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Photosystem 2 efficiency and thylakoid protein pattern in DCMU-treated wheat seedlings during senescence

N. NEDUNCHEZHIAN[*,+], K. MUTHUCHELIAN[**], and M. BERTAMINI[*]

Instituto Agrario de San Michele all' Adige, San Michele all' Adige, Italy[*]
School of Energy, Environment and Natural Resources, Madurai Kamaraj University, Madurai - 625 021, India[**]

Abstract

Changes in various components of photosynthetic apparatus during the 6-d dark incubation at 25 °C of detached control and DCMU-treated *Triticum aestivum* L. leaves were examined. The rate of photosystem 2 (PS2) activity was decreased with increase of the time of dark incubation in control leaves. In contrast to this, DCMU-treated leaves demonstrated high stability by slowing down the inactivation processes. Diphenyl carbazide and NH_2OH restored the PS2 activity more in control leaves than in DCMU-treated leaves. Mn^{2+} failed to restore the PS2 activity in both control and DCMU-treated samples. Similar results were obtained when F_v/F_m was evaluated by chlorophyll fluorescence measurements. The marked loss of PS2 activity in dark incubated control leaves was primarily due to the loss of D1, 33, and 23 kDa extrinsic polypeptides and 28-25 kDa LHCP2 polypeptides.

Additional key words: chlorophyll fluorescence; diphenyl carbazide; donor side; electron transport; $MnCl_2$; NH_2OH; photosystem; senescence; thylakoid protein; *Triticum aestivum*.

Introduction

Changes in photosynthetic parameters during ageing have been studied in great detail in many plant species (Šesták 1977, Thomas and Stoddart 1980). The effects of several physiological and environmental factors on the changes in chloroplasts during ageing of both attached and detached leaves have also been investigated. Leaf senescence may be induced by shading, mineral deficiency, drought, or pathogen infection (Thomas and Stoddart 1980). In the absence of such factors, leaf senescence occurs in age dependent manner in many species (Batt and Woolhouse 1975, Jiang *et al.* 1993).

The mechanism of chloroplast degradation in attached leaves (Choe and Thimann 1975, Biswal and Biswal 1988, Nooden *et al.* 1996) is different from degradation that occurs in dark in detached leaves (Biswal and Mohanty 1978, Nedunchezhian *et al.* 1996). In attached leaves, senescence is influenced by hormones and protein-synthesising capacity of the organelles.

Loss in the total chlorophyll (Chl) and protein contents (Nedunchezhian *et al.* 1995) as well as the disorganisation of the structure of the chloroplast membranes (Dodge 1970, Jiang *et al.* 1993) together with the reduction in ability of photosynthetic CO_2 fixation have been reported (Hernández-Gil and Schaedle 1973). The structural changes in chloroplast during senescence begin with a gradual disintegration of the stroma lamellae followed by the disruption of grana stackings (Dodge 1970). The effects of ageing on the functional characteristics of isolated chloroplasts have also been reported (Harnischfeger 1973). Recently, the loss of PS2 activity during ageing of *Vigna* leaves has been observed (Nedunchezhian *et al.* 1996). Although the general phenomenology of senescence-induced alterations of some of the photosynthetic activities is known, very little is known about the specific sites of their alterations or the sequence of events that occur during these phenomena.

