

Photoinhibition of photosynthesis in *Vitis berlandieri* and *Vitis rupestris* leaves under field conditions

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Abstract

Photoinhibition of photosynthesis was investigated in *Vitis berlandieri* and *Vitis rupestris* 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 PS2, F_v/F_m , markedly declined, F_0 increased significantly in leaves of *V. berlandieri*, while F_0 did not increase in *V. rupestris* leaves. Isolated thylakoids of leaves of *V. berlandieri* showed significant inhibition of whole chain and PS2 activities at midday. A smaller inhibition was observed for *V. rupestris*. 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 species, while DPC and NH_2OH significantly restored PS2 activity in *V. rupestris* 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 *V. berlandieri* and *V. rupestris* leaves. The marked loss of PS2 activity noticed in midday samples was mainly due to the marked loss of D1 protein in *V. berlandieri* while in *V. rupestris* it was the 33 kDa protein.

Additional key words: chlorophyll fluorescence; donor side; electron transport; photosystem 2.

Introduction

Diurnal and seasonal changes of irradiance in the field range from near darkness to levels well in excess of what is required for maximum photosynthesis. Excess irradiation has the potential to damage the photosynthetic apparatus and plants have evolved strategies to minimise its detrimental effects. For example, some plants move their leaves or chloroplasts away from the sun so as to reduce the radiation absorbing area and load (Hirota *et al.* 1983). Others have evolved metabolic processes that reduce the effects of excess photon energy and thus protect the photosynthetic apparatus from permanent damage.

Photoinhibition is most commonly connected with photodamage, a long-term depression of quantum efficiency due to damage to the photosynthetic apparatus as the result of an excess of photosynthetic photon flux density, PPF (Demmig-Adams and Adams 1992, Walters and Horton 1993). Chronic photoinhibition may be considered as the depression of photosynthetic efficiency

from which the plant does not recover even after 3-4 d in shade (Greer and Laing 1992). On the other hand, canopy shade is also not advantageous for photosynthesis of grapevine (Bertamini and Nedunchezian 2001).

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. Ogren and Rosenqvist (1992) argue that moderate photoinhibition is common in field-grown plants. Studies 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 and Rosenqvist 1992, Öquist *et al.* 1992). Lovelock *et al.* (1994) found that rain forest species differ in their

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Abbreviations: Chl – chlorophyll; DCBQ – 2,6-dichloro-*p*-benzoquinone; DCP – 2,6-dichlorophenol indophenol; DPC – diphenyl carbazide; F_0 – minimal fluorescence; F_m – maximum fluorescence; MV – methyl viologen; PPF – photosynthetic photon flux density; PS – photosystem; SDS-PAGE – sodium dodecylsulphate-polyacrylamide gel electrophoresis; SiMo – silicomolybdate.

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capacity to recover from sudden exposure to high PPFD. The capacity for recovery corresponds 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 photosystem 2 (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, Prášil *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 high irradiance 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

Materials and methods

Plants: Leaves of *V. berlandieri* and *V. rupestris* were collected from selected 10-year-old seedlings grown in the field on training system with upright growing shoots (Cordon Royat) condition in Istituto Agrario di San Michele all' Adige, Italy. All measurements were made at five different sunny days (August 2001) and different sampling time intervals during the day, early in the morning (6 h, solar time), midday (12 h, solar time), and evening (19 h, solar time) on sunset. During experimental period the daily maximum PPFD on clear days was 2 000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The maximum temperature was 32–33 °C during the experimental period. PPFD 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, FRG). 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; PPFD was less than 0.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the leaf surface. F_m was measured at 20 kHz with a 1-s pulse of 6 000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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 ($\text{H}_2\text{O} \rightarrow \text{MV}$) and partial reactions of photosynthetic electron transport mediated by PS2 ($\text{H}_2\text{O} \rightarrow \text{DCBQ}$; $\text{H}_2\text{O} \rightarrow \text{SiMo}$) and PS1 ($\text{DCPIP} \rightarrow \text{MV}$) were measured as described by Nedunchezian *et al.* (1997). Thylakoids were suspended at 10 g(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

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.

To our knowledge, the importance of photoinhibition in grapevines has not been evaluated to date in the field. There is evidence from pot experiments that *Vitis vinifera* (Downton 1983) and the related wild grape *Vitis californica* (Gamon and Percy 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 leaves of *Vitis berlandieri* and *Vitis rupestris* 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 *V. berlandieri* and *V. rupestris* leaves under field conditions at different sampling time in a day.

