Photoinhibition of photosynthesis in water deficit leaves of grapevine (*Vitis vinifera* L.) plants

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Abstract

Photoinhibition under irradiance of 2 000 μmol m^{-2} s^{-1} (HI) was studied in detached control (C) and water deficit (WD) leaves of grapevine (*Vitis vinifera* L.) plants. The degree of photoinhibition was determined by means of the ratio of variable to maximum chlorophyll (Chl) fluorescence (F_v/F_m) and electron transport measurements. The potential efficiency of photosystem (PS) 2, F_v/F_m, marginally declined under HI in WD-leaves without significant increase of F_0. In contrast, F_v/F_m ratio declined markedly with significant increase of F_0 in C-leaves. In isolated thylakoids, the rate of whole chain and PS2 activity under HI were more decreased in C- than WD-leaves. The artificial exogenous electron donors diphenyl carbazide, NH_2OH, and Mn^{2+} failed to restore the HI-induced loss of PS2 activity in both C- and WD-leaves. Thus HI operates at the acceptor side of PS2 in both leaf types. Quantification of the PS2 reaction centre protein D1 following HI exposure of leaves showed pronounced differences between C- and WD-leaves. The marked loss of PS2 activity under HI of C-leaves was due to the marked loss of D1 protein of the PS2 reaction centre.

Additional key words: acceptor side; chlorophyll fluorescence; diphenyl carbazide; electron transport; irradiance; Mn; NH_2OH; photosystem 2.

Introduction

Water stress is the most important factor limiting plant growth in the Mediterranean area. Grapevine (*Vitis vinifera* L.) is common crop in these areas, showing the most active growth during spring and summer. Despite its adaptation to these climatic conditions, a combination of soil water stress with frequent environmental constraints such as steep leaf to air water vapour gradients, high irradiance (HI) and temperature causes a progressive reduction of grapevine photosynthesis, plant growth, and grape yield (Lawlor 1976, Chaves and Rodrigues 1987, Schultz 1996, Flexas *et al*. 1998). The overall effect of water stress on vine leaf photosynthesis has been largely examined under different environmental conditions and on different grapevine cultivars (Delgado *et al*. 1995, Flexas *et al*. 1999, 2002). Reductions of net photosynthetic rate (P_N) induced by water stress have usually been related to stomatal closure (Lawlor and Fock 1977, Rodrigues *et al*. 1993, Lawlor and Cornic 2002, Flexas *et al*. 2004).

Drought-induced decrease in photosynthesis is due primarily to stomatal closure, which lowers CO_2 availability in the mesophyll, rather than to direct effect on the capacity of the photosynthetic apparatus (Cornic and Massacci 1996, Escalona *et al*. 1999). Stomatal closure is one of the first responses to soil drying, and a parallel decline in photosynthesis and stomatal conductance under progressive water stress has already been reported (Medrano *et al*. 1997).

P_N declines more rapidly than whole chain electron transport and net O_2 evolution in water stressed plants. Even with P_N close to zero, electron transfer was 80 % of the control value at relative water content (RWC) of 70 %

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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; PFD – photon flux density; P_N – net photosynthetic rate; PS – photosystem; RC – reaction centre; RuBPC – ribulose-1,5-bisphosphate carboxylase; RWC – relative water content; SDS-PAGE – sodium dodecylsulphate-polyacrylamide gel electrophoresis; SiMo – silicomolybdate.

(Tourneux and Peltier 1995). Biehler and Fock (1996) observed a decrease in P_N of 75 % in wheat leaves at –3 MPa but electron transport was only decreased by 10 %, at photosynthetic active radiation of 850 μmol m^{-2} s^{-1}. At very small photon flux (90 μmol m^{-2} s^{-1}), P_N decreased by 50 % but electron transfer was unaffected. Similarly, Cornic (1994) reported for sunflower under a limiting PFD (300 μmol m^{-2} s^{-1}), an 80 % decrease of P_N with no changes in whole chain electron transport rate. Moreover, in this case O_2 could entirely replace CO_2 as an electron acceptor. Generalizing, in saturating photon flux, electron flow exceeds P_N, particularly during drought.

