PHOTOSYNTHETICA 39 (4): 529-537, 2001

Decline of photosynthetic pigments, ribulose-1,5-bisphosphate carboxylase and soluble protein contents, nitrate reductase and photosynthetic activities, and changes in thylakoid membrane protein pattern in canopy shade grapevine (*Vitis vinifera* L. cv. Moscato giallo) leaves

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Abstract

In canopy shade leaves of grapevine (*Vitis vinifera* L. cv. Moscato giallo) grown in the field the contents of chlorophyll (Chl), carotenoids (Car), and soluble protein per fresh mass were lower than in sun leaves. RuBPC activity, *in vivo* nitrate reductase activity (indicator of nitrate utilisation), apparent electron transport rate, and photochemical fluorescence quenching were also significantly reduced in canopy shade leaves. When various photosynthetic activities were followed in isolated thylakoids, canopy shade leaves exerted a marked inhibition of whole chain and photosystem (PS) 2 activity. Smaller inhibition of PS1 activity was observed even in high-level canopy shade (HS) leaves. The artificial exogenous electron donors, DPC and NH_2OH, significantly restored the loss of PS2 activity in HS leaves. Similar results were obtained when F_v/F_m was evaluated by Chl fluorescence measurements. The marked loss of PS2 activity in canopy shade leaves was due to the loss of 47, 43, 33, 28-25, 23, 17, and 10 kDa polypeptides.

Additional keywords: carotenoids; chlorophyll; donor side; electron transport; fluorescence; nitrate reductase; photosystems 1 and 2.

Introduction

Both irradiance (*I*) and spectral quality regulate the photosynthetic properties of higher plants by controlling the activity and composition of the photosynthetic apparatus (Boardman 1977, Anderson 1986, Senger and Bauer 1987). Environmental *I* is critical during leaf expansion for determining the photosynthetic properties of the mature leaf. More recently, controlled environment studies with many plant species have shown that fully expanded leaves retain the capacity to fine-tune photosynthesis in response to change in growth *I* (Davies *et al.* 1986, Chow and Anderson 1987, De la Torre and Burkey 1990). Studies on PAR regulation of photosynthesis and chloroplast biochemistry often fall into one of three categories: (*1*) a comparison of plants from sun and shade habitats, (*2*) the response of plants to growth *I* with a constant spectral quality, or (*3*) the response of plants to spectral quality differences where blue, red, or far-red *I* are compared at low *I*. In these experiments, conditions are often different from those of a natural plant canopy where both the *I* and spectral quality surrounding individual leaves vary throughout the growing season.

Grapevine plants are normally planted at high densities and have many shoots that give rise to canopy closure. During the development of a closed grape canopy, some leaves expand under full sun and later function in extreme shade following the development of leaves at higher nodes. Leaf physiology responds to shade in one of two ways. First, extreme shade conditions can induce rapid senescence of lower canopy leaves several weeks in advance of senescence of the whole plant (Secor *et al.*

Received 1 June 2001, *accepted* 17 October 2001.

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Abbreviations: Car – carotenoids; Chl – chlorophyll; DCBQ – 2,6-dichloro-*p*-benzoquinone; DCPIP – 2,6-dichlorophenol indophenol; DPC – diphenylcarbazide; DTT – dithiothreitol; ETR – apparent electron transport rate; F_0 – minimal fluorescence; F_m – maximum fluorescence; *I* – irradiance; kDa – kilodalton; LHCP – light-harvesting chlorophyll protein; LSU – large subunit; MV – methyl viologen; PAR – photosynthetically active radiation; PS – photosystem; q_P – photochemical fluorescence quenching; q_N – non-photochemical fluorescence quenching; RuBPC – ribulose-1,5-bisphosphate carboxylase; SDS-PAGE – sodium dodecylsulphate-polyacrylamide gel electrophoresis; SiMo – silicomolybdate; SSU – small subunit.

Acknowledgements: This work was in part supported by a grant from the "Provincia Autonoma di Trento" and the "Consiglio Nazionale delle Ricerche" (CNR): project "Analisi e Ricerche per il sistema Agri-Industriale" sub-project "Prometavit".

1984, Wells 1991). A second type of response is acclimation of photosynthesis in shaded leaves that remain on the plant until senescence (Sailaja and Rama Das 2000).

