

# Effects of Polyamines on Chlorophyll and Protein Content, Photochemical Activity, and Chloroplast Ultrastructure of Barley Leaf Discs during Senescence<sup>1</sup>

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ABE S. COHEN<sup>2</sup>, RADOVAN B. POPOVIC, AND SAUL ZALIK<sup>3</sup>

Department of Plant Science, University of Alberta, Edmonton, Alberta T6G 2E3 Canada

## ABSTRACT

The polyamines putrescine, spermidine, and spermine prevent the loss of chlorophyll normally associated with senescence of excised leaf tissue maintained in darkness on water (control). Retention of chlorophyll in barley leaf discs was in the range of 90% 4 days after excision and placement on effective polyamine solutions. In contrast, the loss of soluble protein was hastened with 0.5 millimolar spermidine and spermine treatments but it was retarded by 0.5 millimolar putrescine.

Photosystem I and II activities of chloroplasts from polyamine-treated leaf discs declined more rapidly as compared to the control. Chloroplast ultrastructural changes resulting from the polyamine treatments included the apparent destruction of the envelope, preservation of thylakoid membrane structure, and reduced accumulation of osmiophilic bodies. The influence of polyamines on senescence-related processes may be due to their cationic nature.

## MATERIALS AND METHODS

**Plant Material.** Barley seeds (*Hordeum vulgare* L., cv. Gateway) were allowed to germinate in Vermiculite at 23 C for 7 days under continuous illumination of 600 ft-c. Discs, 4 mm in diameter, were cut from the primary leaves 1 cm below each leaf tip; they were floated on the appropriate test solutions in Petri dishes (30 discs per dish) and maintained in darkness at 23 ± 2 C. Preliminary studies with larger leaf sections resulted in variegation, presumably due to inadequate penetration. Aqueous solutions of the polyamines were neutralized with NaOH to pH 7.0.

**Total Soluble Protein Measurement.** For each treatment the leaf discs were ground in 2 ml of ice-cold 0.02 M Tris-HCl (pH 7.5). The homogenate was centrifuged at 26,000g for 10 min and supernatant protein was determined by the method of Bradford (8). Chl was determined by Arnon's method (4).

**Chloroplast Isolation.** For each treatment 360 leaf discs were ground by mortar and pestle in 10 ml of medium consisting of 0.5 M sucrose, 1 mM MgCl<sub>2</sub>, 0.2% (w/v) BSA, and 67 mM phosphate buffer (KH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>) (pH 8.0). The homogenate was filtered through nylon cloth of 40 μm pore size and centrifuged at 4,000g for 5 min. The chloroplast pellet was resuspended in buffer containing 0.33 M sorbitol, 2 mM EDTA, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, and 50 mM Hepes (pH 7.6) (35).

**Measurement of Photochemical Activity.** PSII and PSI activity were measured using a Clark-type O<sub>2</sub> electrode. For PSI activity O<sub>2</sub> consumption was monitored in a 1-ml reaction chamber containing 3 mM sodium isoascorbate, 0.2 mM TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine), 0.1 mM methyl viologen, 1 to 5 μg of Chl, and 15 μM DCMU in the resuspending buffer. PSII activity was measured by O<sub>2</sub> evolution as described by Delieu and Walker (14).

**Electron Microscopy.** Samples from each treatment were taken for electron microscopy at 48 and 96 h by cutting sections from the discs avoiding the midrib. The sections were fixed in glutaraldehyde in phosphate buffer (pH 7.2) for 3 h at 22 C and postfixed in 2% OsO<sub>4</sub> for 2 h. The tissue was dehydrated in a graded ethanol series and embedded in araldite resin. The material was sectioned and stained with 2% uranyl acetate followed by 0.2% lead citrate (20). The sections were studied with a Philips EM 200 microscope.

## RESULTS

**Chl and Protein Retention.** The effectiveness of the polyamines in preventing the loss of Chl and soluble protein in the leaf discs is shown in Table I. For Chl, a progressive decline in level occurred with the leaf discs floating on water (control). After 24 h 80% of the original Chl was retained but only 16% was found after 96 h. For each of the polyamines at the lowest concentrations tested the Chl loss was similar to that of the control. At higher

Natural and artificially induced senescence of leaf tissue have been used to study the effects of a number of metabolites and growth regulators on protein metabolism and on the structure and photochemical properties of chloroplasts (9, 16, 18, 23, 26, 32, 36). In this respect it has been found that kinetin can retard the characteristic loss of protein and pigments during leaf senescence (7, 15, 22) as well as preserve chloroplast structure (7) and the integrity of the electron transport chain (15). Poovaiah and Leopold (27) have reported that inorganic cations, particularly Ca, can also defer the symptoms of leaf senescence, an effect they attributed to the maintenance of membrane integrity.