Generally, the quantum efficiency of electron transport in photosystem (PS) 2 and photochemical quenching (q_P) decrease only at RWC below *ca*. 75 %. The maximum photochemical efficiency of PS2, given by F_v/F_m, is little affected by decreasing RWC (between 100 and 50 %) in any study indicating absence of photoinhibition (Tezara *et al*. 1995) but does decrease at low RWC (Tezara *et al*. 1995, Tourneux and Peltier 1995). Similarly, maximum quantum yield of PS1 is not affected (Cornic 1994). Thus, with decreasing RWC, the functions of PS2, PS1, and electron transport are largely maintained, even when P_N is strongly inhibited. In contrast, water stress may result in damage to the O_2 evolving complex of PS2 (Canaani *et al*. 1986, Toivonen and Vidaver 1988) and to the PS2 reaction centres, RCs (Havaux *et al*. 1987, He *et al*. 1995, Chen *et al*. 2004, Bertamini *et al*. 2006).

Photon energy is necessary to drive the process of photosynthesis; however, energy absorbed in excess of that required for the saturation of photosynthesis may cause photoinhibition. The photoinhibition occurs at thylakoid level, particularly at PS2 (Cleland *et al*. 1986, Eckert *et al*. 1991). Several investigators view photoinhibition of photosynthesis as a process of stress-induced damage to PS2. This is based on the fact that, as a consequence of photoinhibition, the D1 protein of PS2 RC becomes degraded (Prášil *et al*. 1992, Rintamäki *et al*. 1995). But some recent reports suggest that photoinhibition, first of all, results from the formation of photochemically inactive PS2 RCs, which convert the excitation energy into heat. This down-regulation of PS2 and thermal dissipation is considered as a protective mechanism against HI stress (Aro *et al*. 1993, Gilmore and Björkman 1994). The photoinactivation and impairment of electron transport occurs 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).

We have focused on the photoinhibitory responses in control (C) and water deficit (WD) leaves of grapevine (*Vitis vinifera* L.) plants. The effect of photoinhibition was analyzed with respect to photosynthetic oxygen evolution and potential efficiency of PS2 (F_v/F_m) function by Chl fluorescence. The amount of D1 protein was also analyzed in relation to the functional properties of PS2 after photoinhibition.

Materials and methods

Plants and WD treatment: One-year-old grapevine (*Vitis vinifera* L. cv. Riesling) plants were grown in 10 000 cm^3 pots in a growth chamber. The pots were filled with soil : sand : peat : vermiculite (3 : 1 : 3 : 3) and added with NPK (15 : 15 : 15, by vol.) complete fertilizer. Irradiance at the top of plants was 170 μmol m^{-2} s^{-1} as provided by high pressure sodium lamps (*SON-T AGRO*, *Philips*, Belgium) with a photoperiod of 16/8 h. Air temperature during day and night was kept at 30/20±1 °C and relative humidity at 70 %. All plants were watered daily.

After four weeks, the plants were used for the WD treatment, which was developed gradually by withholding water. After a period of 10 d, leaf water potential reached −2.00±0.04 MPa, measured with a pressure chamber (model *3000*, *Soil Moisture Equipment Co.*, USA). The youngest and fully developed leaves were used for the measurements.

Photoinhibition under controlled conditions: Detached C- and WD-leaves were placed into a controlled-environment chamber equipped with a 24V/250W metal-halide lamp (*H. Walz*, Effeltrich, Germany). The upper leaf surface was exposed to a photosynthetic photon flux density (PPFD) of 2 000 μmol m^{-2} s^{-1}. Air temperature was 20 ºC and relative humidity 66±5 %. The PPFD was measured with quantum sensor (*LI-Cor*, Lincoln, NE, USA). Leaf temperatures, recorded with thermocouple attached to the lower surface, were 27–29 ºC. Discs of 1.6 cm^2 area were punched from the leaf blades after specified times of HI exposure and placed on moist filter paper in petri dishes (temperature 25–27 ºC). The leaf discs were darkened for 5 min before the degree of photoinhibition was determined by fluorescence measurements.

Modulated Chl fluorescence was measured on leaves using a *PAM 2000* fluorometer (*H. Walz*, Effeltrich, Germany). F_0 was measured by switching on the modulated radiation to 0.6 kHz; PPFD at leaf surface was <0.1 μmol m^{-2} s^{-1}. F_m was measured at 20 kHz with 1-s pulses of 6 000 μmol m^{-2} s^{-1} of "white light".

Activities of electron transport: Thylakoid membranes were isolated at 4 °C from each sample of six to eight disks as described by Berthhold *et al*. (1981). Leaf disks were homogenized in an ice cold grinding medium containing 25 mM Tris-HCl pH 7.8, 10 mM NaCl, 5 mm $MgCl_2$, and 330 mM sucrose. The homogenate was filtered rapidly through four layers of *Miracloth* and

thylakoids were collected by centrifugation at 9 000×*g* for 5 min.

