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Photoinhibition of photosynthesis in leaves of grapevine (*Vitis vinifera* L. cv. Riesling). Effect of chilling nights

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Abstract

Photoinhibition of photosynthesis was investigated in control (C) and chilling night (CN) leaves of grapevine under natural photoperiod 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 potential efficiency of photosystem (PS) 2, F_v/F_m was measured at midday, it markedly declined with significant increase of F_0 in CN leaves. In isolated thylakoids, the rate of whole chain and PS2 activity were markedly decreased in CN leaves than control leaves at midday. A smaller inhibition of PS1 activity was also observed in both leaf types. Later, the leaves reached maximum PS2 efficiencies similar to those observed in the morning during sampling at evening. The artificial exogenous electron donors diphenyl carbazide, NH_2OH, and Mn^{2+} failed to restore the PS2 activity in both leaf types at midday. Thus CN enhanced inactivation on the acceptor side of PS2 in grapevine leaves. Quantification of the PS2 reaction centre protein D1 following midday exposure of leaves showed pronounced differences between C and CN leaves. The marked loss of PS2 activity in CN leaves noticed in midday samples was mainly due to the marked loss of D1 protein of the PS2 reaction centre.

Additional keywords: acceptor side; chlorophyll fluorescence; D1 protein; diurnal course of photosynthetic activities; electron transport; Mn; NH_2OH; photosystems 1 and 2; photoinhibition.

Introduction

Light is essential for plant growth and development. However, too much of it may become harmful. The term photoinhibition relates to light-induced inhibition of light reactions of photosynthesis. It results from the absorption of photon energy in excess of the leaf capacity to utilize it for productive electron transport. Photoinhibition is a wide term, ranging from a protective, readily reversible regulatory mechanism of PS2 centres, to the accumulation of irreversible photodamage (Kyle and Ohad 1986, Osmond 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).

Low temperature is a major factor limiting the geographical locations suitable for crop growth and periodically accounts for significant losses in plant production. Short-term exposure of plants to low temperature usually inhibits net photosynthesis due to accumulation of soluble sugars (Ebrahim *et al*. 1998, Hurry *et al*. 1998). The photosystems are the primary targets for chilling-induced photoinactivation. In some chilling-sensitive plant species inhibition of photosynthetic electron transport can occur, despite relatively minimal reductions in F_v/F_m due to net photoinactivation of photosystem (PS) 1 rather than PS2 (Tjus *et al*. 1998, Sonoike 1999, Hendrickson *et al*. 2004).

Low temperature-induced stress limits growth of grapevine (Buttrose 1969), which is an economically important C_3 crop in many parts of the world. However,

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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_m – maximum fluorescence; MV – methyl viologen; PPFD – photosynthetic photon flux density; PS – photosystem; SDS-PAGE – sodium dodecylsulphate-polyacrylamide gel electrophoresis; SiMo – silicomolybdate.
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grapevine leaves in the field remain relatively resilient to low temperature-induced net photoinactivation of PS2 based on sustained, high variable chlorophyll (Chl) fluorescence (Chaumont *et al*. 1997, Flexas *et al*. 2001, Hendrickson *et al*. 2003, 2004). This implies one or more highly efficient energy dissipation mechanism(s) are induced in grapevine leaves by the combination of low temperature and high irradiance.

The damage resulting from excess photon absorption may also be extenuated by other environmental factors. Essentially any perturbation, which decreases the rate of photosynthesis, will lead to an increased susceptibility to excess photon absorption. Exposure to the combination of low temperature and irradiance produces large decreases in both the maximal quantum yield of carbon assimilation (Long *et al*. 1983, Ortiz-Lopez *et al*. 1990). Low temperature affects photoinhibition at several levels. It diminishes the enzymatic reactions of electron transport and carbon metabolism, as well as the stroma-grana lateral diffusion and protein synthesis involved in repair of damaged PS2 centres. Chilling under irradiation enhances photoinhibition in both chilling-sensitive and chilling tolerant plants, and the basic mechanism of photoinhibition, in so far as PS2 is involved, appears to be the same (Krause 1994).