Extensive research on sun-to-shade adaptations in leaves has demonstrated that low *I*-grown plants adapt to the *I*-limiting growth conditions by increasing the photon harvesting ability and alter chloroplast anatomy by increasing the amount of thylakoid membrane regions (Anderson 1986, Anderson *et al.* 1988). Adjustments to the contents of Chl *b*, to light-harvesting complex proteins (LHCP), and to additional changes in RuBPC and Q_B protein contents are mostly regulated in the shade adapted leaves by changes in gene expression at the transcriptional or post-transcriptional level (Senger and Bauer 1987). In the shade, additional changes in leaf photosynthetic performance result from ageing or senescence of the leaves in what has eloquently been described as orderly withdrawal of materials from the general economy of the plant (Woolhouse 1987). Thus the loss of photosynthetic activity with ageing, due to shade, occurs at the same time as Chl breakdown. This is observed as a yellowing of photosynthetic tissues and the orchestrated degradation of chloroplast processes in a step-wise fashion, including membrane proteins, stroma enzymes, and loss of chloroplast integrity (Woolhouse 1987, Nedunchezhian *et al.* 1995).

The grapevine canopy consists of leaves of different ages that are subjected to variable *I* during the entire growing season (Hunter and Visser 1988). According to Boardman (1977) the photosynthetic productivity of a leaf is primarily governed by its position in the plant canopy. Values of sunlight *I* measured in the centre of a dense canopy can be less that 10 μmol m^{-2} s^{-1} although above canopy (ambient) values are over 2 000 μmol m^{-2} s^{-1}. The reason for this large reduction is that grapevine leaves strongly absorb sunlight (Smart *et al.* 1990). Inside a dense canopy, associated with low *I* availability, premature senescence occurs. It was, there-fore, interesting to determine the changes in Chl content of the leaf as well as the relationship, if any, with different photosynthetic activities observed by Hunter and Visser (1988). In the present paper we report the influence of canopy shade on the changes in photosynthetic pigments, ribulose-1,5-bisphosphate carboxylase (RuBPC), soluble proteins, nitrate reductase, photosynthetic activities, and thylakoid membrane protein pattern in field-grown grapevine (*Vitis vinifera* L. cv. Moscato giallo) leaves.

Materials and methods

Plants: Leaves were collected from 15-year-old grapevines (*Vitis vinifera* L. cv. Moscato giallo) grown in the field on double armed pergola training system (sloping trellis with dense canopy) in the vineyards of the Istituto Agrario di San Michele all' Adige (TN), north-eastern Italy. Vineyard management was according to commercial practices used in the Trentino region. In order to simplify the experimental procedure, we classified the same leaf age samples (50-60-d-old) into three groups according to the *I* they received on the leaf surface, the canopy position, and their Chl content per unit area: high shade (HS) leaves were grown at a low canopy position under PAR of less than 15 μmol m^{-2} s^{-1}; low shade (LS) leaves were grown at a middle canopy position with a maximum of 80±25 μmol m^{-2} s^{-1}; sun (S) leaves were grown at a top canopy position and well irradiated (1 500±200 μmol m^{-2} s^{-1}) throughout most of the day and classified as control.

Pigment analysis: The measurement of Chl content using a *Minolta* Chl meter (*SPAD-502*, *Minolta*, Osaka, Japan) was reliably matched with that of conventional extraction using acetone. Chl was extracted with 100 % acetone from liquid N_2-frozen leaf discs and stored at –20 °C. Chl and Car were analysed spectrophotometrically according to Lichtenthaler (1987).

Modulated Chl fluorescence in leaves was measured on the upper surface of the leaf discs using a *PAM-2000* fluorimeter (*Walz*, Effeltrich, Germany) after 30 min dark adaptation. F_0 was measured by switching on the modulated radiation of 0.6 kHz; PAR 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".

Induction kinetics of fluorescence quenching coefficients q_P (photochemical quenching) and q_N (non-photochemical quenching) were determined by the saturation pulse method according to Schreiber *et al.* (1986) using 200 μmol m^{-2} s^{-1} of actinic radiation in air of ambient CO_2 concentration. Apparent electron transport rate (ETR, μmol m^{-2} s^{-1}) was calculated by the method of Genty *et al.* (1989).

