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Senescence retarding effect of metal ions: Pigment and protein contents and photochemical activities of detached primary leaves of wheat

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Abstract

Al^{3+} significantly delayed the loss of chlorophyll (Chl), protein, and carotenoids when compared to K^+ and Mg^{2+} during dark-induced senescence of detached primary leaves of *Triticum aestivum*. Thylakoid membranes isolated from Al^{3+}-treated leaves showed a better retention of photosystem (PS) 2, PS1, and whole chain electron transport activities than thylakoids of K^+- or Mg^{2+}-treated leaves. These ions protected the electron transport activities and restored the DCMU-dependent fluorescence increase of thylakoid membranes in a valency-dependent manner. Al^{3+} also delayed the change of excitation energy distribution during senescence.

Additional key words: aluminium; carotenoids; chlorophyll; DCMU; electron transport; fluorescence emission spectra; leaf senescence; magnesium; potassium.

Introduction

Leaf senescence is a physiologically programmed process, which is characterised by loss of pigments, proteins, lipids, and nucleic acids (Šesták 1985, Grover *et al.* 1986, Smart 1994). At cellular level, the senescing leaves experience a sequential and ordered dismantling of organelles (Nooden *et al.* 1997). Chloroplast is the first organelle that shows ultrastructural changes among the organelles (Biswal and Biswal 1988). During leaf senescence, the photochemical activity limits photosynthesis (Harding *et al.* 1990). The drastic decline in activities of PS2, PS1, and whole chain electron transport (WCE) is reported in several senescing systems (Biswal and Mohanty 1978, Jenkins and Woolhouse 1981, Bricker and Newman 1982, Mc Rae *et al.* 1985, Grover *et al.* 1986, Sabat *et al.* 1989, Prakash *et al.* 1998). Therefore, the onset of leaf senescence obviously curtails the economic yield of crop plants to a significant extent (Frith and Dalling 1980). Hence, delaying leaf senescence has become an agronomically desirable trait (Grover 1993, Quirino *et al.* 2000). Some of the metal ions delay senescence process: Co^{2+} (Yu and Yaang *et al.* 1979, Geetachandra *et al.* 1981), Ni^{2+} (Mishra and Samal 1971), Ag^+ (Aharoni 1985), and Ca^{2+} (Poovaiah and Leopold 1973,Swamy *et al.* 1995).

Al^{3+}, which is one of the most abundant elements on the earth, is a growth-limiting factor in acid soils (Kochian 1995). At low concentrations, Al^{3+} stimulates the electron transport catalysed by PS2 of blue-green algae sphaeroplasts and isolated spinach-beet chloroplasts (Wavare *et al.* 1983). Al^{3+} also stimulates conductance to CO_2 trans-fer and net photosynthetic rate (Lidon *et al.* 1998). Metal ions such as Na^+, K^+, Ca^{2+}, and Mg^{2+} regulate electron transport, photophosphorylation, and energy distribution between the two photosystems of plant photosynthesis (Papageorgiou 1975, Williams 1977). Ca^{2+} and Mg^{2+} stabilise the photosynthetic membranes and activate the oxygen evolution capacity of cyanobacteria preparations and chloroplast membranes by binding to the negatively charged groups on membrane surface (Govindjee and Govindjee 1975, McCarty 1980). The screening of the fixed negative membrane surface charge depends on the cation valency and not on the chemical nature of their co-ions (Barber and Mills 1976). Wavare and Mohanty (1983) found in sphaeroplasts

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Abbreviations: Asc, ascorbate; Car, carotenoids; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DCPIP, 2,6-dichlorophenol indophenol; MV, methyl viologen; p-BQ, p-benzoquinone; PS, photosystem; WCE, whole chain electron transport.
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isolated from the cyanobacterium *Synechococcus cedrorum* that the stimulation of PS2-mediated oxygen evolution increases with the increase in valency of cation. Hence, the delay in loss of photochemical activities by selected cations such as K^+, Mg^{2+}, and Al^{3+} during leaf senescence can be expected as a function of their valency.