The D1 reaction centre protein of PS2 is a target of irradiation-induced damage to the PS2 complex; turnover of the D1 protein is accelerated by increasing irradiance (Aro *et al.* 1993). The hypothesis that degradation of D1 protein may regulate the functioning of the PS2 repair cycle under photoinhibitory conditions has arisen from experiments with higher plants acclimated to different growth irradiances. Like in cold-acclimated spinach, the D1 protein is stabilized in young canopy leaves and in D1 inactivation, and that turnover takes little part in photoinhibition and recovery. Plants that are capable of sustaining high rates of replacement of damaged D1 protein show little reduction in quantum efficiency of O_2 evolution or F_v/F_m (Andersson and Aro 2001). However, in higher plants the D1 repair is generally limited at low temperatures because D1 proteolysis is slowed down, thus preventing the integration of newly synthesized D1 protein and consequently increasing photoinhibitory damage (Aro *et al*. 1990). For grapevine leaves, Chaumont *et al*. (1995) demonstrated that 80 % of initial D1 pool size was retained during a 5 °C and 1 500 μmol m^{-2} s^{-1} treatment over several hours, suggesting that the rate of repair is still considerable at low temperatures in contrast to the results of other C_3 plants.

In the present work we focused on the photoinhibitory response and photosynthetic performance of grapevine plants following their exposure to chilly nights (CN). The effect of photoinhibition was analyzed with respect to photochemical efficiency of PS2 (F_v/F_m), photosynthetic electron transport activities, and D1 protein content in grapevine leaves under natural photoperiod at different sampling time in a day.

Materials and methods

Plants and treatment: One-year old cuttings of *Vitis vinifera* L. cv. Riesling were grown in 10 000 cm^3 pots under natural photoperiod. Plants received full solar irradiance for most of the day in a 12 h photoperiod. The maximum irradiance available at the top of the plant was 1 700 μmol m^{-2} s^{-1} on a clear day. Irradiance and temperature were measured as in Iacono *et al.* (1994). The plants were watered regularly with nutrient solution.

Chilly-night–sunny-day treatments were carried out as follows: potted plants were transferred in the evening into walk-in growth chamber (*Lab line*, model *104 A*, USA) for overnight (18:00 to 06:00) exposure in the dark. On the following morning they were transferred to open full sun light for the rest of the day. Control (C) plants were treated similarly, except for overnight incubation at 23 rather than 6 °C.

All measurements were made at five different sunny days (August 2001) and different sampling time intervals during the day, early in the morning (06:00, solar time), midday (12:00, solar time), and evening (19:00, solar time) on sunset.

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 radiation of 0.6 kHz; photosynthetic photon flux density (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 ($H_2O\rightarrow$ methyl viologen, MV) and partial reactions of photosynthetic electron transport mediated by PS2 ($H_2O\rightarrow$2,6-dichloro-p-benzoquinone, DCBQ; $H_2O\rightarrow$silicomolybdate, SiMo) and PS1 ($DCPIPH_2\rightarrow MV$) were measured as described by Nedunchezhian *et al.* (1997). Thylakoids were suspended at 10 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.

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, 100 μM DCPIP, and thylakoid

membranes equivalent to 20 μg of Chl. Where mentioned, the concentrations of $MnCl_2$, DPC, and NH_2OH were 5, 0.5, and 5 mM, respectively.

Immunological determination of D1 protein was done by Western blotting. Thylakoids were solubilized 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 electro-blotting 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, Jerusalem, Israel). The densitometry analysis of Western blots was performed with a *Bio-Image* analyzer (*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 PS2 photochemical efficiency, measured as F_v/F_m of leaves sampled in the morning was 0.778 and 0.734 for C and CN leaves, respectively (Fig. 1). For leaves measured at midday (13:00 solar time) on a fully sunny day, the F_0 increased significantly in CN leaves while it slightly increased in C leaves. The F_v/F_m ratio decreased by 0.685 in control and 0.528 in CN leaves (Fig. 1). In samples prepared in the evening (19:00, solar time) on sunset, F_0 decayed significantly in CN leaves and F_v/F_m ratio reached values of around 0.776 and 0.730 in C and CN leaves, respectively.

