Photoinhibition and recovery of photosystem 2 in grapevine (*Vitis vinifera* L.) leaves grown under field conditions

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Abstract

Photoinhibition of photosynthesis was investigated in grapevine (*Vitis vinifera* L.) exposed to 2 or 4 h of high irradiance (HI) (1 700-1 800 μmol m^{-2} s^{-1}) leaves under field conditions at different sampling time in a day. The degree of photoinhibition was determined by means of the ratio of variable to maximum chlorophyll fluorescence (F_v/F_m) and photosynthetic electron transport measurements. When the photochemical efficiency of photosystem 2 (PS2), F_v/F_m, markedly declined, F_0 increased in both 2 (HI2) and 4 h (HI4) HI leaves sampled at midday. When various photosynthetic activities were followed on isolated thylakoids, HI4 leaves showed significantly higher inhibition of whole chain and PS2 activity than the HI2 leaves sampled at midday. Later, the leaves reached maximum PS2 efficiencies similar to those observed early in the morning during sampling at evening. The artificial exogenous electron donor Mn^{2+} failed to restore PS2 activity in both variants of leaves, while DPC and NH_2OH significantly restored PS2 activity in HI4 midday leaf samples. Quantification of the PS2 reaction centre protein D1 and 33 kDa protein of water splitting complex following midday exposure of leaves showed pronounced differences between HI2 and HI4 leaves. The marked loss of PS2 activity noticed in midday samples was mainly due to the marked loss of D1 protein in HI2, while in HI4 it was mainly 33-kDa protein.

Additional key words: chlorophyll fluorescence; diurnal course; donor side; electron transport; photosystems 1 and 2; proteins.

Introduction

Photoinhibition of photosynthesis commonly occurs in nature when plants are exposed to solar radiation in excess of photon energy necessary for photosynthetic and photorespiratory processes (Long *et al*. 1994, Krause *et al*. 1995). In most cases, the decrease in photosynthetic competence, observed upon high irradiance (HI) exposure of leaves, is reversible in low irradiance, both under laboratory conditions and in the natural environment. Sustained damage to the leaves, *e.g*. gross pigment destruction, is seldom observed. Based on the recovery kinetics, it has been suggested that photoinhibition of PS2 comprises at least two different processes, one of them related to zeaxanthin formation *via* xanthophyll cycle (Krause *et al*. 1995, Thiele *et al*. 1997), the other to D1 protein inactivation (Leitsch *et al*. 1994). The first process appears to be a down regulation of PS2 that is reversible within 20–60 min; the second process frequently is referred to as photodamage that can be reversed within several hours by D1 protein degradation and re-synthesis (Aro *et al*. 1993).

Although photoinhibition has been extensively investigated under controlled conditions, there is still some doubt whether it is a significant factor for plants growing in the field. Ögren and Rosenqvist (1992) argue that moderate photoinhibition is common in field-grown plants. Studies such as those of Demmig-Adams *et al.* (1989), Greer and Laing (1992), and Lovelock *et al.* (1994) support this notion. Other investigators have found little evidence for photoinhibition defined as either photodamage or photoprotection (Ludlow and Powles 1988, Flexas *et al*. 2001).

Comparative studies have revealed differences in the tendencies of species to become photoinhibited (Ögren