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 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 DCBQ and 200 µM SiMo.

DCPIP photoreduction was determined as the decrease in absorbance at 590 nm using a *Hitachi 557* spectrophotometer (*Hitachi*, Tokyo, Japan). 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 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.

Soluble proteins were extracted by grinding two leaves (0.3-0.5 g fresh mass) in a mortar with 6 cm^3 of 100 mM Tris-HCl, pH 7.8, containing 15 mM $MgCl_2$, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 10 mM PMSF in the presence of liquid nitrogen. Homogenates were filtered through nylon cloth. After centrifugation at 11 000×*g* for 10 min, the concentration of soluble proteins was determined in the supernatant as reported by Bradford (1976).

Extracts and assay of RuBPC activity: Fully expanded leaves were cut into small pieces and homogenised in a grinding medium of 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 5 mM DTT, and 0.25 M EDTA. The extract was clarified by centrifugation at 10 000×*g* for 10 min. The clear supernatant was decanted slowly and used as the RuBPC. The assay of RuBPC activity was measured as described by Nedunchezhian and Kulandaivelu (1991).

Nitrate reductase activity: The fully expanded mature leaf tissue of 100 mg was suspended in a glass vial containing 5 cm^3 of the assay medium consisting of 100 mM KH_2PO_4-KOH, pH 7.0, 100 mM KNO_3, and 1 % (v/v) *n*-propanol. The vial was sealed and incubated in the dark at room temperature of 27 °C for 60 min. Suitable aliquots of the assay medium were removed for nitrate analysis. The amount of nitrate formed was expressed as NO_2^- formed (Jaworski 1971).

SDS-PAGE: Thylakoids and crude leaf extracts of RuBPC were separated using the discontinuous polyacrylamide gel system of Laemmli (1970), with the following modifications. The gels consisted of a 10-18 % gradient of polyacrylamide containing 4 M urea. Samples were solubilised at 20 °C for 5 min in 2 % (m/v) SDS, 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 Lowry *et al.* (1951).

Results

Chl and Car: When expressed on a fresh leaf mass basis, both low (LS) and high (HS) shade leaves showed Chl values lower than the sun (S) leaves (Table 1). A reduction of 38 % was observed in LS leaves as compared to 71 % at HS level. Similar changes were observed for Car (Table 1). The Chl *a/b* ratio was also markedly decreased in shade leaves. In contrast to this, the Car/Chl ratio was higher in canopy shade leaves than in S leaves (Table 1).

Photosynthetic electron transport activities: To obtain information on PS2 activity, the 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 effect of canopy shade was prominent on the variable part of fluorescence without changes in F_0. F_v and F_v/F_m were much lower in canopy shade leaves than in S leaves (Table 1).

However, when photosynthetic electron transport was studied using isolated thylakoids from S, HS, and LS leaves, the rate of $DCPIPH_2 \rightarrow MV$ (PS1) was about 8 % lower in LS and 16 % in HS leaves as compared with S leaves (Table 1). The PS2 activities measured as $H_2O \rightarrow DCBQ$ and $H_2O \rightarrow SiMo$ were about 17 and 29 % lower in LS and 24 and 55 % lower in HS leaves in comparison with S leaves (Table 1). A similar trend was also noticed for whole chain ($H_2O \rightarrow MV$) electron transport (Table 1).

To locate the possible site of inhibition in the PS2 reaction, we followed the DCPIP reduction supported by $MnCl_2$, DPC, and NH_2OH, that donate electrons in the

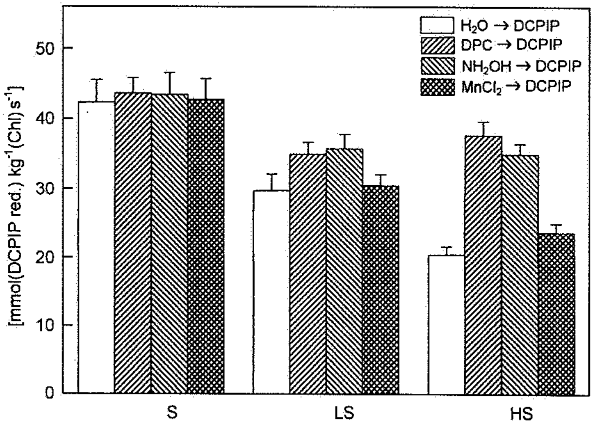

Fig. 1. Effect of various exogenous electron donors on photosystem 2 activity ($H_2O \rightarrow DCPIP$) in thylakoids isolated from sun (S) and different level canopy shade (HS, LS) leaves. Means ± standard errors of 3 replicates.