Materials and methods

Plants and treatment: Healthy seeds of wheat (*Triticum aestivum* L.) were obtained from Acharya N.G. Ranga Agricultural College, Tirupati. The seeds were surface sterilised with 0.1 % $HgCl_2$ for 2 min, thoroughly washed with tap water and then with distilled water. The seeds were imbibed for 6 h before placing seeds on 2-cm thick cotton bed moistened with distilled water. The seedlings were grown in dark for two days. Then the seedlings were exposed to "white light" in a growth chamber, where the photon flux density at the leaf surface was 30-35 μmol m^{-2} s^{-1}, the relative humidity was 60-65 %, and temperature was 25±1 °C. On the 8th day, fully expanded leaf segments (4-5 cm long) were cut from apical region and used for treatment. The four sets in quadruplicate (20 leaf segments in each set) were incubated individually in double distilled water, 50 μM KCl, 50 μM $MgCl_2$, and 50 μM $AlCl_3$ in test tubes. The bases of leaves were immersed in test solutions in complete darkness at 25±1 °C for 96 h. The leaf segments were sampled up to 96 h at regular intervals of 24 h for experiments.

Estimation of pigments and proteins: 0.1 g of leaf segments was homogenised in a pre-chilled mortar and pestle in 10 cm^3 of 80 % acetone. The homogenate was transferred into 15 cm^3 centrifuge tubes, and centrifuged at 50 rps for 2 min. The concentrations of chlorophyll (Chl) *a* and *b* were measured in supernatant after dilution to a total volume of 15 cm^3 according to Arnon (1949). Similarly, the concentration of carotenoids (Car), which had principal absorption in the blue region of the spectrum, was calculated according to Mackinney (1941). The total protein content in leaf segments was estimated following Lowry *et al.* (1951). 0.1 g of leaves was homoge-nised in 10 cm^3 of 10 % trichloroacetic acid (TCA) and incubated at 4 °C for 8 h. The TCA precipitate was ether-dried, dissolved in 1 M NaOH. Protein content was determined using bovine serum albumin (BSA) as standard.

Thylakoid membranes were isolated by a procedure similar to that of Saha and Good (1970) as described in Swamy *et al.* (1995). One g of leaf segments was homogenised in 10 cm^3 of isolation buffer [50 mM HEPES-NaOH (pH.7.8), 400 mM sucrose, 2 mM $MgCl_2$, 5 mM KCl]. The homogenate was filtered through two layers of *Miracloth*, and centrifuged at 83.3 rps for 5 min. The pellet was suspended in wash buffer [5 mM HEPES-NaOH, pH 7.8, 100 mM sucrose, 2 mM $MgCl_2$, 5 mM KCl] and centrifuged briefly at 33.3 rps for 30 s. The supernatant was pelleted again at 63.3 rps for 5 min and the pellet was finally suspended in a minimal volume of suspension buffer [20 mM HEPES-NaOH, pH 7.8, 100 mM sucrose, 2 mM $MgCl_2$, 5 mM KCl]. All operations were carried out at 4 °C in dim light. Chl content was estimated according to Arnon (1949).

Photosynthetic electron transport activities were measured polarographically with Clark-type oxygen electrode (*Hansatech,* U.K.) in 2 cm^3 reaction buffer containing 50 mM HEPES-NaOH, pH 7.5, 100 mM sucrose, 2 mM $MgCl_2$, and 5 mM KCl under saturating irradiance of 900 μmol m^{-2} s^{-1} at 25±1 °C according to Subhan and Murthy (2000). WCE and PS1 mediated activities were measured as molecular consumption. The reaction mixture for WCE contained 0.5 mM methyl viologen (MV) while that for the PS1 determination contained 5 mM ascorbate, 1 mM sodium azide, 0.5 mM MV, 0.1 mM DCPIP, and 10 μM DCMU. PS2-mediated electron transport was studied as molecular oxygen evolution using 0.5 mM p-BQ. Thylakoid membranes equivalent to 40 μg Chl were used in all assays.