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 of Chl. Where mentioned, the concentrations of MnCl_2 , DPC, and NH_2OH were 5.0, 0.5, and 5.0 mM, respectively.

Immunological determination of thylakoid proteins was done 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, Jerusalem, 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 PS2 photochemical efficiency, measured as F_v/F_m of leaves sampled early in the morning (6 h, solar time), was 0.779 and 0.792 for *V. berlandieri* and *V. rupestris*, respectively (Fig. 1). For leaves measured at midday (12 h, solar time) on a fully sunny day, the F_0 increased significantly in *V. berlandieri* while F_0 did not increase in *V. rupestris*. The F_v/F_m ratio decreased to around 0.516 in *V. berlandieri*, and 0.690 in *V. rupestris* (Fig. 1). In samples prepared in the evening (19 h, solar time) on sunset, F_0 decayed significantly in *V. berlandieri* and F_v/F_m reached values of around 0.764 and 0.785 in *V. berlandieri* and *V. rupestris*, respectively.

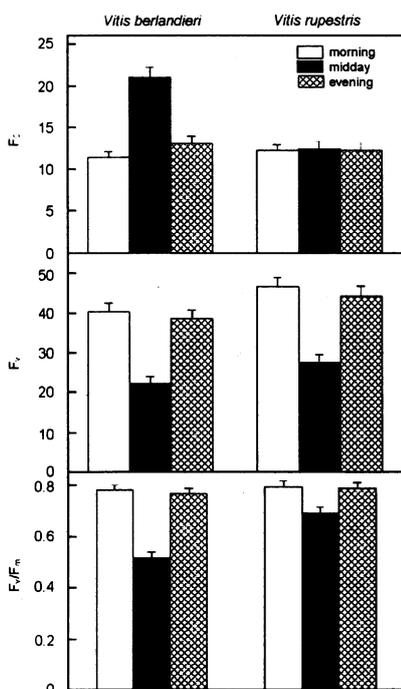


Fig. 1. Changes in the relative levels of fluorescence emitted as minimal fluorescence (F_0) or variable fluorescence (F_v), and the ratio of variable to maximum fluorescence (F_v/F_m) of *Vitis berlandieri* and *Vitis rupestris* leaves at different sampling time intervals in a day. Means \pm standard errors of 5 replicates.

Changes in photosynthetic activities: Photosynthetic electron transport activities were measured in thylakoids isolated from *V. berlandieri* and *V. rupestris* leaves at different sampling times in a day (Fig. 2). In contrast to morning, photosynthetic electron transport from $H_2O \rightarrow DCBQ$ and $H_2O \rightarrow SiMo$, measured at midday, was reduced by about 39 or 4, and 3 or 18 % in *V. berlandieri* and *V. rupestris* leaves, respectively. A significant reduc-

tion of PS2 activity was noticed with DCBQ, but not with SiMo as electron acceptor in *V. berlandieri* (Fig. 2). In contrast, a reduction of PS2 activity was noticed only when the electron acceptor was SiMo in *V. rupestris*. Evening samples reached PS2 activity similar to those observed early in the morning in both plant species (Fig. 2). A small decline in PS1 activity was observed only in *V. berlandieri* leaves sampled at midday (Fig. 2).

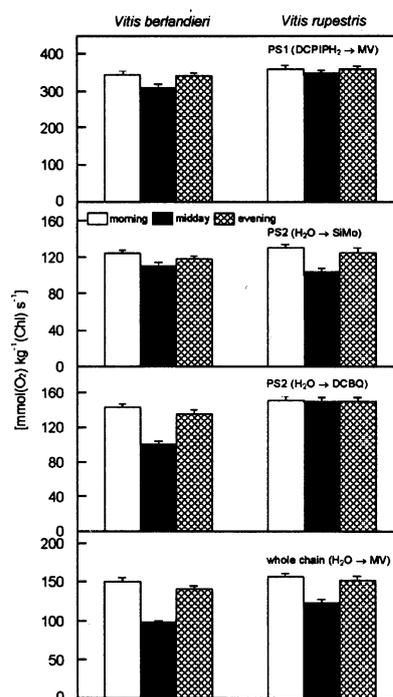


Fig. 2. Changes in the rates of whole chain ($H_2O \rightarrow MV$), PS2 ($H_2O \rightarrow DCBQ$; $H_2O \rightarrow SiMo$), and PS1 ($DCPIP \rightarrow MV$) electron transport activities in thylakoids isolated from *Vitis berlandieri* and *Vitis rupestris* leaves at different sampling time intervals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