Whole chain electron transport (H_2O→methyl viologen, MV) and partial reactions of photosynthetic electron transport mediated by PS2 (H_2O→2,6-dichloro-p-benzoquinone, DCBQ; H_2O→silicomolybdate, SiMo) and PS1 ($DCPIPH_2$→MV) were measured following the method of Nedunchezhian *et al*. (1997) with a Clark-type electrode (*Hansatech*, Kings Lynn, UK) fitted with a circulating water jacket at 27 °C. "Actinic light" from a slide projector placed on the side of the electrode chamber was filtered through 9.5 cm of water. The irradiance was 1 100 μmol m^{-2} s^{-1} at the surface of the water bath cell. Thylakoid membranes were suspended at 10 g(Chl) m^{-3} in an 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 0.5 mM DCBQ, and 0.2 mM SiMo for oxygen evolution (PS2), and 1 mM MV, 2 mM ascorbate, 5 μM DCMU, 1 mM sodium azide, and 100 μM DCPIP for oxygen uptake (PS1). Thylakoid membranes were suspended at 10 g(Chl) cm^{-3} in an 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 1 mM MV and 1 mM sodium azide for whole chain electron transport.

Rate of 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, 0.1 mM DCPIP, and thylakoid membranes equivalent to 20 μg of Chl. Where mentioned, the concentrations of $MnCl_2$, DPC, and NH_2OH were 5.0, 0.5, and 5.0 mM, respectively.

SDS-PAGE: Thylakoid membranes were separated using the polyacrylamide gel system of Laemmli (1970), with following modifications. Gels consisted of a 12–18 % gradient of polyacrylamide containing 4 M urea. Samples of thylakoid membrane preparation were solubilised at 20 °C for 5 min in 2 % (m/v) SDS and 60 mM DTT and 8 % sucrose using SDS-Chl ratio of 20 : 1. Electrophoresis was performed at 20 °C with constant current of 5 mA. Gels were stained in methanol/acetic acid/water (4 : 1 : 5, v/v/v) containing 0.1 % (m/v) Coomassie brilliant blue R and de-stained in methanol/acetic acid/water (4 : 1 : 5, v/v/v). Thylakoid membrane protein was estimated according to the method of Lowry *et al.* (1951).

Immunological determination of thylakoid proteins: The relative contents of certain thylakoid proteins per Chl unit were determined immunologically by Western blotting. Thylakoids were solubilised in 5 % SDS, 15 % glycerol, 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 above 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*, USA] 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, Jerusalem, Israel), The densitometric analysis of Western blots was performed with a *Bio-Image* analyzer (*Millipore Corporation*, Michigan, USA).

Statistical analysis of the physiological responses was tested using a three-way analysis of variance (*ANOVA*) if the data met the assumptions of normality and homoscedasticity. Significant differences were determined by the Student's *t*-test criterion. All the statistical procedures were performed with *SPSS 10.0 for Windows* (*SPSS*, Chicago, IL, USA).

Results

Changes in Chl fluorescence: In order to compare the susceptibility to photoinhibition between C- and WD-leaves, leaf samples were subjected to HI in a controlled-environment chamber for 60 min. C-leaves responded more sensitively to HI than WD-leaves, as indicated by the more pronounced decrease in F_v/F_m ratios of C-leaves (Fig. 1). The treatment with HI for 60 min lead to a decline of about 30 or 16 % in F_v/F_m and elevation of about 23 or 10 % in F_0 in C- and WD-leaves, respectively.

Changes in photosynthetic activities and photoreduction: Photosynthetic electron transport activities were measured in thylakoids isolated from HI-irradiated C- and WD-leaves (Fig. 2). After 60 min of HI, the rate of PS2 activity from H_2O→DCBQ and H_2O→SiMo was decreased by 32 or 5 % in C-leaves and by 12 or 3 % in WD-leaves (Fig. 2). A small inhibition of PS1 activity was also observed in both C- and WD-leaves (Fig. 2).

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 HI-treated C- and WD-leaves. Wydrzynski and Govindjee (1975) have shown that $MnCl_2$, DPC, and NH_2OH donate electrons in the PS2 reaction. The PS2 activity was reduced to *ca*. 30 and 14 % in C- and WD-leaves, respectively, when water served as electron donor (Fig. 3). A similar trend was also found using DPC, NH_2OH, and $MnCl_2$ as donor in both leaf types.