Chl fluorescence emission of thylakoid membranes equivalent to 20 μg of Chl suspended in a buffer containing 50 mM HEPES-NaOH, pH 7.5, 100 mM sucrose, 2 mM $MgCl_2$, and 5 mM KCl was estimated in absence and presence of 10 μM DCMU at 25 °C using the *Jasco FP.777* spectrofluorometer with an excitation wavelength of 440 nm. The emission and excitation slit widths were 5 nm. Chl *a* fluorescence emission spectra at low temperature (77 K) were recorded for the samples prepared by mixing equal volumes of thylakoid suspension and 60 % glycerol to give final concentration of 5 g(Chl) m^{-3}. The samples were taken into micro-capillary tubes and frozen quickly in a specially constructed Dewar flask which fits in the cuvette position of *Hitachi MPF4* spectrofluorometer. Then, the samples were excited at 432 nm and emission was collected from 650 to 750 nm. The slit widths of excitation and emission monochromators were 8 and 2 nm, respectively.

Results and discussion

Loss of Chl (*a*+*b*) is an index of progress in leaf senescence and therefore the effects of K^+, Mg^{2+}, and Al^{3+} on total Chl loss were primarily studied. Chl (*a*+*b*) content steadily declined to 26 % at 96 h of dark incubation, which was arrested by the metal ions. Al^{3+} caused retention of 33 % of total Chl when compared to 29 and 30 % retention by K^+ and Mg^{2+}, respectively, at 96 h (Table 1). A loss of 64 % of protein at 96 h was minimised in metal ion-treated leaves (Table 1). Only a 30 % loss was observed in Al^{3+}-treated leaves when compared to 46 and 39 % in K^+- or Mg^{2+}-treated leaves, respectively. This indicates valency-dependent protection

Table 1. Effects of KCl, $MgCl_2$, and $AlCl_3$ on contents of chlorophyll (Chl) *a*, *b*, and (*a*+*b*) [g(Chl) kg^{-1}(f.m.)], carotenoids (Car) [g(Chl) kg^{-1}(f.m.)], and total protein [g(protein) kg^{-1}(f.m.)] in primary leaves during dark incubation. Values are means ± SD (n = 4). The concentrations of each metal ion were 50 μM. Values in parenthesis indicate percentage of residual Chl and protein.