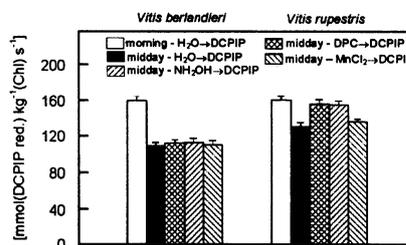


Fig. 3. Effect of various exogenous electron donors on PS2 activity ($H_2O \rightarrow DCPIP$) in thylakoids isolated from *Vitis berlandieri* and *Vitis rupestris* leaves sampling at midday. Means \pm standard errors of 5 replicates.

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 *V. berlandieri* and *V. rupestris*. Wydrzynski and Govindjee (1975) found that $MnCl_2$, DPC, NH_2OH , and HQ could donate the electrons to PS2. In our experiments, PS2 activity was reduced to about 31 and 18 % in *V. berlandieri* and *V. rupestris*, when water served as electron donor (Fig. 3). A similar trend was also found using $MnCl_2$ as donor in both plant species. A significant restoration of PS2 mediated DCPIP reduction was observed when NH_2OH and DPC were used as electron donor in *V. rupestris*. In contrast, the PS2 activity was not restored using either DPC or NH_2OH in *V. berlandieri* leaves (Fig. 3).

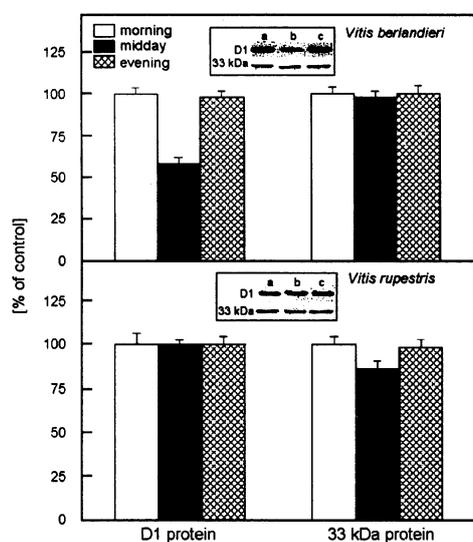


Fig. 4. Relative D1 and 33 kDa protein contents of thylakoids isolated from *Vitis berlandieri* and *Vitis rupestris* leaves at different sampling time intervals in a day. Inserts: Lane a, morning; lane b, midday; lane c, evening.

Changes in contents of D1 and 33 kDa proteins by immunoblot: Photoinhibition of PS2 induces breakdown of the D1 protein (Andersson and Styring 1991, 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 (Smith *et al.* 1990,

Discussion

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

A good PS2 activity was measured as the F_v/F_m ratio in both leaves when sampled early in the morning. In contrast, when sampling was made at midday, the F_v/F_m was markedly decreased in *V. berlandieri*. The PS2 photochemistry of both *V. berlandieri* and *V. rupestris* leaves

Lütz *et al.* 1992). Photoinhibition of PS2 activity in thylakoids of *V. berlandieri* and *V. rupestris* leaves was compared with changes in the relative contents of D1 and 33 kDa proteins as determined by Western blotting followed by quantification by the *Bio-Image* apparatus (Fig. 4). The relative contents of D1 and 33 kDa proteins decreased to 42 and 2 % in *V. berlandieri*, and to 3 and 14 % in *V. rupestris* leaves sampled at midday. In the evening, the leaves reached net D1 or 33 kDa protein contents that were similar to those observed in early morning (Fig. 4).

In Fig. 5 relative D1 protein contents and F_v/F_m ratios are compared after photoinhibitory treatments (midday) of *V. berlandieri* and *V. rupestris* leaves. In the *V. rupestris* leaves, no significant D1 degradation could be attributed to the action of photoinhibitory PPFD, even when F_v/F_m ratios had decreased to 10-13 % of the controls. The *V. berlandieri* leaves showed a strong decrease in D1 protein content together with the decline of F_v/F_m ratio by about 38 % (Fig. 5).