PS2 reaction (Wydrzynski and Govindjee 1975), in thylakoids of S, HS, and LS leaves (Fig. 1). In HS leaves the PS2 activity was reduced to about 50 % of that in S leaves when water or $MnCl_2$ served as electron donor. In contrast to this, a significant restoration of PS2 mediated DCPIP reduction was observed when NH_2OH and DPC were used as electron donors in both types of shade leaves (Fig. 1).

q_P, ETR, and q_N: The canopy shade induced an impaired PS2 photosynthetic electron transport measured with thylakoids *in vivo* (Table 1). Gradual increases of q_P and ETR and decreases of q_N were observed in S leaves (Fig. 2), while a slow increase of q_P and ETR (in particular in HS) and an increase of q_N that reached a steady state (with no decrease) in both types of shade leaves was observed (Fig. 2). After 280 s, q_P, q_N, and ETR were 0.206, 0.448, and 7.700 μmol m^{-2} s^{-1} in HS leaves (Fig. 2). Using an *I*-response curve between 5 to 280 μmol m^{-2} s^{-1} (PAR) served (Fig. 2). After 280 s, q_P, q_N, and ETR were 0.206, in S, HS, and LS leaves, a linear increase of ETR and the value of 70 μmol m^{-2} s^{-1} at maximum *I* was found in S leaves (Fig. 3). In contrast, a slow increase of ETR was observed in LS and HS leaves. The ETR saturation was reached at 200 μmol m^{-2} s^{-1} in LS and at 100 μmol m^{-2} s^{-1} in HS leaves (Fig. 3).

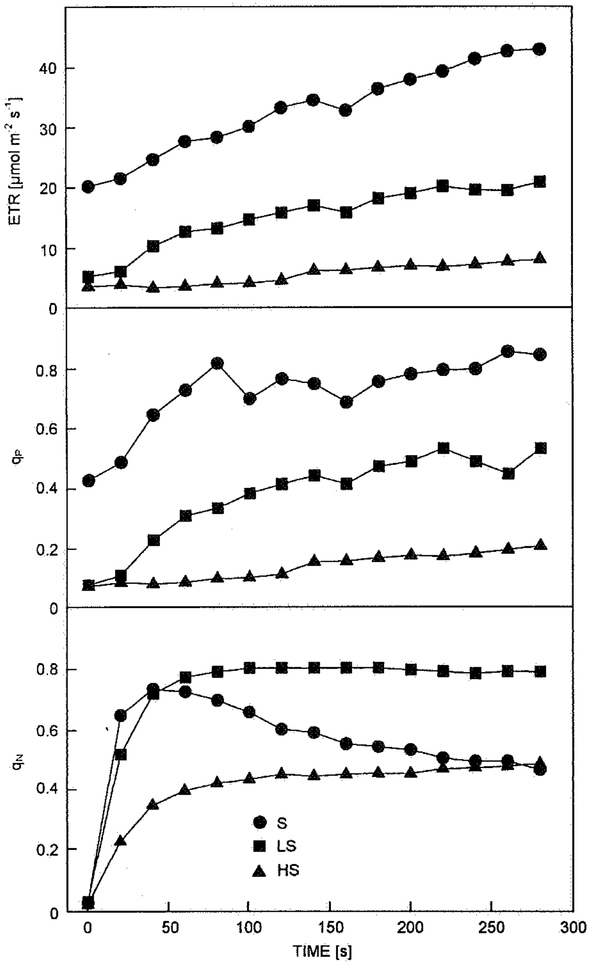

Fig. 2. Induction kinetics of fluorescence quenching coefficients q_P (photochemical quenching) and q_N (non-photochemical quenching) and apparent electron transport rate, ETR [μmol m^{-2} s^{-1}] determined from sun (S) and different level canopy shade (HS, LS) leaves. Values for a typical single experiment are shown.

