Alterations in electron transport characteristics during senescence of *Cucumis* cotyledonary leaves. Analysis of the effects of inhibitors

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Abstract

Cotyledonary leaves of *Cucumis sativus* cv. Poinsette exhibited senescence-induced losses in chlorophyll (Chl) and protein contents within three weeks since germination. Chl and protein concentrations in cotyledonary leaves approached maximum on 6th d after germination and they declined to 50 and 41 %, respectively, by the 20th day of growth. Activities of both photosystem (PS) 2 and PS1 decreased by 33 and 31 %, respectively, on the 20th day, compared to the control 6th day. Changes in sensitivity of PS2 to inhibitors like atrazine and dibromothymoquinone and sensitivity of PS1 to KCN accompanied the changes in PS2 and PS1 activities. Hence both the acceptor side of PS2 and the donor side of PS1 are affected by senescence-induced changes in cucumber cotyledonary leaves.

Additional key words: atrazine; cucumber; DCMU; dibromothymoquinone; KCN; photosystems 1 and 2; plant age; thylakoid proteins.

Introduction

Senescence is a physiologically programmed process during which metabolites are remobilized from older to younger plant parts (Stoddart and Thomas 1982). Chloroplasts, among all the cellular organelles, are most sensitive to leaf senescence (Biswal and Biswal 1988). During foliar senescence, loss of photosynthetic pigments is one of the most visible changes, and measurement of Chl loss is commonly used as

Received 13 November 1997, *accepted* 18 December 1997.
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Abbreviations: ASC, ascorbate; Cyt b_6/f, cytochrome b_6/f; DAD, diaminodurene; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenol indophenol; MV, methylviologen; PC, plastocyanin; PQ, plastoquinone; PS, photosystem.
Acknowledgements: This work is supported by a grant from DAE-BRNS project (Project No: 4/3/95-R&D-II) to Prasanna Mohanty. We thank Dr. A.S. Bhagwat, BARC for helpful suggestion. We also thank the Dean, SLS, JNU, for providing the necessary facilities.

a measure of progress of senescence. The thylakoid photochemical activities limit photosynthesis during senescence (Harding *et al.* 1990). Jenkins and Woolhouse (1981) showed in *Phaseolus* that the electron transport activities of PS1 and PS2 declined during leaf senescence by 25 and 33 %, respectively. Similarly, in several other senescing systems, drastic decline in PS2, PS1, and whole chain electron transport activities were reported (Biswal and Mohanty 1976, McRae *et al.* 1985, Sabat *et al.* 1985, Grover *et al.* 1986, Sabat *et al.* 1989). Bricker and Newman (1982) found ~18 % higher loss in PS1 electron transport activity as compared to that of PS2 during senescence of cotyledonary chloroplasts in soybean. Holloway *et al.* (1983) suggest that PQ limits the electron transport activity during leaf maturity. However, Sabat *et al.* (1985) observed that the decline in rates of whole chain electron transport was greater than that of rates of either PS2 or PS1, suggesting that plastocyanin (PC) limits the electron transport rates during senescence. Thus, the two mobile electron carriers seem to be involved in the senescence induced losses in photoelectron transport activities. All the above findings suggest that leaf senescence induces a general loss in photochemical activity. Possible reason for decline in the photosynthetic activity during senescence may be alterations in the electron-donating or electron-accepting ability of electron transport carriers (Mohanty 1987). However, specific alterations in the thylakoid membrane proteins that mediate electron transfer have not yet been fully characterized. Although temporal sequence of loss of electron transport carrier proteins has been investigated (Ben-David *et al.* 1983, Roberts *et al.* 1987), detailed investigation of these aspects is needed to explain the loss in photosynthetic ability during leaf senescence. Since losses in pigments and proteins including thylakoid proteins occur in senescing leaves, we studied the extent of change in sensitivity towards selected inhibitors of photosystems in cotyledonary leaves of *Cucumis*.

Materials and methods

Plants: Seeds of *Cucumis sativus* cv. Poinsette were surface sterilised with 0.1 % mercuric chloride for 5 min and then rinsed thoroughly with distilled water. The cleaned seeds were placed in glass bottles (24×10 cm) on 2 cm thick cotton bed moistened with ~ 250 cm^3 of autoclaved mineral nutrient medium (Arora and Saradhi 1995) containing all essential macronutrients and micronutrients. Seedlings were raised in a growth chamber maintained at 25±2 °C under continuous "white" fluorescent radiation of 35-40 μmol m^{-2} s^{-1} and relative humidity of 85-90 %. Plants were grown for 3 weeks, and cotyledonary leaves were harvested at 6, 10, 15, and 20 d after germination. The cotyledonary leaves showed maximal electron transport activities and pigment contents on the 6th day and exhibited sign of senescence by the 20th day.