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[+]Author for correspondence. *Permanent address*: Govt. HSS, Vellimedupettai - 604 207, Tindivanam, India; e-mail: nedu@yahoo.com
Abbreviations: Chl – chlorophyll; DCBQ – 2,6-dichloro-*p*-benzoquinone; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP – 2,6-dichlorophenol indophenol; DPC – diphenyl carbazide; DTT – dithiothreitol; F_0 – minimal fluorescence; F_m – maximum fluorescence; kDa – kilodalton; LHCP – light-harvesting chlorophyll protein; PPFD – photosynthetic photon flux density; PS – photosystem; SDS-PAGE – sodium dodecylsulphate-polyacylamide gel electrophoresis; SiMo – silicomolybdate.
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A number of environmental conditions elicit photosystem stoichiometry adjustments and changes of the Chl antenna size of the photosystems in the thylakoid membrane chloroplasts (Riethman and Sherman 1988). The ability of photosynthetic organisms to respond to sub-lethal doses of herbicide is of great practical as well as fundamental importance. Triazine and urea-type herbicides block photosynthetic electron transport by occupying the plastoquinone-binding site of D1. The response of chloroplasts to sub-lethal concentration of herbicides has often been studied (Mannan and Bose 1985, Bose *et al.* 1992, Zer and Ohad 1995, Nedunchezhian *et al.* 1997, Komenda and Masojídek 1998, Kowalczyk *et al.* 1998). Invariably, herbicides cause changes in the structure and composition of thylakoids, including greater grana size and increased content of unsaturated lipids (Bose *et al.* 1992). Concomitantly, a lower Chl *a/b* ratio and lower β-carotene/xanthophyll ratio has been reported (Nauš and Melis 1992). Plants grown under 100 μM DCMU had a minimum altered pigment composition but significant changes in the photosynthetic electron transport reactions (Kulandaivelu and Annamalainathan 1991, Komenda *et al.* 2000).

In this report, we characterised the changes of photosynthetic ability of wheat thylakoids caused by stress-induced senescence. The effect of DCMU on photosynthetic activities of thylakoids was also studied. We report some specific sequential alteration in the site(s) for entry of electrons between water and PS2 reaction centres of photosynthetic apparatus during the dark stress period. The effect of DCMU, which seems to protect the donor side of PS2 from dark induced senescence, is also described.

Materials and methods

Plants and DCMU treatment: Wheat (*Triticum aestivum* L.) seedlings were grown on three layers of coarse filter paper in a glass Petri plate at 25 °C under "white fluorescent light" (1 600 μmol m^{-2} s^{-1}) provided by a bank of cool day fluorescent lamps and 14/10 h light/dark regime. 20 cm^3 of 100 μM DCMU was added initially. Thereafter, only water was added periodically.

Detached leaves incubated in the dark: About 10-cm-long segments of fully expanded primary leaves from 10-d control and DCMU-treated seedlings were cut and floated on 25 cm^3 of double distilled water (15 segments per plate). The plates were covered and kept in darkness at 25 °C for 10 d. Samples were removed at every 2-d interval and used for assays.

Total Chl and protein contents were estimated spectrophotometrically according to the methods of Arnon (1949) and Lowry *et al.* (1951), respectively.

Isolation of thylakoids and PS2 membranes from control and DCMU-treated seedlings was made according to Mannan and Bose (1985) and Berthold *et al.* (1981), respectively.

Measurement of PS2 activity: Oxygen evolution (PS2 activity) was measured following the method of Noorudeen and Kulandaivelu (1982) with a Clark-type electrode (*Hansatech*) fitted with a circulating water jacket at 27 °C. Actinic radiation 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. Thylakoids and PS2 membranes 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, 200 μM SiMo, and 2 mM $K_3Fe(CN)_6$.

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 PS2 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.

Modulated Chl fluorescence in leaves and PS2 membranes was measured on leaf discs using a *PAM 2 000* fluorometer (*H. Walz*, Effeltrich, Germany). F_0 was measured by switching on the light modulated at 0.6 kHz; PPFD was less than 0.1 μmol m^{-2} s^{-1} at the leaf surface. F_m was measured at 20 kHz with a 1-s pulse of 6 000 μmol m^{-2} s^{-1} of "white light". Modulated Chl fluorescence on isolated PS2 membranes at room temperature was measured with the same device in 0.7 cm^3 of reaction mixture containing 50 mm Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 100 mM sucrose, and 10 μg Chl equivalent PS2 membranes. The integrated measuring irradiance (480 nm) was 0.15 μmol m^{-2} s^{-1}, red actinic irradiance (650 nm) was 100 μmol m^{-2} s^{-1}.

SDS-PAGE: Thylakoids and PS2 membranes were separated using the discontinuous polyacrylamide gel system of Laemmli (1970), with the following modifications. 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).