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Abbreviations: Chl – chlorophyll; DCBQ – 2,6-dichloro-*p*-benzoquinone; DCPIP – 2,6-dichlorophenol indophenol; DPC – diphenyl carbazide; F_0 – minimal fluorescence; F_v – variable fluorescence; HI – high irradiance; MV – methyl viologen; PPFD – photosynthetic photon flux density; PS – photosystem; SDS-PAGE – sodium dodecylsulphate-polyacrylamide gel electrophoresis; SiMo – silicomolybdate.
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and Rosenqvist 1992, Öquist *et al*. 1992). Lovelock *et al.* (1994) found that rain forest species differ in their capacity to recover from sudden exposure to high irradiance. The capacity for recovery appears to correspond to the succession role of the species, such that species commonly found in gaps and open habitats recover to a greater extent than species found in the forest understory (Lovelock *et al.* 1994). Several investigators view photoinhibition of photosynthesis as a process of stress-induced damage of PS2. This view is based on the fact that, as a consequence of photoinhibition, the D1 protein of PS2 reaction centre becomes degraded (Kyle *et al.* 1984, Prasil *et al.* 1992, Rintamaki *et al.* 1995). But some recent reports suggest that photoinhibition, first of all, results from the formation of photochemically inactive PS2 centres, which convert the excitation energy into heat. This down regulation of PS2 and thermal dissipation is considered as a protective mechanism against HI stress (Cleland *et al.* 1986, Aro *et al.* 1993, Gilmore and Björkman 1994). The photoinactivation and impairment of electron transport occur at the acceptor and donor sides of PS2, although inactivation of the acceptor side may be the main mechanism for the impairment of electron transport (Eckert *et al.* 1991, Aro *et al.* 1993). This evidence comes largely from the *in vitro* studies. But the mechanism of photoinhibition *in vivo* may be more complicated, and the dominating mechanism of inactivation *in vivo* is not clear.

The importance of photoinhibition in *Vitis* species in the field has been evaluated in our previous study (Bertamini and Nedunchezhian 2002) that did not include *Vitis vinifera*. There is evidence from pot experiments that *Vitis vinifera* (Downton 1983) and the related wild grape *Vitis californica* (Gamon and Pearcy 1990) are susceptible to photoinhibition when stressed by water shortage and high temperature. The object of our work was to study photoinhibition and the process of recovery in 2 h (HI2) and 4 h (HI4) HI exposure of grapevine leaves grown under field conditions at different sampling times. We measured photochemical efficiency of PS2 (F_v/F_m), photosynthetic electron transport activities, and D1 and 33 kDa proteins in grapevine (*Vitis vinifera* L.) leaves under field conditions at different sampling time in a day.

Materials and methods

Plants and experimental design: Leaves of grapevine (*Vitis vinifera* L.) were collected from selected 10-y-old seedlings grown under field condition on training system with upright growing shoots (Cordon Royat) condition in Istituto Agrario di San Michele all' Adige, Italy. In order to simplify the experimental procedure, we proceeded to classify the leaf samples into two groups according to the irradiance received on the surface of the leaf (1 700-1 800 μmol m^{-2} s^{-1}). All measurements were made at five different sunny days (August 2002) and at a different sampling time during the day, early in the morning (06:00), midday (12:00), and evening (19:00, always solar time) on sunset. During the experimental period, the daily maximum PPFD on clear days was approximately 1 700-2 000 μmol m^{-2} s^{-1} and the maximum temperature was around 32–33 °C. Irradiance and temperature were measured as in Iacono *et al.* (1994).

Modulated Chl fluorescence in leaves was measured on leaf discs using a *PAM 2000* fluorometer (*H. Walz*, Effeltrich, Germany). Before the measurements, the leaves were dark adapted for 30 min. F_0 was measured by switching on the modulated irradiation of 0.6 kHz; PPFD was less than 0.1 μmol m^{-2} s^{-1} at the leaf surface. F_m was measured at 20 kHz with a 1-s pulse of 6 000 μmol m^{-2} s^{-1} of "white light".

Activities of electron transport: Thylakoid membranes were isolated from the leaves as described by Berthold *et al.* (1981). Whole chain electron transport ($H_2O \rightarrow MV$) and partial reactions of photosynthetic electron transport mediated by PS2 ($H_2O \rightarrow DCBQ$; $H_2O \rightarrow SiMo$) and PS1 ($DCPIPH_2 \rightarrow MV$) were measured as described by Nedunchezhian *et al.* (1997). Thylakoids were suspended at 100 mg(Chl) m^{-3} in the assay medium containing 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM NH_4Cl, and 100 mM sucrose supplemented with 500 μM DCBQ and 200 μM SiMo.