Parameter	Treatment	Dark incubation [h] 0	24	48	72	96
Chl (*a*+*b*)	Control	2.46±0.10 (0)	1.91±0.05 (78)	1.26±0.08 (51)	0.90±0.08 (37)	0.65±0.04 (26)
	KCl		2.25±0.07 (92)	1.61±0.10 (65)	1.10±0.07 (45)	0.70±0.06 (29)
	$MgCl_2$		2.24±0.08 (91)	1.46±0.12 (59)	1.07±0.06 (44)	0.73±0.05 (30)
	$AlCl_3$		2.40±0.05 (98)	2.22±0.07 (90)	1.31±0.08 (53)	0.82±0.04 (33)
Chl *a*	Control	1.80±0.08 (0)	1.47±0.04 (82)	0.96±0.05 (53)	0.67±0.04 (37)	0.47±0.02 (26)
	KCl		1.78±0.06 (99)	1.23±0.05 (68)	0.74±0.05 (41)	0.48±0.04 (27)
	$MgCl_2$		1.76±0.05 (98)	1.10±0.08 (61)	0.77±0.05 (43)	0.52±0.02 (29)
	$AlCl_3$		1.80±0.03 (0)	1.76±0.04 (98)	0.94±0.03 (52)	0.58±0.01 (32)
Chl *b*	Control	0.48±0.03 (0)	0.44±0.01 (92)	0.30±0.02 (63)	0.24±0.02 (50)	0.18±0.01 (38)
	KCl		0.45±0.02 (94)	0.39±0.01 (81)	0.34±0.02 (71)	0.21±0.02 (44)
	$MgCl_2$		0.46±0.01 (96)	0.34±0.02 (71)	0.29±0.02 (60)	0.21±0.01 (44)
	$AlCl_3$		0.47±0.01 (98)	0.46±0.02 (96)	0.36±0.02 (75)	0.23±0.01 (48)
Car	Control	0.061±0.004 (0)	0.058±0.003 (95)	0.047±0.002 (77)	0.032±0.002 (52)	0.029±0.001 (48)
	KCl		0.059±0.004 (97)	0.050±0.002 (82)	0.045±0.001 (74)	0.036±0.002 (59)
	$MgCl_2$		0.060±0.004 (98)	0.055±0.003 (90)	0.045±0.001 (74)	0.041±0.001 (67)
	$AlCl_3$		0.059±0.003 (97)	0.057±0.001 (93)	0.050±0.003 (82)	0.045±0.002 (74)
Protein	Control	26.66±2.10 (0)	24.70±1.03 (93)	17.14±0.80 (64)	14.28±0.30 (54)	9.50±0.50 (36)
	KCl		24.80±1.50 (93)	20.05±0.70 (75)	18.10±0.50 (68)	14.28±0.46 (54)
	$MgCl_2$		26.00±1.50 (98)	22.86±0.50 (86)	20.48±0.30 (77)	16.20±0.40 (61)
	$AlCl_3$		26.60±1.00 (0)	25.71±0.40 (96)	20.86±0.90 (78)	19.05±0.50 (72)

of Chl and proteins. Selective loss of pigments denotes structural alterations in individual photosystems. The loss in Chl *a* contents corresponds to changes in PS1 (Myers *et al.* 1980, Fujita and Murakami 1987). Therefore, studying the concentrations of Chl *a* and Chl *b* under the influence of these ions provides an insight into the stability of thylakoid membrane organisation during leaf senescence. The degradation profile of Chl *a* under the influence of these metal ions was similar to the loss of Chl (*a*+*b*) (Table 1). The retention of Chl *a* was higher in Al^{3+}-treated leaf segments at 96 h of dark incubation than in K^+- or Mg^{2+}-treated leaves. Generally, the preferential degradation of Chl *a* is a characteristic feature of leaf senescence. However, in our conditions the concentration of Chl *b* also decreased equally with ~10 % difference to Chl *a* (Table 1), indicating degradation of Chl *a*/*b* associated polypeptides, namely light-harvesting complex 2 (LHC2), which after ribulose-1,5-bisphosphate carboxylase constitutes the major portion of total chloroplast protein (Grover 1993). A significant protection of Chl *b* (48 %) by Al^{3+} and 44 % each in K^+- and Mg^{2+}-treated leaves at 96 h suggests stabilisation of LHC2. On the other hand, Car which protect photochemical reactions from free radicals generated in stress conditions (Niyogi *et al.* 1997) were maintained at higher concentrations throughout dark incubation (Table 1) when compared to Chl. This showed the relative stability of Car over Chl during senescence as observed by Grover *et al.* (1986). A decrease of about 52 % in Car content in control leaf at 96 h can be attributed to enzyme degradation (Srichandan *et al.* 1989) and/or to the damaging effect of the reactive oxygen species generated in the chloroplasts (Swain *et al.* 1990). The 52 % loss in Car at 96 h was also influenced by metal ions (Table 1) as shown by retention of 59, 67, and 74 % of Car at 96 h by K^+, Mg^{2+}, and Al^{3+} ions, respectively, suggesting the influence of metal ions in providing further stability to Car.