Immunoblotting experiment: The relative content of some thylakoid proteins was determined immunologically by Western-blotting. Thylakoids were first 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 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 buffer that contained 0.05 % *Tween-20*, a secondary antibody [Anti-Rabbit IgG (whole molecule) Biotin Conjugate, *Sigma*] was allowed to react in 1 % gelatine for 2 h. The D1 protein was detected using polyclonal antiserum against spinach D1 protein (kindly provided by Prof. I. Ohad, Jerusalem, Israel) and the antibody against the 33 kDa protein of the water-splitting system (gift from Dr. R. Barbato, Padova, Italy). The bands were quantified by densitometry using the *Bio-image* apparatus (*Millipore*, Michigan, USA).

Results

The visible symptom of ageing induced by dark stress in detached wheat leaves was the gradual yellowing of the leaf. DCMU prevented the appearance of yellow colour, as the leaf segments of DCMU-treated seedlings remained green even 6 d after incubation (results not shown). The control leaf segments became almost yellow by the sixth day of dark incubation. Thus for all our experiments the leaf segments were incubated for 6 d.

Changes in PS2 reaction: 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. At the beginning of the experiment the values of F_v/F_m in the control and DCMU-treated leaves were 0.87 and 0.75, respectively (Table 1). After 6 d of dark incubation, the F_v/F_m ratio decreased to around 0.53 in control leaves and to 0.70 in DCMU-treated leaves. F_0 was marginally increased in both types of leaves. A similar increase in F_0 has been observed in leaves in anaerobic condition or in leaves from plants grown in deficiency of Mg and S. In both cases, the increase of F_0 was ascribed to the presence of reduced Q_A^- (Šetlík *et al.* 1990, Godde and Dannehl 1994).

Table 1. Changes in the relative levels of fluorescence emitted as minimal fluorescence (F_0), variable fluorescence (F_v), and the ratio of variable to maximum fluorescence (F_v/F_m) in the leaves from control and DCMU-treated leaves incubated for 6 d in the dark. F_0 was measured by switching on the modulated radiation 0.6 kHz; PPFD was less than 0.1 μmol m^{-2} s^{-1} at the leaf surface. F_m was measured at 20 kHz with a 1 s pulse of 6 000 μmol m^{-2} s^{-1} of "white light".

Treatment	[d]	F_0	F_v	F_v/F_m
Control	0	2.00±0.05	16.20±0.45	0.87±0.02
	6	2.70±0.06	3.00±0.05	0.53±0.01
DCMU	0	4.00±0.10	12.00±0.25	0.75±0.02
	6	4.30±0.11	9.80±0.18	0.70±0.02

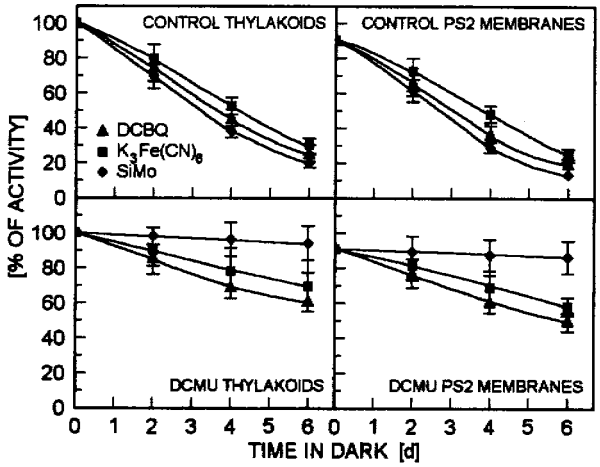

Fig. 1. Changes in the rate of photosystem (PS) 2 electron transport activity in thylakoids and PS2 membranes isolated from control and DCMU-treated leaves at different days of ageing under dark incubation. The 100 % values are [mmol(O_2) kg^{-1}(Chl) s^{-1}]: $H_2O \rightarrow$DCBQ 42, 27; $H_2O \rightarrow K_3Fe(CN)_6$ 36, 22; $H_2O \rightarrow$SiMo 25, 24 for thylakoids, and $H_2O \rightarrow$DCBQ 54, 28; $H_2O \rightarrow K_3Fe(CN)_6$ 46, 26; $H_2O \rightarrow$SiMo 32, 28 for PS2 membranes isolated from control and DCMU-treated leaves, respectively. Values represent averages of 3 experiments. All values are significant at ±5 % level.