DCPIP photoreduction was determined as the decrease in absorbance at 590 nm using a *Hitachi 557* spectrophotometer. The reaction mixture (3 cm^3) contained 20 mM Tris-HCl, pH 7.5, 5 mm $MgCl_2$, 10 mM NaCl, 100 mM sucrose, 100 μm DCPIP, and thylakoid membranes equivalent to 20 μg Chl. Where mentioned, the concentration of $MnCl_2$, DPC, and NH_2OH were 5.0, 0.5, and 5.0 mM, respectively.

Immunological determination of thylakoid proteins: The relative contents of certain thylakoid proteins per mg Chl were determined immunologically by Western blotting. Thylakoids were solubilised in 5 % SDS, 15 % glycerine, 50 mM Tris-HCl (pH 6.8), and 2 % mercaptoethanol at room temperature for 30 min. The polypeptides were separated by SDS-PAGE as described by Laemmli (1970) and proteins were then transferred to nitrocellulose by electroblotting for 3 h at 0.4 A. After saturation with 10 % milk powder in TBS buffer (pH 7.5), the first antibody in 1 % gelatine was allowed to react overnight at room temperature. After washing with TBS containing 0.05 % *Tween-20*, the secondary antibody [*Anti-Rabbit IgG* (whole molecule) *Biotin Conjugate*, *Sigma*] was allowed to react in 1 % gelatine for 2 h. For detection of D1 protein a polyclonal antiserum against spinach D1 protein

was used (kindly provided by Prof. I. Ohad, Jerusalum, Israel), and the antibody against the 33 kDa protein of the water-splitting system was a gift from Dr. Barbato, Padova, Italy. The densitometric analysis of Western blots was performed with a *Bio-Image* analyser (*Millipore Corporation*, Michigan, USA).

Results

Changes in Chl fluorescence: To obtain information on PS2 activity, F_v/F_m, which reflects the quantum yield of PS2 photochemistry (Krause and Weis 1991), was determined *in vivo* using leaf discs which had been dark adapted for 30 min. The photochemical efficiency of PS2, measured as F_v/F_m of leaves sampled early in the morning (6 h, solar time), was 0.792 and 0.795 for HI2 and HI4 leaves, respectively (Fig. 1). For leaves measured at midday (12 h, solar time) on a fully sunny day, the F_0 increased in both HI2 and HI4 leaves. The F_v/F_m ratio decreased to 0.634 in HI2 leaves and to 0.517 in HI4 leaves, respectively (Fig. 1). In samples prepared in the evening (19:00, solar time) on sunset, F_0 decayed significantly in HI2 and F_v/F_m reached values of 0.791 and 0.793 in HI2 and HI4 leaves, respectively.