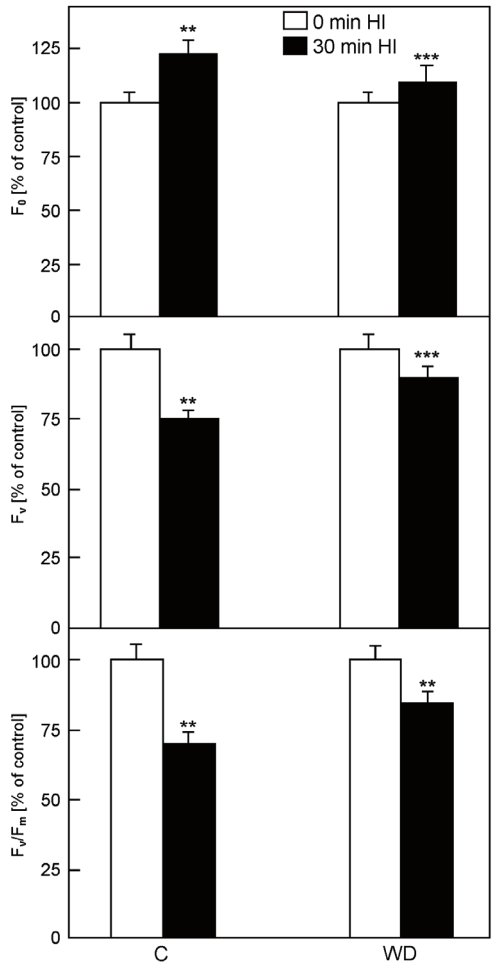

Fig. 1. Changes in relative fluorescence emitted as minimal fluorescence (F_0), maximal fluorescence (F_m), and the ratio of variable to maximal fluorescence (F_v/F_m) of control (C) and water deficit (WD) leaves treated for 60 min by high irradiance (HI). Control values for F_0, F_v, and F_v/F_m were 0.410, 1.455, 0.780 and 0.400, 0.945, 0.702 in C- and WD-leaves, respectively (means ± S.E.; $n = 5$, $^{***}p<0.001$, $^{**}p<0.01$).

Changes in D1 protein by immunoblot: Photoinhibition of PS2 induces breakdown of the D1 protein (Prášil *et al*. 1992). In systems without protein biosynthesis this can be seen directly as a loss in D1 protein content. In intact plant the correlation between D1 protein content and activity of PS2 is more complex (Lütz *et al*. 1992). Photoinhibition induced inhibition of PS2 activity in thylakoids of C- and WD-leaves was compared with changes in the relative content of D1 protein determined by Western blotting followed by quantification by the *Bio-Image* apparatus (Fig. 4). The relative content of D1 protein in HI-treated leaves decreased by 25 % in C-leaves and by 8 % in WD-leaves.

In Fig. 5, relative D1 protein contents and F_v/F_m ratios are compared after photoinhibitory treatments of C- and WD-leaves. In the WD-leaves, no significant D1 degradation could be attributed to the action of photoinhibitory irradiation, even when F_v/F_m ratios had decreased by 13–16 % of the controls. The C-leaves showed a strong decrease in D1 protein content together with the decline of F_v/F_m to about 60 % of its value in untreated leaves (Fig. 5).

Discussion

We found that exposure to HI produces differential loss of photosynthetic activity and potential efficiency of PS2 (F_v/F_m), the C-leaves being more sensitive to HI than the WD-leaves.

The decline in F_v/F_m (used here as a convenient measure of photoinhibition) indicates a reduction in potential PS2 efficiency. In many studies, a close correlation of the F_v/F_m ratio with the quantum yield of photosynthetic O_2 evolution or CO_2 assimilation under limiting irradiance has been reported (Krause and Weis 1991). The reduction of F_v/F_m in HI-treated C- and WD-leaves was mainly caused by a decline of F_v and increase of F_0. An increase of F_0 may be induced by the inactivation of part of PS2 RCs (Critchley and Russell 1994, Yamane *et al*. 1997). Our experimental results from C-leaves are in accordance with this idea. When F_0 increased in C-leaves under HI, some PS2 RCs lost their photochemical activity as indicated by a marked decline in the photochemical efficiency of PS2 (F_v/F_m). Bolhar-Nordenkampf *et al*. (1991) observed relatively low F_v/F_m ratios.