The rate of PS2 activity of thylakoids and PS2 membranes was decreased with increase of the time of dark incubation in both control and DCMU-treated leaves (Fig. 1). After 6 d, photosynthetic electron transport from $H_2O \rightarrow$DCBQ, $H_2O \rightarrow K_3Fe(CN)_6$, and $H_2O \rightarrow$SiMo was

reduced by about 73, 67, and 80 % in control leaves and by 34, 28, and 0 % in DCMU-treated leaves, respectively.

To locate the possible site(s) of inhibition in the PS2 reaction, we followed the DCPIP photoreduction supported with various artificial electron donors in PS2 membranes isolated from dark incubated control and DCMU-treated leaves. According to Wydrzynski and Govindjee (1975), $MnCl_2$, DPC, and NH_2OH donate electrons in the PS2 reaction. In our experiment, the electron transport activity of PS2 isolated from 6-d dark incubated control and DCMU-treated leaves was reduced to about 82 and 52 %, respectively, when water served as electron donor (Fig. 2). A similar trend was found using

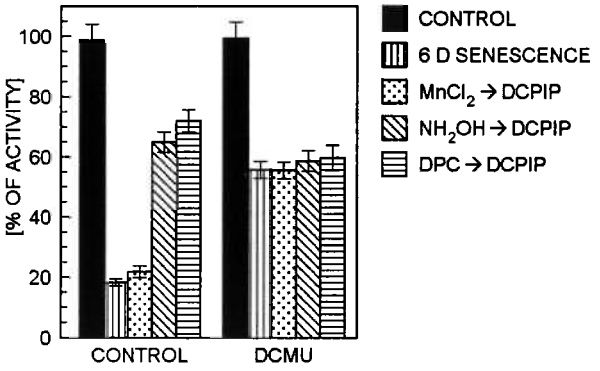

Fig. 2. Effect of various exogenous electron donors on photosystem 2 (PS2) activity in PS2 membranes isolated from control and DCMU-treated leaves at 6 d of ageing under dark incubation. The 100 % values are [mmol(O_2) kg^{-1}(Chl) s^{-1}]: H_2O→DCPIP 54, 31; $MnCl_2$→DCPIP 49, 29; DPC→DCPIP 54, 31; NH_2OH→DCPIP 56, 32 for control and DCMU-treated leaves, respectively. Values represent averages of 3 experiments. All values are significant at ±5 % level.

$MnCl_2$ as a donor, while using DPC and NH_2OH a significant restoration of PS2 mediated DCPIP reduction was observed in dark incubated control leaves. In contrast to this, in dark incubated DCMU-treated leaves PS2 activity was not restored, using neither DPC nor NH_2OH (Fig. 2).

These results agree with measurements obtained by modulated Chl fluorescence with various exogenous electron donors (Fig. 3). The addition of DPC and NH_2OH to control PS2 membranes induced a 75-80 % increase of variable fluorescence (F_v) (Table 2). The F_v/F_m ratio also increased from 0.25 to 0.54. In this experiment F_0 was also slightly increased (Fig. 3). These results indicated that during ageing of control leaves, the water splitting system is inhibited and, at least in part, the changes in the Q_A^- re-oxidation kinetics may be related to donor side limitation. On the other hand, during ageing of DCMU-treated leaves F_0 increased and F_m did not change by addition of electron donors (Fig. 3).

Table 2. Changes in the relative fluorescence emitted as minimal fluorescence (F_o), variable fluorescence (F_v), and the ratio of variable to maximum fluorescence (F_v/F_m) in PS2 membranes isolated from control and DCMU-treated leaves with or without electron donors. Concentrations of $MnCl_2$, DPC, and NH_2OH were 5.0, 0.5, and 5.0 mM, respectively. The integrated measuring irradiance (480 nm) was 0.15 μmol m^{-2} s^{-1}, red actinic (650 nm) irradiance was 100 μmol m^{-2} s^{-1}.