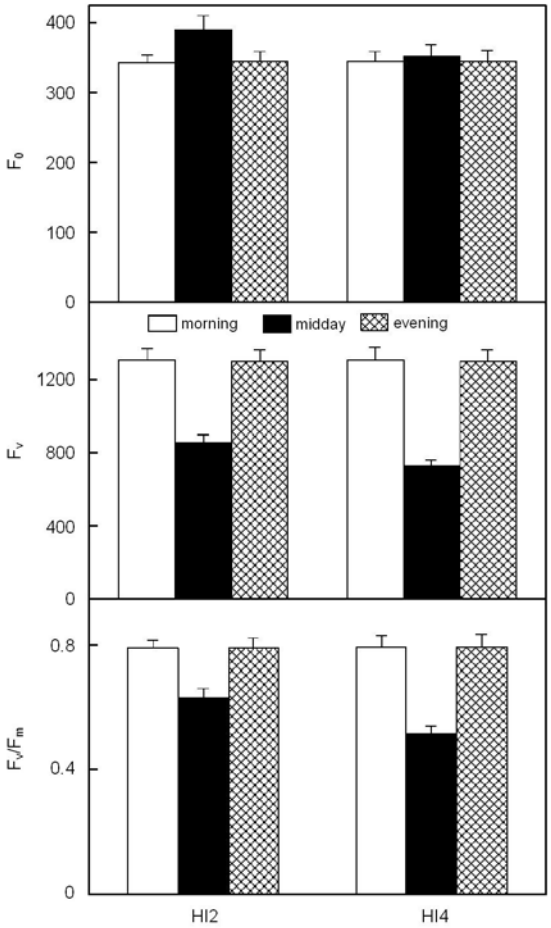

Fig. 1. Changes in the relative levels of fluorescence emitted as minimal fluorescence (F_0) or variable fluorescence (F_v) and the ratio of variable to maximum fluorescence (F_v/F_m) of HI2 and HI4 leaves at different sampling time intervals in a day. Means ± standard errors of 5 replicates.

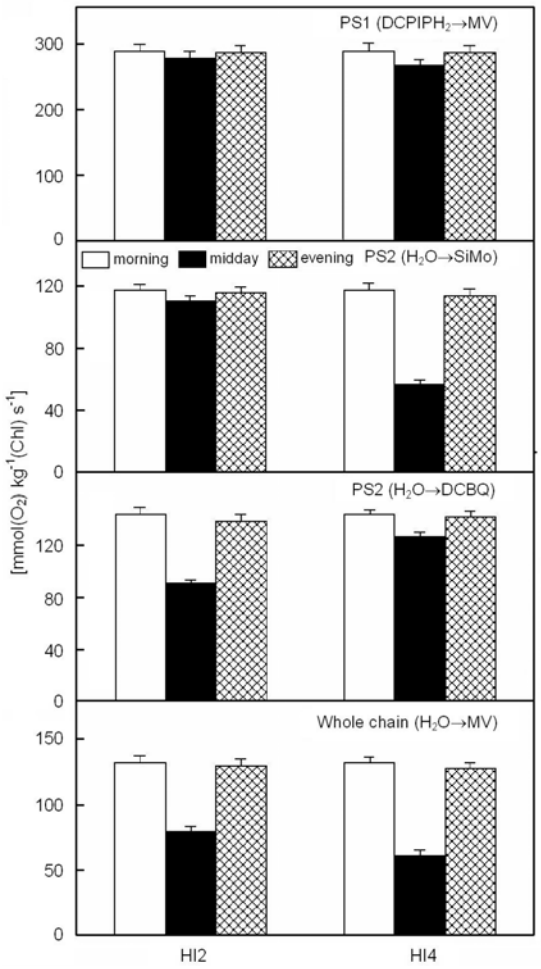

Fig. 2. Changes in the rates of whole chain ($H_2O \rightarrow MV$), PS2 ($H_2O \rightarrow DCBQ$; $H_2O \rightarrow SiMo$), and PS1 ($DCPIPH_2 \rightarrow MV$) electron transport activities in thylakoids isolated from HI2 and HI4 leaves at different sampling time intervals in a day. Means ± standard errors of 5 replicates.

Changes in photosynthetic activities: Photosynthetic electron transport activities were measured in thylakoids isolated from HI2 and HI4 leaves at different sampling times in a day (Fig. 2). Photosynthetic electron transport from $H_2O \rightarrow DCBQ$ and $H_2O \rightarrow SiMo$, measured at midday, was reduced by about 37 and 4 %, and by 12 and 52 % in HI2 and HI4 leaves, respectively. In HI2 the reduction of PS2 activity was significant with DCBQ as electron acceptor but not with SiMo (Fig. 2). In contrast, a marked reduction of PS2 activity with SiMo was noticed in HI4. Evening samples reached PS2 activity similar to those observed early in the morning in both variants (Fig. 2). A small decline in PS1 activity was observed