Chl and protein contents: Chls were extracted in chilled 100 % dimethylformamide in dark, and their amounts were estimated according to Porra *et al.* (1989). Leaf proteins were precipitated with 10 % trichloroacetic acid, dissolved in 1 M NaOH, and the

total amount of proteins was estimated according to Lowry *et al.* (1957) using bovine serum albumin (*Sigma*, fraction V) as standard.

Thylakoid membranes were isolated by a procedure similar to that of Nakatani and Barber (1977) as described in Vani *et al.* (1996). The cotyledonary leaves at various stages of growth (plants 6, 10, 15, and 20 d old) were homogenized in ice-chilled isolation medium containing 0.4 M sorbitol, 15 mM Tricine-KOH (pH 7.8), and 10 mM sodium chloride (buffer A). The homogenate was filtered through four layers of *Miracloth* and centrifuged at 3 000×*g* for 5 min. Supernatant and most of the loose pellet was discarded, and the thylakoid pellet was washed in buffer containing 10 mM Tricine (pH 7.8), 10 mM sodium chloride, and 5 mM magnesium chloride (buffer B), and finally resuspended in suspension buffer C (buffer B with added 0.1 M sorbitol).

Electron transport activities of isolated thylakoids were measured polarographically in terms of oxygen consumption or evolution at 25 °C and ~700 μmol(photon) m^{-2} s^{-1}, using Clark type oxygen electrode (*Hansatech*, U.K.) as described by Alia *et al.* (1992) and Bala *et al.* (1996). Irradiance was measured using a *LI-COR* model *LI-189* quantum meter. PS2 activity was assayed as oxygen evolution in 1 cm^3 of reaction mixture of 100 mM Hepes-KOH (pH 7.5), 10 mM sodium chloride, 5 mM magnesium chloride, 100 mM sorbitol, 1 mM ammonium chloride, and 100 μM DCBQ, with thylakoids containing 10 μg Chl. The effect of varying concentrations of DBMIB and atrazine on PS2 activity was tested. PS1 activity was measured as oxygen consumption in 1 cm^3 of reaction mixture that contained chloroplasts with 5 μg Chl, 10 μM DCMU, 50 μM methyl viologen (MV), 5 mM ascorbate, 5 mM sodium azide, 100 μM DCPIP, 10 mM sodium chloride, 5 mM magnesium chloride, 100 mM sorbitol, and 100 mM Tricine-KOH (pH 7.8), as described by Sabat *et al.* (1989). The effect of varying concentrations of KCN (pH 7.0) on photosystems was assayed in 1 cm^3 reaction mixture containing 100 mM Tricine-KOH (pH 7.8), 10 mM sodium chloride, 5 mM magnesium chloride, 100 mM sorbitol, 0.4 mM diaminodurene, 5 mM L-ascorbate, and 50 μM MV (Izawa *et al.* 1973).

Results and discussion

The PS2 activity of *Cucumis* cotyledons measured at 2 d intervals gradually increased from the 2nd d after germination to a maximal value on the 6th d, and then gradually declined (values not shown). A 50 % decline in total Chl content was observed in 20 d-old cotyledonary leaves in relation to the 6 d-old cotyledonary leaves taken as control (Table 1). Contents of both Chl *a* and *b* declined at almost the same rate, although there was a marginal increase in Chl *a/b* ratio. The loss in total cotyledonary leaf proteins was about 40 % on the 20th day in comparison to the 6th day (Table 2). The thylakoid protein/Chl ratio showed only marginal changes, and thus the losses in Chl and proteins were almost parallel.

The thylakoids of 6 d-old cotyledonary leaves exhibited PS2-catalyzed O_2 evolution with DCBQ, functioning near to Q_A, the stable primary acceptor of PS2

Table 1. Changes in chlorophyll (Chl) contents [g kg^{-1}(d.m.)] of *Cucumis* cotyledonary leaves as a function of leaf age. Values are means of three independent experiments, with standard deviations.

Plant age [d]	Chl *a*	Loss [%]	Chl *b*	Loss [%]	Chl (*a*+*b*)	Loss [%]	Chl *a*/*b*
6 (control)	21.14±0.69	0	7.44±0.31	0	28.58±0.97	0	2.84±0.07
10	16.40±0.40	22.4	5.27±0.19	30.1	21.67±0.58	24.2	3.11±0.01
15	13.29±0.96	37.1	4.18±0.24	43.0	17.47±1.18	38.9	3.18±0.10
20	10.76±0.33	49.1	3.50±0.24	53.0	14.26±0.56	50.1	3.07±0.11

(Duysens and Sweers 1963, Satoh *et al.* 1992). With progress of cotyledon age, the activity of PS2 steadily declined (between days 6 and 20 by 30-33 %) (Fig. 1*A*).

