPFD of 200 $\mu mol.m^{-2}.s^{-1}$.

Gas exchange measurements

When measuring the photosynthetic net CO_2 assimilation rates P_N and the stomata conductance (gH_2O) of attached soybean leaves in dependence of the applied irradiance, we found that, with progressing opening of the stomata as a response to the increasing PPFD, the net CO_2 assimilation increased. This was shown by a clear linear correlation of P_N to gH_2O (Fig. 3A), cf. also Ball et al. (1987) concerning this aspect. The net CO_2 assimilation rates P_N, measured after a 25 min light exposure, showed a typical light saturation curve (Fig. 3B). Light saturation of photosynthetic CO_2 assimilation was obtained at a PPFD of ca. 1000 $\mu mol.m^{-2}.s^{-1}$ and a gH_2O value of 245. It is amazing that the soybean plants grown at a medium PPFD of 300 to 400 $\mu mol.m^{-2}.s^{-1}$

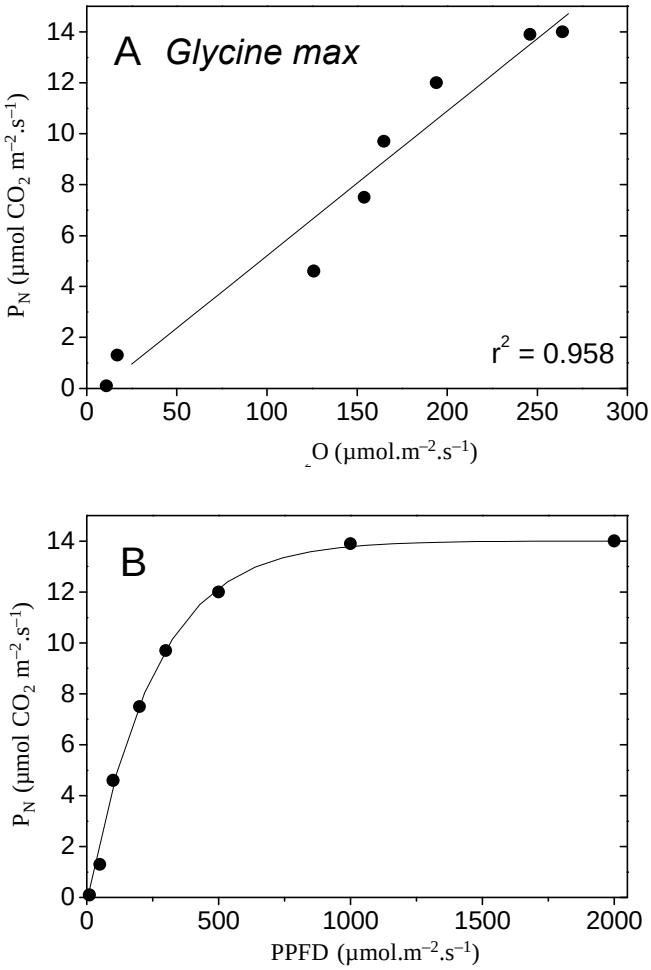


Fig. 3. Correlation of net CO_2 assimilation rates P_N **A)** with stomatal conductance (gH_2O-values) and **B)** with irradiance (PPFD) in soybean leaves measured after an illumination period of 25 min.

exhibited this high light saturation of photosynthetic CO_2 assimilation near 1000 µmol.$m^{-2}.s^{-1}$ measured here during 20 to 30 min illumination periods.

A decline of the P_N-rates after a 25 min exposure to excess high-light of 2000 µmol.$m^{-2}.s^{-1}$ could not be observed (Fig. 3B) indicating that on the whole leaf level an essential photoinhibition of the photosynthetic apparatus had obviously not occurred. This is, however, in contrast to the results obtained via the kinetic Chl fluorescence measurements which indicated a decline in the photochemical quenching coefficient qP, and in the photochemical efficiency of photosystem 2 in the illuminated

state F'_v/F'_m (Fig. 2A). In fact, the rise in non-photochemical Chl fluorescence quenching qN was caused to a major part by the photoinhibitory component qI (Fig. 2B). This apparent contradiction appears to be caused by the fact that the Chl fluorescence kinetics were excited and measured from the adaxial upper leaf surface of soybean leaves. One has to consider in this respect that, in contrast to the CO_2-assimilation measurements, the Chl fluorescence is detected by a low intensity measuring light (0.1 $\mu mol.m^{-2}.s^{-1}$) and thus is only representative for the chloroplasts of the most upper part of the palisade cells but not for all cells of the leaf.

The net CO_2 assimilation rates, in turn, reflect the situation of the whole leaf comprising all chloroplasts; those in the densely packed palisade parenchyma cells and those in the spongy parenchyma cells which are separated by large aerial interspaces. As the P_N rates did not indicate a decline or a photoinhibition e. g. when going from a PPFD of 1000 to 2000 $\mu mol.m^{-2}.s^{-1}$, this indicated that a partial photoinhibition of some chloroplasts (increase in qI) next to the adaxial upper surface of the leaf did not affect the overall photosynthetic capacity and rates of the green soybean leaf. The morphological properties of the leaf cause light scattering, determine the optical leaf properties and the light distribution within the leaf and thus can reduce the incident light to non-photoinhibitory levels (Vogelmann, 1993). A partial photoinhibition of the most upper chloroplasts in the palisade parenchyma cells, which still absorb and dissipate light energy, thus protect the remaining leaf chloroplasts from photoinhibition. These results demonstrate that the Chl fluorescence signatures measured on the adaxial upper leaf side do not reflect the physiological condition of the whole leaf chloroplasts. A similar result was previously obtained in maple leaves (*Acer platanoides* L.), in which case a full photoinhibition had been assumed in the Chl fluorescence kinetics of the adaxial leaf side but only a partial decline of the P_N rates could be observed (Schindler and Lichtenthaler, 1996).

These observations indicate that one has to be extremely cautious in judging a possible photoinhibition, decline in photosynthetic electron transport and CO_2 assimilation solely by measurements of the Chl fluorescence kinetics of only one leaf side. In fact, in a preliminary study (data not shown) we found that the Chl fluorescence kinetics measured at the abaxial lower leaf side can still be fairly normal when those of the adaxial upper leaf side already indicate a strong photoinhibition. As a consequence, if one applies Chl fluorescence measurements, one needs to determine the Chl fluorescence signatures of the adaxial upper leaf side as well as those of the abaxial lower leaf side to obtain reliable information on the physiological situation of the whole leaf.

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