Table 2. Effects of KCl, $MgCl_2$, and $AlCl_3$ on photochemical activities: whole chain electron transport ($H_2O \rightarrow MV$) [mmol (O_2 nocsumed) kg^{-1}(Chl) s^{-1}], photosystem (PS) 2 ($H_2O \rightarrow$p-BQ) [mmol(O_2 evolved) kg^{-1}(Chl) s^{-1}], and PS1 ($DCPIPH_2 \rightarrow MV$) [mmol (O_2 nocsumed) kg^{-1}(Chl) s^{-1}]. The concentration of metal ion was 50 μM. Values are mean±SD ($n = 4$). Values in parenthesis indicate percentage of residual electron transport activities.

Parameter	Treatment	Dark incubation [h] 0	24	48	72	96
WCE	Control	28±2 (0)	22±2 (79)	17±1 (61)	11±1 (39)	-
	KCl		24±1 (86)	19±1 (68)	13±1 (46)	-
	$MgCl_2$		24±2 (86)	19±1 (68)	15±1 (54)	-
	$AlCl_3$		27±1 (96)	22±1 (79)	19±1 (68)	-
PS2	Control	49±3 (0)	44±4 (90)	38±3 (78)	23±2 (47)	18±1 (37)
	KCl		48±2 (98)	45±2 (92)	32±4 (65)	19±2 (39)
	$MgCl_2$		48±3 (98)	45±4 (92)	38±3 (78)	21±2 (43)
	$AlCl_3$		49±5 (0)	47±3 (96)	45±3 (92)	28±3 (57)
PS1	Control	136±8 (0)	127±7 (93)	120±7 (88)	113±5 (83)	107±4 (79)
	KCl		127±8 (93)	123±6 (90)	116±4 (85)	106±7 (78)
	$MgCl_2$		128±6 (94)	123±5 (90)	118±5 (87)	114±6 (84)
	$AlCl_3$		133±7 (98)	132±5 (97)	130±3 (96)	117±3 (86)

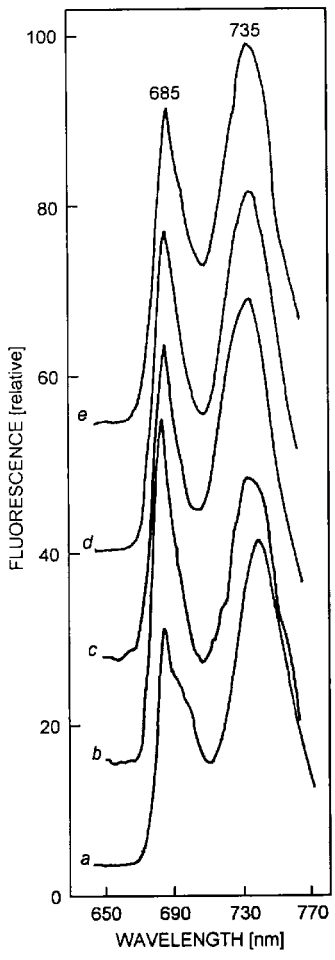

Fig. 1. Low temperature chlorophyll fluorescence emission spectra of leaf thylakoid membranes treated as follows: *a* - 0 h control, *b* – 72 h dark control, *c* – 72 h KCl, *d* – 72 h $MgCl_2$, *e* - 72 h $AlCl_3$.

To relate the persistence of pigments and proteins by these metal ions to photochemical activities of thylakoid membranes, WCE, PS1, and PS2 were assayed. WCE was decreased to 39 % of 0 h control activity at 72 h (Table 2). The activity was delayed in K^+-, Mg^{2+}-, and Al^{3+}-treated leaf segments as they caused retention of 46, 54, and 68 % of 0 h control activity at 72 h. p-BQ-supported PS2 activity was decreased to 37 % of 0 h control leaf activity at 96 h. However, the activity loss was minimised to 43 % by Al^{3+} when compared to 57 % by K^+ and 61 % by Mg^{2+} (Table 2). While PS1 activity was slightly decreased in dark control leaves (to 79 % of 0 h control activity at 96 h), PS1 activity loss was also delayed by these ions as the leaf segments retained 84 and 86 % of 0 h control activity by applying Mg^{2+} and Al^{3+}, respectively, at 96 h (Table 2). Hence, the progressive loss in electron transport activities as observed by Jenkins and Woolhouse (1981), Bricker and Newman (1982), Grover *et al.* (1986), Sabat *et al.* (1989), and Prakash *et al.* (1998) was delayed by these metal ions. This could be due to stabilisation of thylakoid membrane organisation *via* screening of negatively charged surfaces of thylakoid membranes (Govindjee and Govindjee 1975, McCarty 1980). Further, the activities were better retained at 72 h with metal ions of increasing valency. This indicates a possible role of electrostatic repulsive and/or attractive forces in photochemical activities (Barber 1982).