Table 2. Age dependent changes in content of total proteins [g kg^{-1}(d.m.)] and in the ratio of thylakoid proteins/chlorophyll (Chl). Values are means of three independent experiments, with standard deviations.

Plant age [d]	Total proteins	Loss [%]	Thylakoid protein/Chl
6 (control)	0.34±0.01	0	9.56
10	0.26±0.01	23.4	9.81
15	0.23±0.00	33.7	9.93
20	0.20±0.01	41.2	10.30

The PS1 activity monitered as MV reduction and $DCPIPH_2$ oxidation by molecular oxygen also exhibited a 28-31 % loss of activity (Fig. 1*B*). This similar loss of both PS1 and PS2 activities was different from losses in soybean cotyledonary leaves

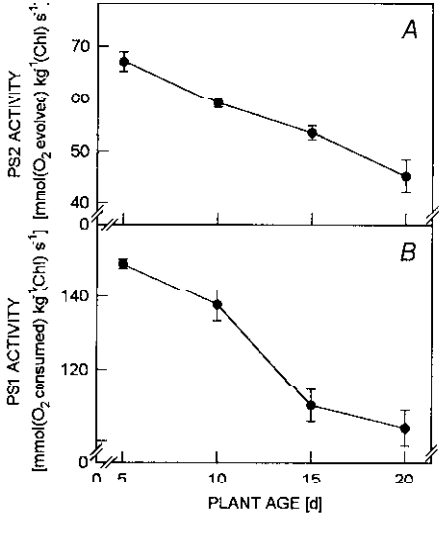

Fig. 1. Changes in photosystem (PS) 2 (*A*) and PS1 (*B*) electron transport activities of *Cucumis sativus* cotyledonary leaves as a function of age. PS2 activity was assayed as $H_2O \rightarrow DCBQ$ at saturating irradiance of ~700-800 μmol m^{-2} s^{-1}. Vertical bars represent standard deviations of the mean value of three different experimental observations.

(Bricker and Newman 1982) in which losses of PS1 activity were larger than those in PS2. Losses in PS2 and PS1 activities during senescence were found also by

Harnischfeger (1973), Biswal and Mohanty (1976), Jenkins and Woolhouse (1981), Sabat *et al.* (1989) - cf. Šesták (1985).

Atrazine, similar to DCMU, inhibits electron flow at the acceptor side of PS2 (Büchel 1972, Tischer and Strotmann 1977, Jursinic and Stemler 1983, Rutherford *et al.* 1984). Our measurements of DCBQ-catalyzed O_2 evolution at rate-saturating irradiance (Fig. 2*A*) showed loss in PS2 activity increasing with concentration of atrazine in both 6 and 20 d-old thylakoids. The inset shows the changes in ratio of uninhibited (V_0)/inhibited (V_i) rates *versus* log atrazine concentration for estimating the change, if any, in I_{50} value as described by Sharma *et al.* (1989) for senescing leaf thylakoids: this value increased from 0.22 μM on the 6th day to 0.25 μM on the 20th day. This suggests a decrease in sensitivity to atrazine due to leaf senescence. Foliar senescence induces alteration at the PS2 acceptor side besides the donor side (Sabat *et al.* 1989).