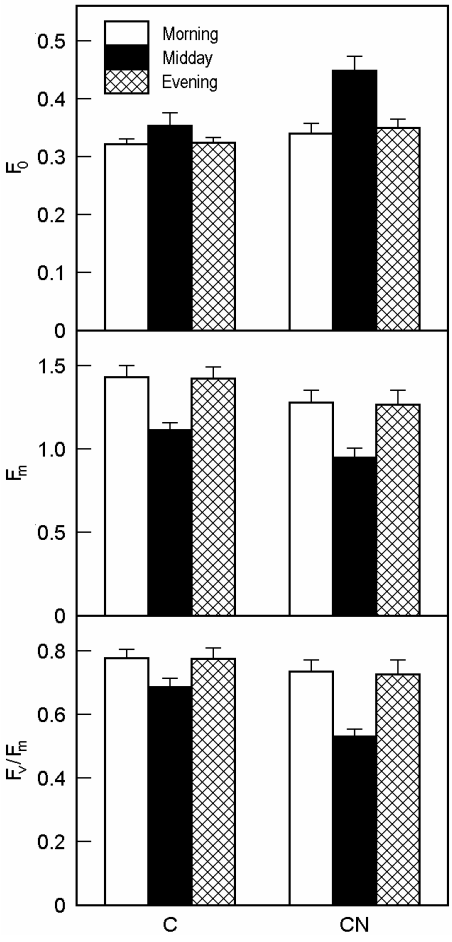

Fig. 1. Changes in the relative levels of fluorescence emitted as minimal fluorescence (F_0), maximal fluorescence (F_m), and the ratio of variable to maximal fluorescence (F_v/F_m) of control (C) and chilly night (CN) leaves at different sampling time intervals in a day (mean±S.E.; n = 5).

Changes in photosynthetic activities (Fig. 2): Photosynthetic electron transport from H_2O→DCBQ and H_2O→SiMo, measured at midday, was reduced by about 18 and 2 % and 38 and 4 % in C and CN leaves, respectively. A significant reduction of PS2 activity was noticed with DCBQ, but not with SiMo as electron acceptor in CN leaves than in C leaves. Evening samples reached PS2 activity similar to those observed early in the morning in both leaf variants (Fig. 2). A small inhibition of PS1 activity was also observed in C and CN 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) C and CN leaves. Wydrzynski and Govindjee (1975) showed that $MnCl_2$, DPC, NH_2OH, and HQ could donate the electrons to PS2. The PS2 activity was reduced to about 19 and 35 % in C and CN leaves, when water served as electron donor. The PS2 activity was not restored using donors of $MnCl_2$, DPC, and NH_2OH in both leaf variants (Fig. 3).

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 (Lutz *et al.* 1992). Photoinhibition-induced inhibition of PS2 activity in thylakoids of C and CN leaves was compared with changes in the relative content of D1 protein as determined by Western

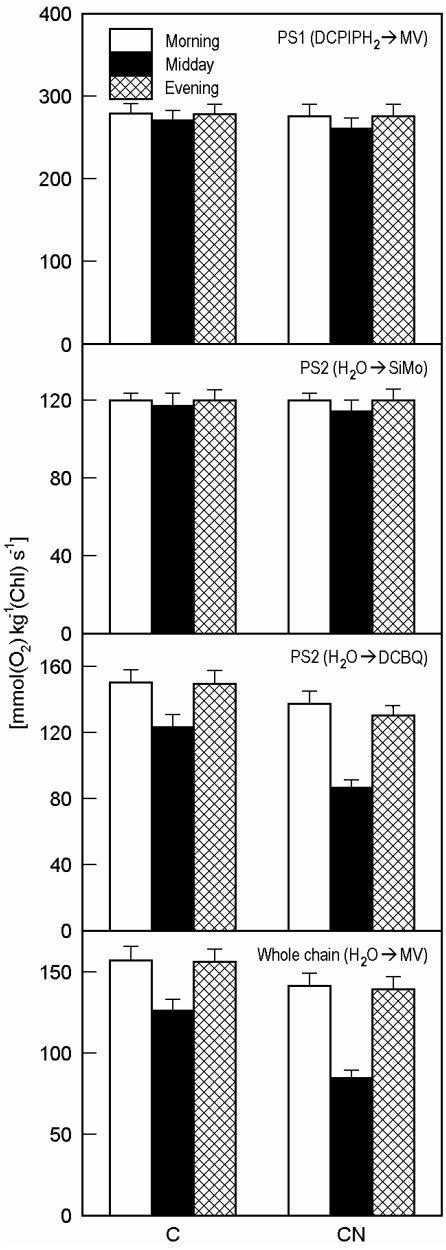

Fig. 2. Changes in the rates of whole chain (H_2O→MV), PS2 (H_2O→DCBQ; H_2O→SiMo), and PS1 ($DCPIPH_2$→MV) electron transport activities in thylakoids isolated from control (C) and chilly night (CN) leaves at different sampling time intervals in a day (mean±S.E.; n = 5).

blotting followed by quantification by the *Bio-Image* apparatus (Fig. 4). The relative content of D1 protein decreased to 14 and 32 % in C and CN leaves sampled at midday, respectively. At evening, the leaves reached net D1 protein contents that were similar to those observed at early morning (Fig. 4).