Cations influence changes in photochemistry of PS1 and PS2, causing an increase in room temperature fluorescence emission associated with PS2 (Homann 1969, Murata 1969) and regulation of energy distribution (Williams 1977). At room temperature, Chl *a* fluorescence emission at 683 nm decreased to 69 % in 72 h control leaf thylakoids (Table 3).

Table 3. Effects of metal ions on chlorophyll (Chl) fluorescence [relative] at 25 °C and 77 K. Thylakoid membranes were isolated from 0 and 72 h control and 72 h metal chloride-treated leaf segments equivalent to 20 μg(Chl) in presence and absence 10 μM DCMU in 1 cm^3 of suspension buffer containing 50 mM HEPES-NaOH, pH 7.5, 2 mM $MgCl_2$, 5 mM KCl, and 100 mM sucrose. KCl, $MgCl_2$, or $AlCl_3$ concentrations were 50 μM.

Fluorescence emission at		Control 0 h	72 h	KCl 72 h	$MgCl_2$ 72 h	$AlCl_3$ 72 h
25 °C	F_{685} – DCMU	61	42	48	54	57
	F_{685} + DCMU	100	48	70	83	91
	F_{685} + DCMU/F_{685} – DCMU	1.64	1.14	1.46	1.54	1.60
77 K	F_{735}/F_{685}	1.43	0.85	1.10	1.09	1.21

The emission was retained in K^+-, Mg^{2+}-, and Al^{3+}-treated leaf thylakoids. K^+, Mg^{2+}, and Al^{3+} caused 79, 89, and 93 % retention of fluorescence emission, respectively, at 72 h. DCMU, an inhibitor of electron transfer at the level of Q_A, caused an enhancement of fluorescence emission at 683 nm by 64 % in 0 h control leaf thylakoids (Table 3). The enhancement of fluorescence emission by DCMU declined to 14 % in 72 h control leaf thylakoids. This may be due to a decreased rate of charge separation and/or an increased rate of charge recombination. However, K^+, Mg^{2+}, and Al^{3+} caused 44, 54, and 60 % enhancement of fluorescence emission at 72 h (Table 3), suggesting the restoration of normal function of PS2 by these ions during leaf senescence in dark. At 77 K, thylakoids isolated from 72 h dark control leaves showed loss of fluorescence emission at 735 nm (originating in PS1) when compared to the emission at 685 nm (originating in PS2) (Fig. 1, Table 3) as the ratio F_{735}/F_{685} decreased to 0.85 at 72 h from 1.43 of 0 h control spectra. This may be due to smaller proportion of Chl associated with PS1 (Jenkins *et al.* 1981). This is consistent with the decrease in leaf Chl *a* concentration at 72 h (Table 1). However, the ratio F_{735}/F_{685} was restored to 1.10, 1.09, and 1.21 by K^+, Mg^{2+}, and Al^{3+}, respectively (Table 3), suggesting that the extent of excitation energy distribution may be protected by Al^{3+} in favour of PS1. Thus, Al^{3+} marginalises the effects of dark-induced alterations in pigments, proteins, and photochemical activities of detached wheat primary leaves significantly when compared to K^+ or Mg^{2+}.

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