Treatment		F_0	F_v	F_v/F_m
Control	0 d	1.90±0.04	3.60±0.11	0.65±0.02
	6 d ageing	2.10±0.08	0.70±0.01	0.25±0.01
	$MnCl_2$	2.10±0.06	1.10±0.02	0.34±0.01
	DPC	2.10±0.07	2.20±0.08	0.51±0.02
	NH_2OH	2.10±0.07	2.50±0.10	0.54±0.02
DCMU	0 d	3.10±0.08	2.30±0.09	0.43±0.01
	6 d ageing	3.50±0.10	1.80±0.05	0.34±0.01
	$MnCl_2$	3.50±0.10	1.70±0.04	0.33±0.01
	DPC	3.50±0.11	1.70±0.04	0.33±0.01
	NH_2OH	3.50±0.10	1.70±0.05	0.33±0.02

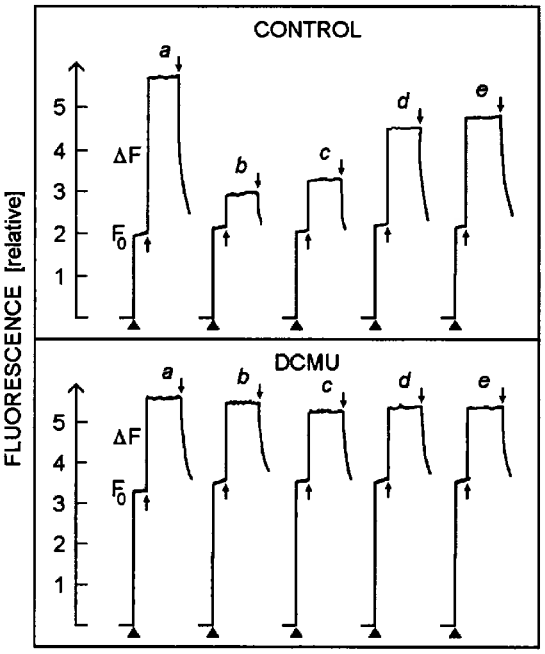

Fig. 3. Room temperature chlorophyll (Chl) fluorescence induction curves from PS2 membranes of control and DCMU-treated leaves. *a* (0 d) and *b* (6 d), without donors; *c*, $MnCl_2$; *d*, NH_2OH; *e*, DPC. The Chl concentration was 10 μg m^{-3}. Switching on the measuring irradiation (480 nm, 0.15 μmol m^{-2} s^{-1} and actinic radiation (650 nm, 100 μmol m^{-2} s^{-1}) on ↑ and ↓ off, respectively.

Changes in thylakoid and PS2 membrane proteins: Since changes in photosynthetic electron transport activities could be caused primarily by the changes or reorganisation of thylakoid components, the polypeptide

profiles of thylakoids and PS2 membranes were analysed by SDS-PAGE. After 6 d of leaf incubation, the contents of 33, 28-25, and 23 kDa polypeptides were significantly reduced in control leaves, while the loss of these polypeptides was less pronounced in DCMU-treated leaves (Fig. 4).