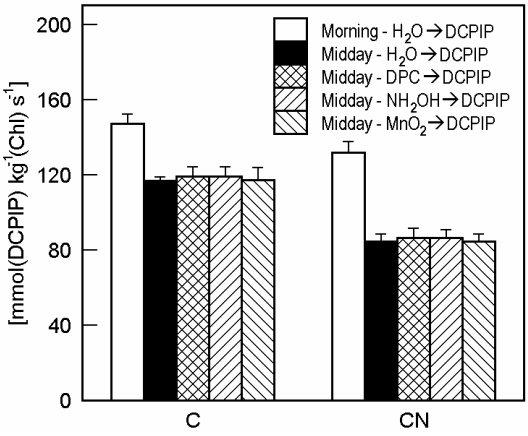

Fig. 3. Effect of various exogenous electron donors on photosystem 2 (PS2) activity (H_2O→DCPIP) in thylakoids isolated from control (C) and chilly night (CN) leaves sampling at midday (mean±S.E.; n = 5).

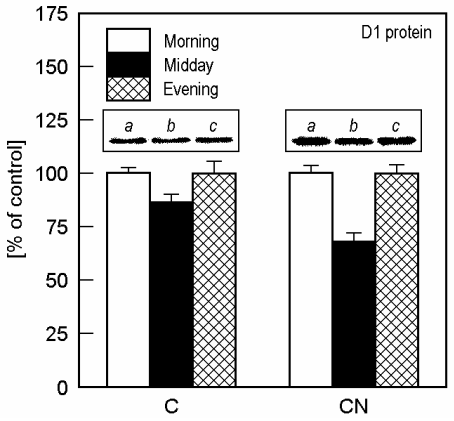

Fig. 4. Relative D1 protein content of thylakoids isolated from control (C) and chilly night (CN) leaves at different sampling time intervals in a day: *a*, morning; *b*, midday; *c*, evening.

In Fig. 5 the relative D1 protein contents and F_v/F_m ratios are compared after photoinhibitory treatments (midday) of C and CN leaves. In the C leaves, no significant D1 protein degradation could be attributed to the action of photoinhibitory irradiation, even when F_v/F_m ratios had decreased to 10–12 %. In contrast, CN leaves showed a strong decrease in D1 protein content together with the decline of F_v/F_m ratio by about 28 % (Fig. 5).

Discussion

Many plants are tolerant to high irradiance and their photosynthetic capacity is inhibited only when high irradiance is accompanied by additional stress such as the absence of sufficient CO_2 (Morot-Gaudry *et al*. 1986). A combination of water stress and high irradiance caused decrease in PS2 efficiency in *Nerium oleander* (Demmig *et al*. 1988). Similarly, in our experiments exposure to CN followed by high irradiance at midday induced severe

inhibition of photosynthetic activity and potential efficiency of PS2 (F_v/F_m) in grapevine leaves.

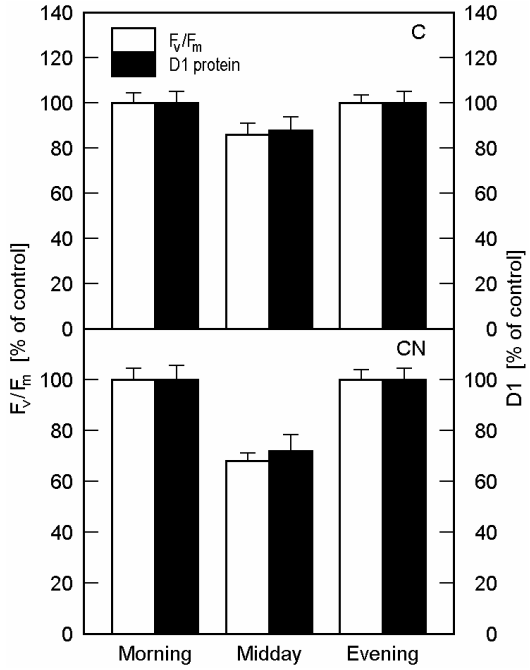

Fig. 5. Quantification of D1 protein and degree of photoinhibition in control (C) and chilly night (CN) leaves sampled at different time intervals in a day. The F_v/F_m ratios were determined as a measure of photoinhibition. % of untreated controls (morning) (mean±S.E.; n = 5).