Analysis of electron transport activities in thylakoids isolated from HI-treated C-leaves showed that the oxygen evolution was inhibited markedly when the electron acceptor used was DCBQ, but not SiMo. This is mainly due to HI induced changes on the reducing side of PS2, *i.e*. due to photoinhibition. This is also supported by our Chl fluorescence studies where F_0 was markedly increased (Šetlík *et al*. 1990, Endo *et al*. 1998). Similar trend was also observed in WD-leaves but less inhibition had occurred. A relationship between F_v/F_m and PS2 electron transport activity in thylakoids isolated from photoinhibited leaves has been shown by Schnettger *et al*. (1994).

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 HI-treated both C- and WD-leaves. Among the artificial electron donors tested $MnCl_2$, DPC, and NH_2OH donate electrons directly to the RC of PS2 (Wydrzynski and Govindjee 1975). Addition of $MnCl_2$, DPC, and NH_2OH did not restore the HI-induced loss of PS2 activity in both C- and WD-leaves. Thus HI induces changes on the acceptor side of PS2 only in both leaf types (Eckert *et al*. 1991, Aro *et al*. 1993).

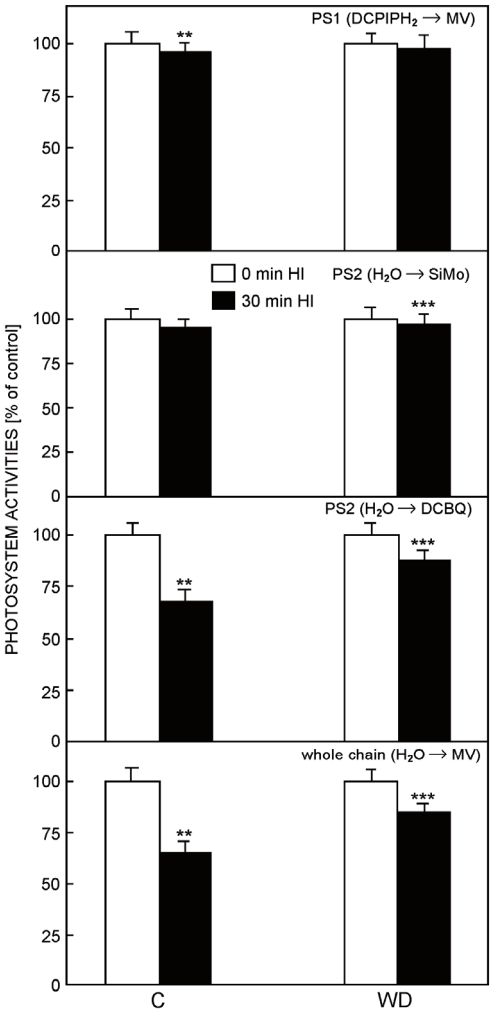

Fig. 2. Changes in the rates of whole chain ($H_2O \rightarrow MV$), PS2 ($H_2O \rightarrow DCBQ$; $H_2O \rightarrow SiMo$), and PS1 ($DCPIPH_2 \rightarrow MV$) electron transport activities in thylakoids isolated from high irradiance (HI) treated control (C) and water deficit (WD) leaves. The 100 % values are [mmol(O_2) kg^{-1}(Chl) s^{-1}]: $DCPIPH_2 \rightarrow MV$ 311.8, 286.8; $H_2O \rightarrow DCBQ$ 142.1, 85.3; $H_2O \rightarrow SiMo$ 102.4, 81.9; $H_2O \rightarrow MV$ 154.6, 85.1 for thylakoids isolated from C- and WD-leaves, respectively (means ± S.E.; $n = 5$, $^{***}p<0.001$, $^{**}p<0.01$).

The loss of PS2 activity could only partially be ascribed to functional inhibition of PS2 since F_v/F_m was reduced by about 30 and 16 % in HI-treated C- and WD-leaves, respectively. We therefore assume that it was mainly due to loss of PS2 RCs on a Chl basis. This was confirmed by the immunological determination of the PS2 RC protein of D1. It is often thought that photoinhibition is a result of marked loss of D1 protein in C-leaves. So it occurs only when the rate of damage to D1 protein exceeds the rate of its repair (Barber 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 (Anderson and Aro 1994).