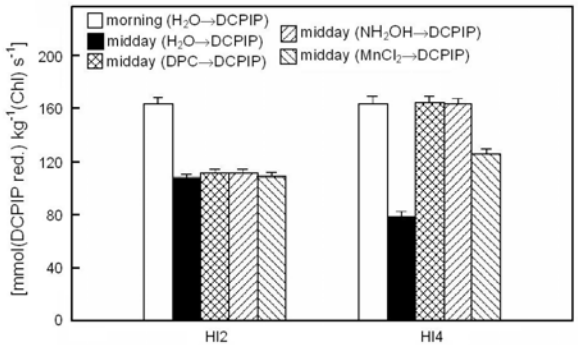

Fig. 3. Effect of various exogenous electron donors on PS2 activity ($H_2O \rightarrow DCPIP$) in thylakoids isolated from HI2 and HI4 leaves sampled at midday. Means ± standard errors of 5 replicates.

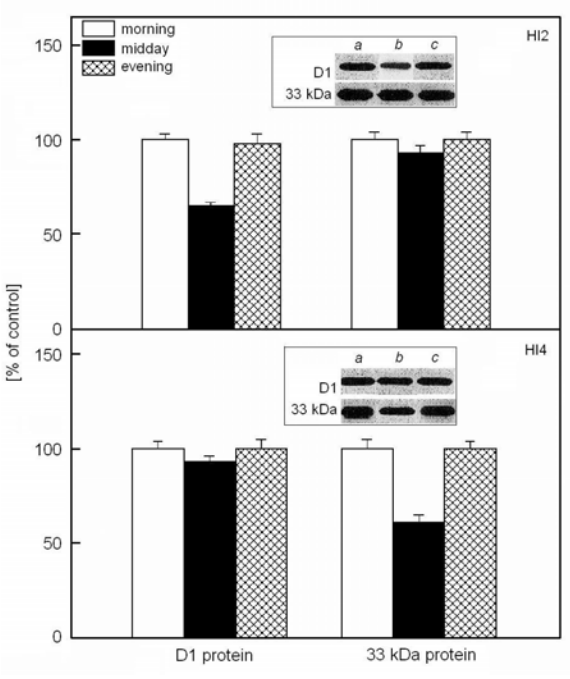

Fig. 4. Relative D1 and 33 kDa protein contents of thylakoids isolated from HI2 and HI4 leaves at different sampling time intervals in a day. Lane *a*, morning; lane *b*, midday; lane *c*, evening.

only in HI2 and HI4 leaves sampled at midday (Fig. 2).

Changes in DCPIP photoreduction rate: To locate the possible site(s) of inhibition in the PS2 reaction, we followed the DCPIP photoreduction supported by various exogenous electron donors in thylakoids isolated from photoinhibited midday leaves of HI2 and HI4. Wydrzynski and Govindjee (1975) have shown that $MnCl_2$, DPC, NH_2OH, and HQ could donate the electrons to PS2. Fig. 3 shows the electron transport activity of PS2 in the presence and absence of three of the above compounds. In comparison with morning values, PS2 activity was reduced by about 34 and 52 % in HI2 and HI4 leaves when water served as electron donor. A similar trend was also found using $MnCl_2$ as electron donor in both variants. A significant restoration of PS2 mediated DCPIP reduction was observed if DPC or NH_2OH were used as electron donor in HI4. In contrast, the PS2 activity was not restored using either DPC or NH_2OH in HI2 leaves (Fig. 3).