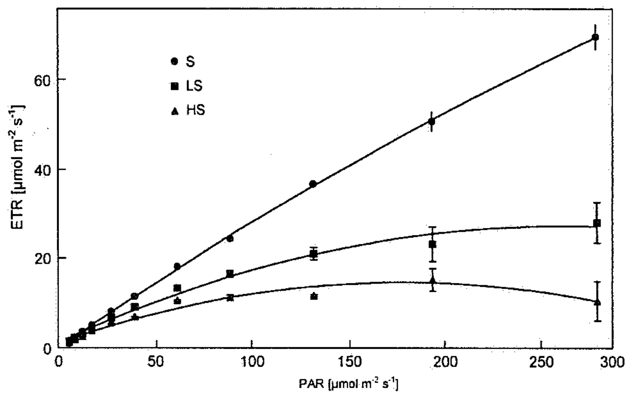

Fig. 3. Irradiance (PAR) response curve of apparent electron transport rate (ETR) measured in the steady state (13 min) at a PAR from 5 to 280 μmol m^{-2} s^{-1} of actinic irradiance. Means ± standard errors of 3 replicates.

Thylakoid membrane proteins: Since the differences in photosynthetic electron transport activities could be caused primarily by the changes or re-organisation of thylakoid components, the polypeptide profiles of S, HS, and LS thylakoids were analysed by SDS-PAGE. In comparison with S-leaves, a specific loss in the contents of polypeptides 47, 43, 33, 28-25, 23, 17, and 10 kDa was found in HS leaves (more pronounced) and LS leaves (Fig. 4*A*).

RuBPC activity and soluble proteins (Table 1): When the enzyme activity in crude leaf extracts was expressed on a protein basis, significantly less RuBPC activity was observed in HS (by 46 %) and LS (by 29 %) leaves than in S leaves. A similar trend was also noticed for soluble proteins (Table 1). The contents of both LSU (55 kDa) and SSU (15 kDa) of RuBPC were markedly affected in HS and LS leaves (Fig. 4*B*). The reduction was larger with increase of the canopy shade.

***In vivo* nitrate reductase activity** expressed on a fresh matter basis was decreased by 30 % in LS and 64 % in HS leaves in comparison with S leaves. When expressed per Chl unit, the enzyme activities were reduced by 42 and 71 %, respectively (Table 1). The S leaves incubated with 15 mM KNO_3 tended to have more induced nitrate reductase activity than canopy shade leaves (Table 1).

Table 1. Physiological and relative fluorescence induction parameters, rates of photosystem activities [nmol(O_2) kg^{-1}(Chl) s^{-1}], and nitrate reductase activities (in relation to various reference units or with and without KNO_3 fertilisation) of grapevine leaves collected from sun (S) and low (LS) or high (HS) shade canopy positions. For details see Materials and methods. Car = carotenoids, Chl = chlorophyll, PS = photosystem. Figures in parentheses are percent reduction with reference to respective sun leaves Means ± standard errors of 5 (10 for fluorescence parameters and 3 for nitrate reductase activities) replicates.