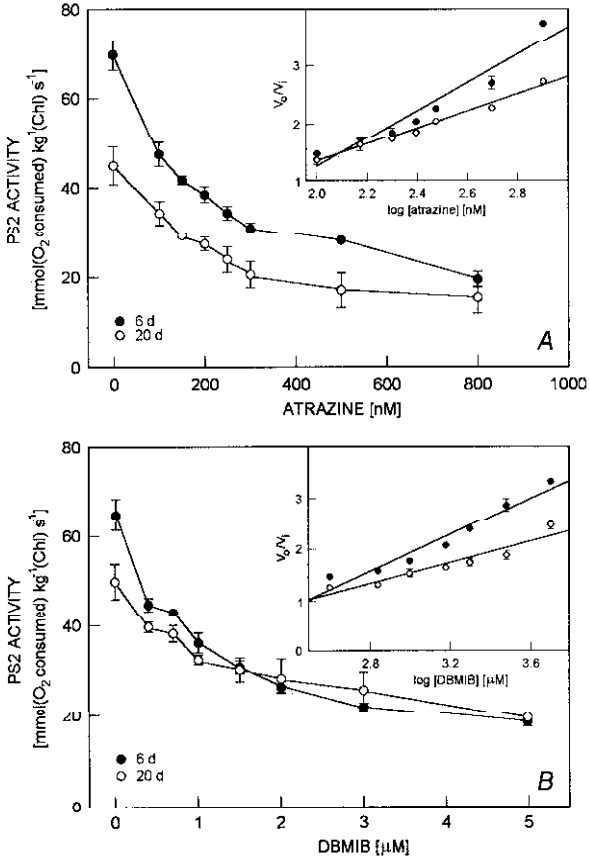

Fig. 2. Effect of varying concentrations of atrazine (*A*) and DBMIB (*B*) on photosystem 2 (PS2) electron transport rates of thylakoids from 6 (●) and 20 (○) d-old cotyledonary leaves of cucumber. *Insets*: Rates of uninhibited/inhibited (V_0/V_i) activities plotted as a function of log of atrazine concentrations. The experiments were done in three different sets of samples. Standard deviations are shown as vertical bars.

Dibromothymoquinone (DBMIB) inhibits electron flow from PQ to Cyt b_6f complex (Trebst *et al.* 1973). We, therefore, monitored the changes in sensitivity of senescing leaf thylakoids to DBMIB. The pattern of inhibition (Fig. 2*B*) was similar to that induced by atrazine, but the differences between young mature and senescing

cotyledons were much smaller. The V_0/V_i *versus* log[DBMIB] plot (Fig. 2*B*, *inset*) demonstrates the increase in I_{50} for DBMIB from 1.27 μM in 6 d-old cotyledonary leaves to 3.4 μM in 20 d-old ones. This suggests a decrease in sensitivity to DBMIB due to plant senescence. Thus, our results show that acceptor side of PS2 is altered as ageing proceeds.

KCN inhibits photosynthetic electron transport at more than one site (Ouitrakul and Izawa 1973). However, the major site of KCN inhibition is the PS1 donor side where plastocyanin (PC) feeds electrons to PS1 (Izawa *et al.* 1973). PS1 activity as monitored by MV reduction and consequent O_2 uptake with reduced 2,6-dichlorophenol indophenol ($DCPIPH_2$) as donor showed a nearly 28-31 % loss (Fig. 1) during senescence. We also tried to ascertain if the extent of inhibition of PS1 catalyzed O_2 uptake in 6 and 20 d-old leaves was altered upon KCN inhibition when ascorbate (ASC)-reduced diaminodurene (DAD), which feeds electrons close to PC (Ouitrakul and Izawa 1973), was used as electron donor. We found a small increase in activity of both 6 and 20 d thylakoids at low concentration of KCN and then a decrease (Fig. 3). At high (80 mM) KCN concentration, the 6 d thylakoids showed 60 % inhibition, while the 20 d thylakoids registered only a 40 % inhibition. The inset shows the residual PS1 activity in the presence of increasing concentration of KCN. Hence the senescing cotyledonary leaf thylakoids were less sensitive to KCN.

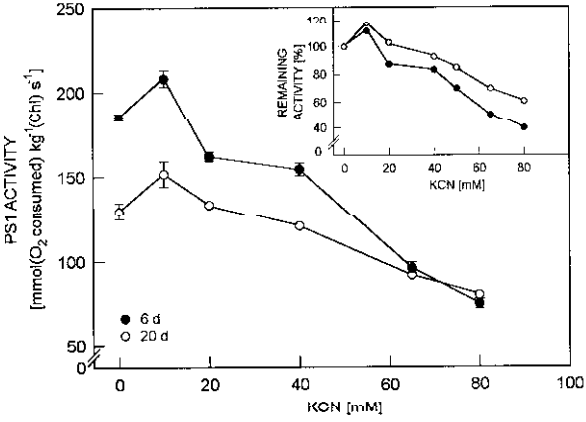

Fig. 3. Effect of varying concentrations of KCN on photosystem I electron transport rates (DAD/ASC→MV) of thylakoids from 6 (●) and 20 (○) d-old cotyledonary leaves of cucumber. *Inset*: % residual activity as function of KCN concentration. The rates of PS1 activity in the absence of KCN were adjusted to 100 %. The experiments were done in three different sets of samples. Standard deviations are shown as vertical bars.

In summary, our evaluation of relative efficiency of photosystem inhibitors indicates that during leaf senescence alterations of the PS2 acceptor and PS1 donor sides occur, and these changes involve mobile electron carriers such as PQ and PC.

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