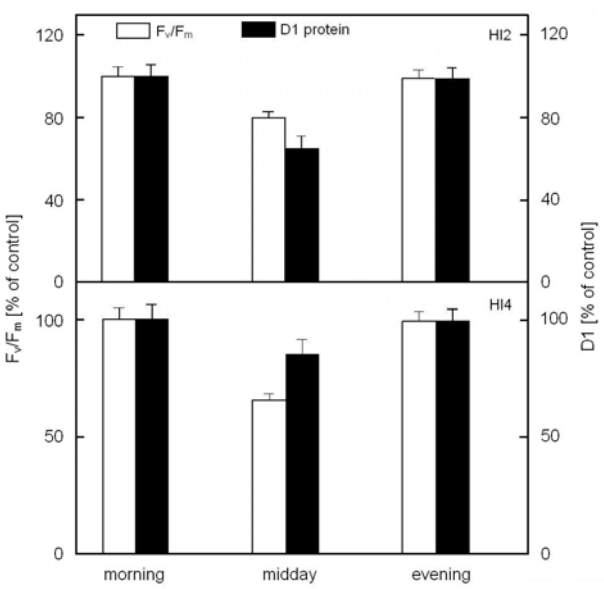

Fig. 5. Quantification of D1 protein and degree of photoinhibition in HI2 and HI4 leaves sampled at different time intervals in a day. The F_v/F_m ratios were determined as a measure of photoinhibition. Means ± standard errors of 5 replicates.

Changes in contents of D1 and 33 kDa proteins determined by immunoblot: Photoinhibition of PS2 induces breakdown of the D1 protein (Andersson and Styring 1991, Prasil *et al.* 1992). In systems without protein biosynthesis this is seen directly as a loss of D1 protein. In intact plant the correlation between D1 protein content and activity of PS2 is more complex (Smith *et al.* 1990, Lutz *et al.* 1992). Photoinhibition induced decline in PS2 activity in thylakoids of HI2 and HI4 leaves was compared with changes in the relative contents of D1 and 33 kDa proteins as determined by Western blotting followed by quantification using the *Bio-Image* apparatus (Fig. 4). In comparison to morning values, the contents of D1 and 33 kDa proteins decreased by 35 and 2 % in HI2 leaves and by 5 and 39 % in HI4 leaves sampled at midday. In the evening, the leaves reached net D1 or 33 kDa protein contents that were similar to those observed early in the morning (Fig. 4).

In Fig. 5 relative D1 protein contents and F_v/F_m ratios are compared after photoinhibitory treatment (mid-day) of HI2 and HI4 leaves. In HI4 leaves, no significant D1 degradation could be attributed to the action of photoinhibitory irradiation, even if F_v/F_m ratios decreased by 32-35 % of the control. The HI2 leaves showed a strong decrease in D1 protein content together with the decline of F_v/F_m ratio by about 20 % (Fig. 5).

Discussion

The grapevine leaves with exposed undisturbed positions in a field-grown canopy frequently exhibited a depression in F_v/F_m in the afternoon. The suggested responsible mechanism is photoinhibition of the well-known type with primary damage residing in PS2.

A good PS2 activity was found as the F_v/F_m ratio in both variants of leaves, when sampled early in the morning. In contrast, when sampling was made at midday, the F_v/F_m ratio decreased markedly in HI4 leaves. The PS2 photochemistry of both HI2 and HI4 leaves was also dependent on the time of sampling, decreasing from morning to midday, being minimal at midday, and increasing again from midday to evening (sunset). In the evening, HI2 leaves reached maximum PS2 photochemical efficiencies similar to those observed early in the morning. The rate of recovery agrees with other reports on photoinhibition in higher plants. For instance, Ögren *et al.* (1984) found that complete recovery of photosynthesis in *Lemna* plants from 60 % photoinhibition took about 10 h. The decrease in F_v/F_m ratio was mainly due to decrease of variable fluorescence (F_v) in HI4, while in HI2 it was induced by decrease of F_v and marked increase of F_0. An increase of F_0 may be induced by the inactivation of part of the PS2 reaction centres (Govindjee 1990, Critchley and Russell 1994, Yamane *et al.* 1997). Our experimental results from HI2 leaves are in accordance with this idea. Similar depressions in F_v/F_m ratio throughout the day have been already reported for several plant species (Demmig-Adams and Adams 1992, Iacono *et al.* 1995, Morales *et al.* 2000). From these studies we can conclude that plants require various times for recovering sufficient PS2 photochemistry after midday depression (Demmig-Adams and Adams 1992).