Parameter	S	LS	HS
Chl *a* [g kg^{-1}(f.m.)]	2.380±0.198	1.430±0.160 (40)	0.620±0.028 (74)
Chl *b* [g kg^{-1}(f.m.)]	0.880±0.040	0.590±0.025 (33)	0.330±0.022 (63)
Chl (*a*+*b*) [g kg^{-1}(f.m.)]	3.260±0.082	2.010±0.060 (38)	0.940±0.065 (71)
Chl *a/b*	2.71	2.44	1.90
Car [g kg^{-1}(f.m.)]	0.800±0.040	0.600±0.030 (25)	0.310±0.030 (62)
Car/Chl	0.24	0.29	0.32
Soluble proteins [g kg^{-1}(f.m.)]	46.2±3.4	33.7±3.10 (27)	24.1±2.5 (48)
RuBPC [nmol(CO_2) kg^{-1}(protein) s^{-1}]	52.7±4.5	37.4±3.31 (29)	28.4±2.8 (46)
F_0	115.0±3.1	112.0±3.0	110.0±2.8
F_v	520.0±8.4	280.0±4.6	190.0±3.1
F_v/F_m	0.818±0.040	0.714±0.035	0.633±0.030
Whole chain ($H_2O \rightarrow$ MV)	39.7±2.1	28.1±1.4 (30)	16.6±1.3 (52)
PS1 ($DCPIPH_2 \rightarrow$ MV)	92.3±4.2	84.9±3.7 (8)	77.6±3.9 (16)
PS2 ($H_2O \rightarrow$ DCBQ)	45.1±1.7	37.4±1.3 (17)	34.3±1.8 (24)
PS2 ($H_2O \rightarrow$ SiMo)	34.5±2.1	24.5±1.2 (29)	15.4±0.9 (55)
Nitrate reductase [nmol(NO_2) kg^{-1}(f.m.) s^{-1}]	68.18±1.04	47.7±0.46 (30)	24.81±0.25 (64)
Nitrate reductase [nmol(NO_2) kg^{-1}(protein) s^{-1}]	2.41±0.22	1.83±0.02 (24)	1.45±0.11 (40)
Nitrate reductase [nmol(NO_2) kg^{-1}(Chl) s^{-1}]	40.31±0.41	23.3±0.18 (42)	11.71±0.20 (71)
Nitrate reductase [nmol(NO_2) kg^{-1}(f.m.) s^{-1}] −15 mM KNO_3	38.12±3.11	31.25±1.88 (18)	25.14±2.10 (34)
Nitrate reductase [nmol(NO_2) kg^{-1}(f.m.) s^{-1}] +15 mM KNO_3	80.14±3.45	51.28±2.33 (36)	31.84±2.40 (60)

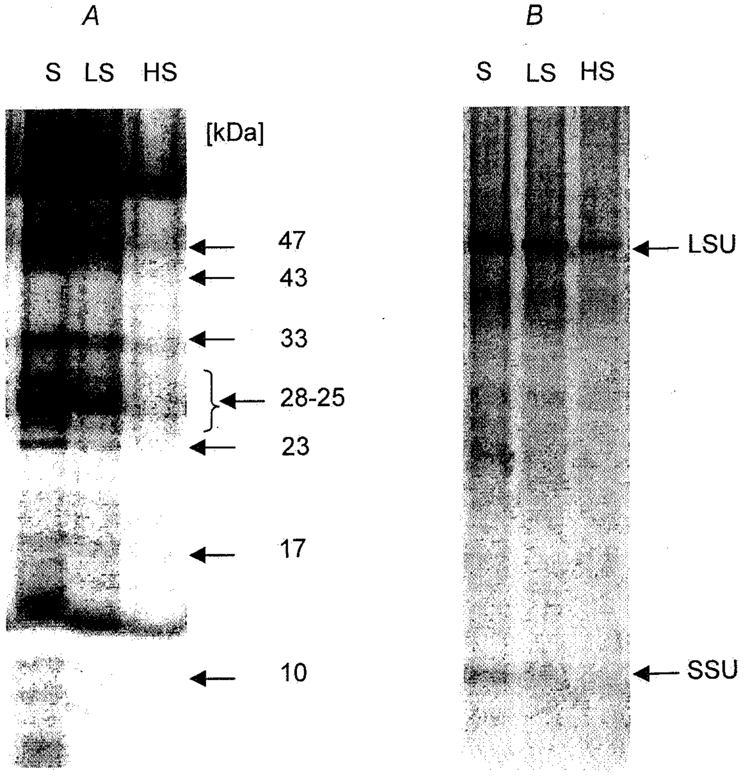

Fig. 4. Coomassie Brilliant Blue stained polypeptide profiles of thylakoid membrane proteins (*A*) and crude leaf extracts (*B*) isolated from sun (S) and low (LS) or high (HS) level canopy shade leaves. Gel lanes were loaded with equal amounts of protein (100 μg).