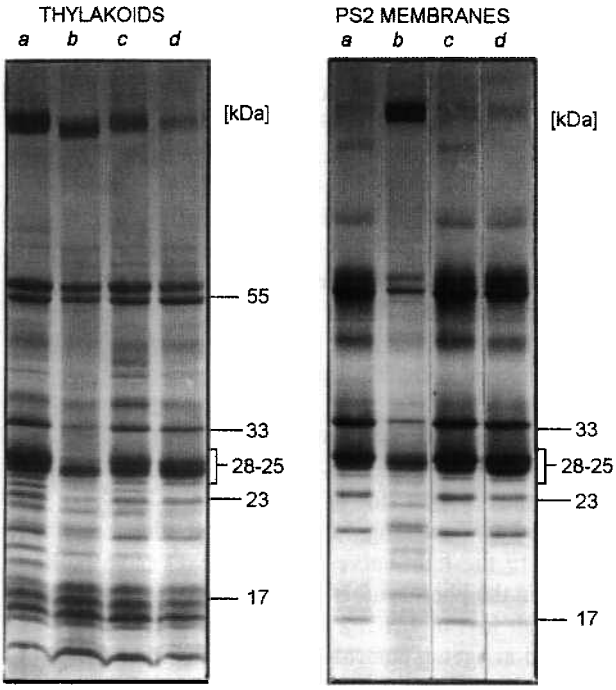

Fig. 4. Coomassie blue stained polypeptide profiles of thylakoids and PS2 membranes isolated from control and DCMU-treated leaves. Gel lanes were loaded with equal amount of proteins (100 μg). *a*, 0 d control; *b*, 6 d control; *c*, 0 d DCMU; *d*, 6 d DCMU.

Changes in D1 and 33 kDa proteins followed by immunoblot: Photoinactivation 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. In intact plant the correlation between D1 protein content and activity of PS2 is more complex (Smith *et al.* 1990, Lutz *et al.* 1992).

To study ageing, the contents of D1 protein per Chl in control and DCMU-treated leaves were determined by Western-blotting (Fig. 5). The relative content of D1 protein decreased by 30 and 67 % in control and DCMU-treated leaves. Besides the changes in D1 protein, we also observed a very clear and substantial decrease in the concentration of 33 kDa protein of water-splitting system in ageing leaves. This protein was more drastically diminished in control leaves than in the DCMU-treated leaves (Fig. 5).

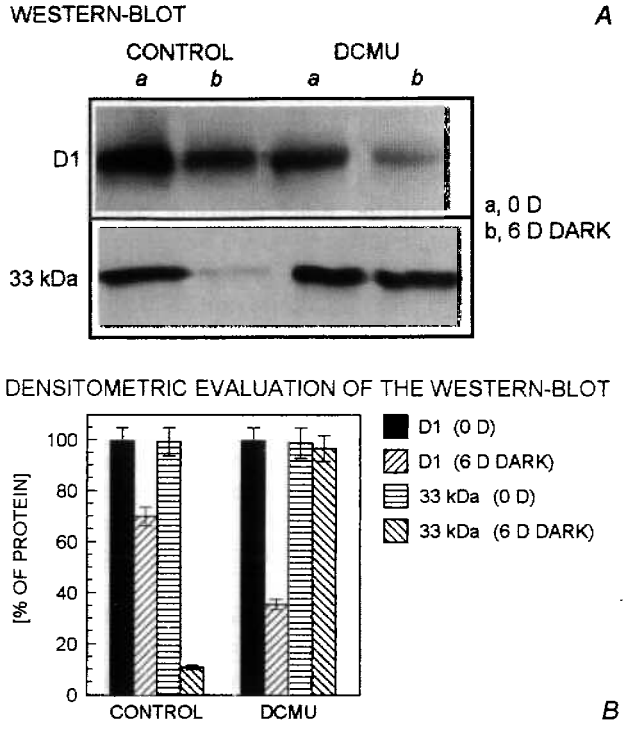

Fig. 5. *A*: Degradation of D1 and 33 kDa proteins in control and DCMU-treated leaves. Each lane was loaded to equal amounts of chlorophyll (5 μg). *B*: Histogram: *Bio-image* densitometric evaluation.

Discussion

Analysis of PS2 activity in thylakoids and PS2 membranes induced by ageing of DCMU-treated leaves showed that oxygen evolution was inhibited when the electron acceptor was DCBQ or ferricyanide, but not inhibited when electron acceptor was SiMo. This indicates that DCMU-treated leaves were further affected only on the acceptor side of PS2 during senescence. In contrast, the rate of PS2 activity was decreased faster when SiMo was used than with DCBQ in dark-adapted control leaves. This indicates that the donor side of PS2 is more impaired than the acceptor side during senescence in control leaves.