Analysis of the electron transport activities in thylakoids isolated from midday leaves of HI2 showed that oxygen evolution was inhibited markedly when the electron acceptor was DCBQ, but not when it was SiMo. This clearly indicates that HI2 leaves are affected at the reducing side of PS2 due to photoinhibition. This was also supported by our Chl fluorescence study where F_0 markedly increased. In contrast to this, in thylakoids isolated from HI4, the rate of PS2 activity observed with SiMo was lower than that observed with DCBQ. This indicates that the donor side is more impaired than the acceptor side of PS2. The extent of variable fluorescence (F_v) was reduced markedly in HI4 leaves without affecting the F_0 level. This is characteristic for the inhibition of donor side of PS2. If the acceptor side of PS2 is photoinhibited, the F_0 level is significantly increased (Allakhverdiev *et al.* 1987, Šetlík *et al.* 1990).

To locate the possible site of inhibition in the PS2 reaction, we followed the DCPIP photoreduction supported by various exogenous electron donors in thylakoids isolated from midday leaves of HI2 and HI4 variants. Among the artificial electron donors tested, DPC and NH_2OH donate electrons to the PS2 reaction centre (Wydrzynski and Govindjee 1975). A significant restoration of PS2 activity was observed in HI4 leaves after the addition of DPC and NH_2OH. This is in agreement with findings that water-oxidising system is sensitive to HI (Veeranjaneyulu *et al.* 1999), while using neither DPC nor NH_2OH in HI2 leaves did not restore the loss of PS2 activity. Hence HI2 leaves are mainly affected on the acceptor side of PS2 due to photoinhibition. Similar observations were made for field grown *Schefflera arboricola* leaves adapted to different irradiances (Schiefthaler *et al.* 1999).

The loss of PS2 activity in HI2 and HI4 leaves at photoinhibition was mainly due to loss of PS2 reaction centres or water-splitting complex. This could be confirmed by the immunological determination of the PS2 reaction centre D1 protein and 33 kDa protein of the water-splitting complex.

It is often thought that photoinhibition is a result of marked loss of D1 protein, so it occurs only when the rate of damage to D1 protein exceeds the rate of its repair (Kyle *et al.* 1984, Ohad *et al.* 1985, Barber 1995, Carpentier 1995). Moreover, the fluorescence parameter F_v/F_m is a good measure of photoinhibition, and a decrease in F_v/F_m under photoinhibitory conditions is often attributed to the loss of D1 protein. Our study with HI2 leaves supports this fact. Both increased F_0 and decreased F_v/F_m caused by HI during midday could almost completely recover in the evening (Fig. 1). Marked losses of D1 protein also occurred after HI treatment during midday and they completely recovered at sunset. In contrast, no marked loss of D1 protein was found in HI4 leaves after irradiation on midday.

As shown by D1 protein quantification, even strong photoinhibition of HI4 leaves was not related to inactivation and degradation of the D1 protein in the PS2 reaction centre. In contrast, in HI2 leaves substantial amount of inactivated D1 was coincident with photoinhibition. HI acclimation probably results in various degrees of D1 stabilisation (Thiele *et al.* 1997).

From the results we suggest that HI inactivated either the acceptor side or donor side of PS2 in grapevine leaves which depended on the duration of irradiation of leaf surface. At 2-h duration of HI in grapevine leaves, acceptor side of PS2 was more impaired than its donor side. This is mainly due to the reduction of F_v/F_m with significant increase of F_0, marked loss of PS2 activity by using electron acceptor DCBQ, and decline in D1 protein content. In contrast to this, after 4 h of HI the donor side of PS2 was more impaired than the acceptor side. This was due to marked reduction of F_v/F_m without increase of F_0, marked loss of PS2 activity by using electron acceptor SiMo, and loss of the 33-kDa protein. Our results also suggest that photoinactivation of PS2 is not correlated at all with net loss of D1 in HI4 leaves and photoinhibition represents the formation of inactive centres. These

centres are apparently capable of dissipating excess excitation energy and thereby exert a regulatory protective function (Krause 1988, Smith *et al*. 1990, Flexas *et al*. 2001).

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