Discussion

The distribution and number of leaves are physiologically significant because the level of attachment of a branch complex to the main stem represents that point where all the leaves of each branch complex cumulatively contribute to the photosynthetic production of the main stem (Masarovičová and Minarčič 1984). In our experiment, the contents of Chl and Car were significantly decreased in canopy shade grown leaves in comparison with S leaves. Both Chl *a* and Chl *b* contents were lower and canopy shade probably also enhanced the chlorophyllase activity in leaves. An increase in the Car/Chl ratio and a decrease in the Chl *a/b* ratio was also observed in both HS and LS leaves. Because all Chl is non-covalently attached to either reaction centre or LHC (Green 1988), the differences in Chl *a/b* indicated that shade affected the distribution of Chl between the Chl-protein complexes. Also an increase of Car/Chl is due to the relatively faster decrease of Chl than Car. These results indicated that HS position induced rapid senescence in grapevine leaves. Similar results were found in dark-adapted *Vigna* seedlings during senescence (Nedunchezhian *et al.* 1995).

The S leaves showed a good PS2 activity, measured as the F_v/F_m ratio. Increase of canopy shade in leaves lead to decrease in F_v/F_m ratio: HS leaves showed the lowest F_v/F_m. The extent of F_v was reduced markedly in canopy shade leaves without affecting the F_0 level. This is characteristic for inhibition of the donor side of PS2. If the acceptor side of PS2 is photoinhibited, the F_0 is increased (Allakhverdiev *et al.* 1987, Šetlík *et al.* 1990).

Fluorescence kinetics analysis showed that shade, induced by dense canopy, reduced the apparent electron transport rate in leaves; these were not capable of relaxation of the membrane energisation. Quenching analysis showed rather slow and incomplete reoxidation of Q (less q_N) instead of a relaxation of membrane energisation (q_N rose to 0.8 in the steady state of LS leaves) like the effect of the specific ATPase inhibitor tentoxin reported by Schreiber and Bilger (1987). The *I*-response curve showed that the ETR was saturated at a low *I* in both HS and LS leaves. These results show an incapability to utilise the ambient PAR and to produce photoassimilates in canopy shade leaves. An increase of q_N is probably due to an accompanied increase of leaf zeaxanthin and antheraxanthin contents in canopy shade leaves. Similar results were found in field-grown iron-deficient leaves of pear (Morales *et al.* 1994).

Analysis of various electron transport activities, measured by using electron acceptors in thylakoids isolated from HS and LS leaves, showed an inhibition of the whole chain of electron transport activity by over 50 % in HS leaves; only a marginal effect on PS1 mediated reactions was noticed. Canopy shade leaves did not produce any significant changes in the rate of PS1 activity. Thus shade has an action site(s) in the PS2 reaction. Similar large reduction in PS2 activity was reported in low *I*-grown plants of *Atriplex* (Boardman *et al.* 1975) and *Picea* (Lewandowska *et al.* 1976). Analysis of electron transport in thylakoids isolated from LS and HS leaves showed that O_2 evolution was significantly inhibited when SiMo was used as electron acceptor, but not significantly inhibited when the electron acceptor was DCBQ. Since DCBQ accepts the electrons directly from Q_A^- (Cao and Govindjee 1990), the rates measured represent the true rate of photochemistry by PS2, uninfluenced by the PQ pool. This indicates that the donor side of PS2 is more impaired in canopy shade leaves.

In order to locate the possible site of canopy shade induced inhibition, we measured PS2 mediated DCPIP reduction in the presence of various artificial exogenous electron donors acting at the oxidising side of PS2. Among the artificial electron donors tested, DPC and NH_2OH were more effective in restoring the lost PS2 activity in HS leaves. This indicates that canopy shade leaves induced changes only on the donor side of PS2, prior to the NH_2OH donation side, and perhaps close to or after the DPC donation side. The present results agree with the findings that the water-oxidising system is sensitive to ageing (Biswal and Biswal 1988, Nedunchezhian *et al.* 1995) and that addition of DPC partially restores PS2 activity in aged chloroplasts (Misra and Biswal 1982, Nedunchezhian *et al.* 1996). In this connection the differences in shade-induced photosynthetic characteristics might have been at least partially affected by different ages of the compared leaves (see Šesták 1985).