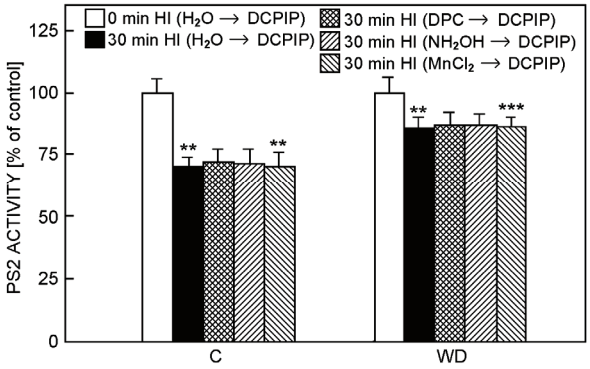

Fig. 3. Effects of exogenous electron donors on PS2 activity ($H_2O \rightarrow DCPIP$) in thylakoids isolated from 60 min high irradiance (HI) treated control (C) and water deficit (WD) leaves. The 100 % values are [mmol(DCPIP) kg^{-1}(Chl) s^{-1}]: $H_2O \rightarrow DCPIP$ 145.5, 94.6; $DPC \rightarrow DCPIP$ 149.6, 95.8; $NH_2OH \rightarrow DCPIP$ 149.2, 95.6; $MnCl_2 \rightarrow DCPIP$ 146.4, 94.6 for thylakoids isolated from C- and WD-leaves, respectively (means ± S.E.; $n = 5$, $^{***}p<0.001$, $^{**}p<0.01$).

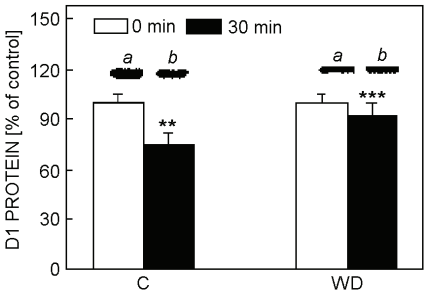

Fig. 4. Contents of D1 protein as determined by Western blotting in high irradiance (HI) irradiated control (C) and water deficit (WD) leaves. Lane *a*, 0 min HI; lane *b* 60 min HI. Each lane was loaded with equal amount of chlorophyll (5 μg). The protein amounts were obtained by densitometric evaluation of the Western blots (means ± S.E.; $n = 5$, $^{***}p<0.001$, $^{**}p<0.01$).

However, as shown by D1 protein quantification, even photoinhibition of WD-leaves does not seem to be related to loss of the D1 protein in the PS2 RC. In contrast, in C-leaves (Fig. 5), substantial D1 degradation was coincident with photoinhibition. This confirms our hypothesis that in WD-leaves, similar to cold-acclimated spinach (Thiele *et al*. 1996), the treatments with excess photons show that the D1 protein is not susceptible to photoinactivation.

Hence we suggest that high degree of photoinhibition in the C-leaves indicated by a strong decrease in the F_v/F_m ratio, an increase of F_0, marked inhibition of PS2 activity, and significant loss of D1 protein probably reflect the reaction of photosynthetic system to excess absorbed photon energy. In contrast, less inhibition of all parameters was observed in WD-leaves during HI treatment. We conclude that WD-grown leaves are tolerant to HI and C-leaves are more susceptible to it.

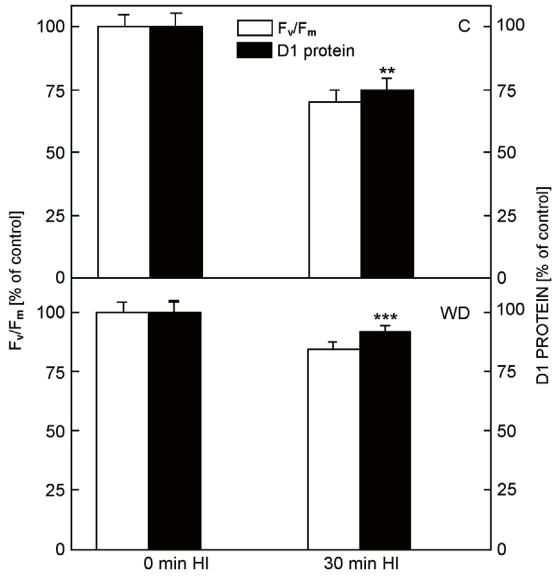

Fig. 5. Quantification of D1 protein and degree of photoinhibition in control (C) and water deficit (WD) leaves treated with high irradiance (HI). Data are given in % of non-photoinhibited controls (means ± S.E.; $n = 5$, $^{***}p<0.001$, $^{**}p<0.01$).

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