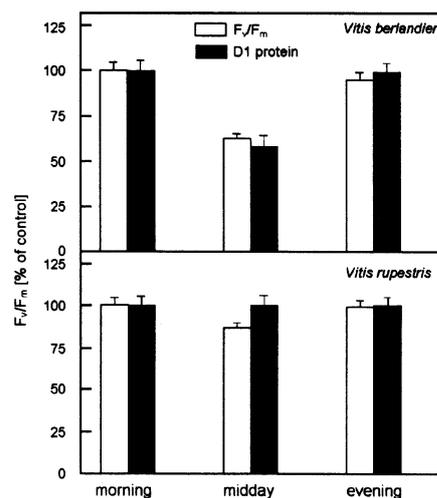


Fig. 5. Quantification of D1 protein and degree of photoinhibition in *Vitis berlandieri* and *Vitis rupestris* leaves sampled at different time intervals in a day. The F_v/F_m ratios were determined as a measure of photoinhibition. % of untreated controls (morning samples). Means \pm standard errors of 5 replicates.

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, *V. berlandieri* leaves reached maximum PS2 photochemical efficiencies similar to those observed early in the morning. The rate of recovery agrees well with other reports on photoinhibition in higher plants, for instance in Ögren *et al.* (1984) where complete recovery of photosynthesis by *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 *V. rupestris* while in *V. berlandieri* it was decrease of F_v and marked increase of F_0 . An increase of F_0 may be induced by the inactivation of part of PS2 reaction centres (Govindjee 1990, Critchley and Russell 1994, Yamane *et al.* 1997). Our results from *V. berlandieri* leaves are in accord 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 conclude that plants require variable times for recovering sufficient PS2 photochemistry after midday depressions (Demmig-Adams and Adams 1992).

When analysing the electron transport activities in thylakoids isolated from midday leaves of *V. berlandieri*, it appeared that the oxygen evolution was inhibited markedly when the electron acceptor used was DCBQ, but not when it was SiMo. This clearly indicates that *V. berlandieri* leaves are affected at the reducing side of PS2 due to photoinhibition. This is also supported by our Chl fluorescence studies where F_0 markedly increased. In contrast to this, in thylakoids isolated from *V. rupestris* the rate of PS2 activity observed with SiMo is lower than the one 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 *V. rupestris* leaves without affecting the F_0 level. This is characteristic for 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 *V. berlandieri* and *V. rupestris*. Among the artificial electron donors tested, DPC and NH_2OH donate electrons in the PS2 reaction centre (Wydrzynski and Govindjee 1975). A significant restoration of PS2 activity was observed in *V. rupestris* by the addition of DPC and NH_2OH . This is in good agreement with findings that water-oxidising system is sensitive to high PPFD (Veeranjaneyulu *et al.* 1998), while in *V. berlandieri* leaves by using DPC or NH_2OH the loss of PS2 activity was not restored. Thus the *V. berlandieri* 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 PPFD (Schieffthaler *et al.* 1999).

The loss of PS2 activity in *V. berlandieri* and *V. rupestris* leaves during 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 water-splitting complex.

Photoinhibition is usually explained by a 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 1997). Moreover, the fluorescence parameter F_v/F_m may be 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 *V. berlandieri* leaves agrees with such case. Both increased F_0 and the decreased F_v/F_m caused by high PPFD during midday could almost completely recover in the evening (Fig. 1). Marked losses of D1 protein also occurred, as induced by high PPFD during midday and completely recovered in the evening (sunset). In contrast, no loss of D1 protein amount was found in *V. rupestris* after high PPFD on midday.

As shown by D1 protein quantification, even strong photoinhibition of *V. rupestris* leaves does not seem to be related to inactivation and degradation of the D1 protein in the PS2 reaction centre. In contrast, in *V. berlandieri* leaves substantial D1 inactivated appeared to be coincident with photoinhibition. Depending on species and leaf age, acclimation to high PPFD probably results in differing degrees of D1 stabilisation (Thiele *et al.* 1997).

We suggest that *V. berlandieri* is more susceptible to photoinhibition as compared with *V. rupestris*. This is mainly due to the marked reduction of F_v/F_m with significant increase of F_0 , marked loss of PS2 activity and D1 protein content. Our results also suggest that photoinactivation of PS2 is not correlated at all with net 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 (Krause 1988, Smith *et al.* 1990, Flexas *et al.* 2001). We also conclude that high PPFD induces changes on the acceptor side of PS2 in *V. berlandieri* and on the donor side in *V. rupestris*.

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