Supporting evidence for the damage to PS2 activity was obtained from the analysis of thylakoid polypeptides: a comparison of thylakoids of canopy shade leaves with those of the S leaves showed specific losses of 47, 43, 33, 28-25, 23, 17, and 10 kDa polypeptides. The loss was more pronounced in HS leaves. The three extrinsic proteins of 33, 23, and 17 kDa associated with the lumen surface of the thylakoid membranes are required for optimal functioning of the oxygen evolving machinery. The three proteins are present in equi-molar amounts (Murata *et al.* 1984, Enami *et al.* 1994), but it is still disputed whether one copy or two copies of each of the proteins are associated with the PS2 unit (Murata *et al.* 1984, Millner *et al.* 1987). Solubilisation of the proteins is associated with partial or total inactivation of O_2 evolution. In particular, removal of the 33 kDa protein from PS2 membrane preparations by treatments with $CaCl_2$ or NaCl (Enami *et al.* 1994) results in strong inhibition of O_2 evolution; this loss is subsequently restored by reconstitution of the protein depleted membranes (Kuwabara *et al.* 1985). Marked reduction in the contents of 33, 23, and 17 kDa polypeptides accompanied the observed loss of PS2 activity in canopy shade leaves. The 10-kDa polypeptide is highly enriched in the grana region of the chloroplast. Immunoprecipitation and nearest-neighbour analysis suggest that the 10-kDa polypeptide is close to both the 23 and 33 kDa polypeptides (Ljungberg *et al.* 1984). These extrinsic proteins located on the inner thylakoid surface have a possible role in oxygen evolution. This could be the major reason for the marked loss of oxygen evolution in canopy shade leaves. Similar observations were made by Nedunchezhian *et al.* (1995, 1996) on dark-adapted *Vigna* seedlings during senescence and ageing of isolated chloroplasts. From the results we have also confirmed that canopy shade induced changes on the donor side of PS2.

Thylakoid stacking, energy distribution, and any damage to the LHC have multiple effects on the photosynthetic system. In our experiment, a significant loss of LHCP2 (28-25 kDa) polypeptides was observed in HS leaves. This could be one of the reasons for the observed marked loss of PS2 activity and induced yellowing in the leaves.

The plants grown under the shade have a relatively low content of soluble proteins as observed in the present study; this agrees with similar reports (Paliwal *et al.* 1986, Givnish 1988, Burkey *et al.* 1997). The soluble protein content was reduced markedly in shade leaves, which might be due to decrease in synthesis of RuBPC, the major soluble protein of leaf. A loss of leaf protein in canopy shade leaves would be partially accounted for by damaged chloroplasts or would be the result of inhibition of protein synthesis.

The reduction in the overall photosynthetic rate correlated well with the decreased RuBPC activity in canopy shade leaves. A marked reduction of RuBPC activity was observed in HS leaves. Such reduction was due to inhibition of protein synthesis induced by shade. The low photosynthetic rate in canopy shade leaves may be attributed to the lower activity of photosynthetic enzymes rather than to the other *I* conditions which include mainly RuBPC of the reductive pentose pathway (Usuda *et al.* 1985, Mae *et al.* 1993). Also the carboxylating enzymes are not fully activated at low *I*, and the degree of activation may regulate the flux of carbon through the photosynthetic pathway (Usuda *et al.* 1985). This is supported by SDS-PAGE analysis of crude leaf extracts of RuBPC proteins shown by a marked loss of both LSU and SSU polypeptides in canopy shade leaves. This loss of LSU and SSU is one of the reasons for marked loss of RuBPC activity in canopy shade leaves.

A drastic reduction of *in vivo* nitrate reductase activity of canopy shade leaves may reflect a balance between synthesis and activation on the one hand, and degradation or inactivation on the other. The increase of *in vivo* nitrate reductase activity in the nitrate replenished plants might be due to the increased uptake of nitrate by the roots during high *I*. Apparently, nitrate taken up by the roots during sun conditions is transported to the shoot where it is stored in the vacuoles and made available for active enzyme reduction. Even though an adequate amount of NO_3^- was externally supplied, the low-*I* plants failed to induce the synthesis of the active nitrate reductase enzyme. The decreased nitrate reductase might reflect the reduction in nitrate uptake by the roots. This decline in uptake might be due to the feed back inhibition of amino acids formed in the leaf blades and transported from there to the shoot (Clarkson 1986).

Our results suggest that canopy shade decreases contents of photosynthetic pigments, ribulose-1,5-bisphosphate carboxylase and nitrate reductase activities, photosynthetic electron transport activities, and contents of thylakoid membrane proteins, and induces rapid senescence or ageing in grapevine leaves. Canopy shade also induced marked changes mainly on the donor side of PS2.

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