Among the artificial electron donors tested, DPC donates electrons directly to the reaction centre, while NH_2OH to a site between Z_1 and Z_2 (Wydrzynski and Govindjee 1975). DPC and NH_2OH restored PS2 activity markedly in ageing control leaves and partially in DCMU treated leaves; this is supported by earlier findings that the water-oxidising system is sensitive to ageing (Biswal and Biswal 1988, Nedunchezhian *et al.* 1996). These

results were also confirmed by measurement of modulated Chl fluorescence (see also Roháček and Barták 1999). After addition of DPC and NH_2OH in PS2 membranes from 6-d aged control leaves, a marked increase in F_v was observed, whereas F_0 marginally increased. Hence the site of senescence is on the oxidising side of PS2, prior to the NH_2OH donation side and perhaps close to or after the DPC donation side. This is supported by our earlier reports in *Vigna* seedlings (Nedunchezhian *et al.* 1995, 1996). In contrast to this, in PS2 membranes from aged DCMU-treated leaves a slight increase of F_0 without any change in F_m was observed when exogenous electron donors were added. The loss of PS2 activity could only partially be ascribed to a functional inhibition of PS2 since F_v/F_m was reduced by about 41 and 8 % in control and DCMU-treated leaves, respectively. We therefore assume that it was mainly due to the loss of PS2 centres or water oxidation complex on a Chl basis. Immunological determination of the PS2 reaction centre polypeptides D1 and 33 kDa of the water oxidising complex polypeptide confirmed this fact.

The degradation of the D1 protein may be caused by damage to the PS2 reaction centre protein (Andersson and Styring 1991, Prášil *et al.* 1992). We found loss of D1 protein that corresponds fairly well with the values from the electron transport experiments. The loss of D1 protein was accompanied by a similar significant decrease in the content of 33 kDa protein of the water splitting system, showing that the whole PS2 is rapidly degraded under prolonged dark stress. A similar phenomenon was observed by Schuster *et al.* (1986) and by Miyao *et al.* (1995) under photoinhibition.

The most likely explanation for the inactivation of electron transport activity is that the related protein(s) is(are) exposed at the thylakoid surface (Seidler 1994). From the polypeptide profiles of ageing control and DCMU-treated thylakoids and PS2 membranes, a significant loss of 33, 28-25, and 23 kDa polypeptides was observed in control leaves and smaller losses in DCMU-treated leaves. Three extrinsic proteins of 33, 23, and 17 kDa associated with the lumenal surface of thylakoid membranes are required for optimal functioning of the oxygen-evolving machinery. The three proteins are present in equimolar 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. Removal of the 33 kDa protein from PS2 membrane preparations by treatments with $CaCl_2$ and NaCl (Enami *et al.* 1994) results in strong inhibition of O_2 evolution and the 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 during ageing of control leaves. Since the DCMU-treated leaves showed only marginal reduction in contents of these polypeptides, this is one of the reasons for higher PS2 activity than in control leaves (Nedunchezhian *et al.* 1996, Komenda *et al.* 2000). The observed loss of PS 2 activity in DCMU-treated leaves is mainly due to the loss of D1 protein. Thus we confirmed that senescence induced marked changes at the donor side of PS2 in control leaves, while in the DCMU-treated leaves similar changes occurred only on the acceptor side of PS2.

Thylakoid stacking, energy distribution, and any damage to the light-harvesting complexes have multiple effects on the photosynthetic system. In our experiment, a significant loss of LHCP2 (28-25 kDa) polypeptides was observed in ageing control leaves. This could be a reason for the observed marked loss of PS2 activity and yellowish colour in control leaves. In contrast, no changes in LHCP polypeptides and greenish colour were observed even after 6-d dark incubation in DCMU-treated leaves.

From the above results we conclude that (*1*) the decrease in the activity of PS2 during *in vivo* ageing is smaller in DCMU-treated seedlings than in the control ones, (*2*) the recovery of PS2 activity by artificial exogenous donors DPC and NH_2OH is larger in control leaves than in the DCMU-treated leaves, and (*3*) the contents of 33, 28-25, and 23 kDa polypeptides are more drastically reduced in control leaves than in the DCMU-treated ones. In contrast, the content of D1 protein was significantly decreased in DCMU-leaves but not in control leaves.

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