A good PS2 activity was measured as the F_v/F_m ratio in both leaf variants, when sampled in the morning. In contrast, when sampling was made at midday, the F_v/F_m ratio was markedly decreased in CN leaves. Similarly, the measured F_v/F_m ratio decreased by approximately 55 % in *Ilex aquifolium* leaves exposed to 550 µmol m^{-2} s^{-1} at 8 °C (Groom *et al*. 1991). The PS2 photochemistry of both C and CN leaves was also dependent on the time of sampling made, decreasing from morning to midday, being minimal at midday and increasing again from the midday to evening (sunset). In the evening, both leaves reached maximum PS2 photochemical efficiencies similar to those observed early in the morning. This suggests that photosynthesis in vine leaves is a robust process even in non-acclimated plants grown in largely suboptimal conditions. The rate of recovery agrees well with other reports on photoinhibition in *Vitis vinifera* (Chaumont *et al*. 1995). The decrease of F_v/F_m ratio was mainly due to decrease of variable fluorescence (F_v) and marked increase of F_0 in CN leaves. It has been proposed that an increase of F_0 may be induced by the inactivation of part of PS2 reaction centres (Critchley and Russell 1994, Yamane *et al.* 1997). Our experimental results from CN 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.* 1994). These studies show that plants require variable 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 showed that the oxygen evolution was inhibited markedly when the electron acceptor used was DCBQ, but not when it was SiMo in both C and CN leaves. This clearly indicates that both leaf variants were affected at the reducing side of PS2, more extensively in CN leaves due to high irradiance. This is also supported by our Chl fluorescence studies where F_0 markedly increased. If the acceptor side of PS2 is photoinhibited the F_0 level is significantly increased (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 both C and CN. Among the artificial electron donors tested $MnCl_2$, DPC, and NH_2OH donate electrons in the PS2 reaction centre (Wydrzynski and Govindjee 1975). Using DPC, NH_2OH, and $MnCl_2$ in both C and CN leaves did not restore the loss of PS2 activity. Hence CN leaves were severely affected on the acceptor side of PS2 by 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 C and CN leaves during photoinhibition was mainly due to loss of PS2 reaction centre D1 protein. This could be confirmed by the immunological determination of the PS2 reaction centre D1 protein. It is often thought that photoinhibition is a result of marked loss of D1 protein in CN leaves, so it occurs only when the rate of damage to D1 protein exceeds the rate of its repair (Ohad *et al*. 1985, Carpentier 1995). D1 protein repair is also a highly important process at low temperature in grapevine leaves. This is surprising given that the D1 repair process has been reportedly suppressed by low temperature in *Capsicum* (Lee *et al*. 1999) and even completely inhibited at 4 °C in pumpkin (Salonen *et al*. 1998). Cold acclimation of plants includes a de-saturation of glycerolipids within the thylakoid membrane that accelerates the processing of precursor D1 protein (Murata and Nishiyama 1998). It is therefore expected that cold-acclimated field grown vines would have an even greater resilience to photoinactivation at low leaf temperatures (Hendrickson *et al*. 2004). Moreover, the fluorescence parameter F_v/F_m is considered 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 CN leaves is similar to such case. Both increased F_0 and the decreased F_v/F_m caused by high irradiance during midday could almost completely recover in the evening (Fig. 1). Marked losses of D1 protein also occurred, as induced by high irradiance during midday and completely recovered in the evening

(sunset).

Thus our results suggest that CN treatment markedly enhances photodamage, which develops in sun exposed vine leaves on the following day. We found that the observed decrease in PS2 efficiency caused by CN is due to both marked decrease of F_v/F_m and loss of the PS2 reaction centre D1 protein. Our results also suggest that photoinactivation of PS2 was correlated well with loss of D1 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 (Smith *et al*. 1990, Flexas *et al*. 2001). We also conclude that CN increased the photoinhibition in grapevine plants during high irradiance in midday.

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