The stabilization of membrane integrity by Ca and the naturally occurring organic cations, the polyamines, has been shown with osmotically sensitive forms of different bacteria (19, 30). More recently, polyamines have also been found to be capable of retarding the progressive senescence of protoplasts isolated from oat leaves (1, 17). There are many examples from both prokaryotes and eukaryotes of polyamine involvement in the regulation of macromolecular synthesis (6, 10, 13, 31), and it has been shown that polyamines can replace the Mg<sup>2+</sup> requirement for protein synthesis in a barley *in vitro* system (12). The present study was undertaken to determine the effects of polyamines on Chl and protein retention, photochemical activity, and chloroplast ultrastructure of barley leaf discs during senescence.

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<sup>2</sup> Present address: Department of Plant Science, University of Manitoba, Winnipeg, Manitoba R3T 2N2 Canada.

<sup>3</sup> To whom reprint requests should be addressed.

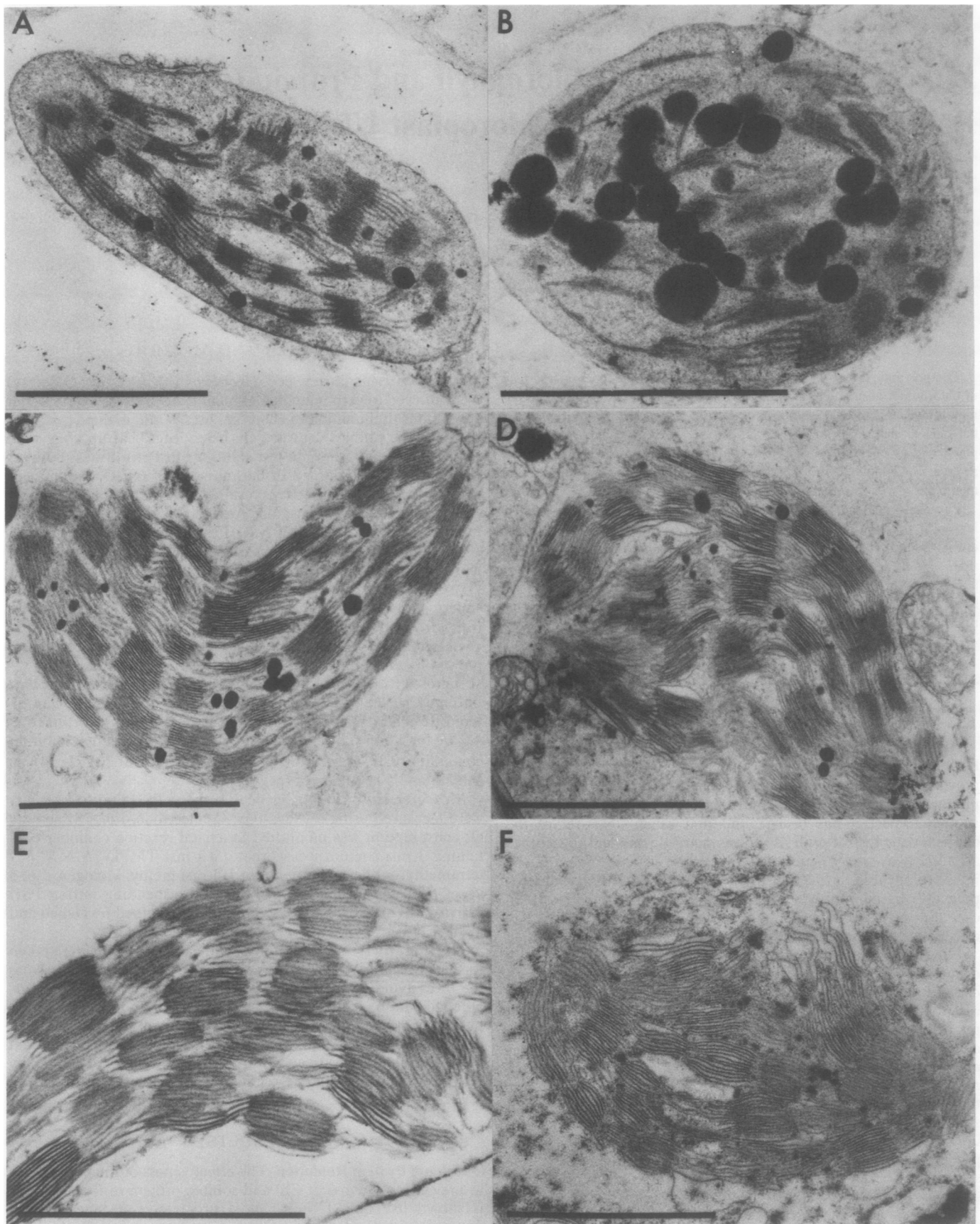


FIG. 1. Ultrastructure of chloroplasts of barley leaf discs floated on water or polyamine solutions in the dark. A: water control, 48 h; B: water control, 96 h; C: 0.5 mM putrescine, 48 h; D: 0.5 mM putrescine, 96 h; E: 0.05 mM spermine, 48 h; F: 0.05 mM spermine, 96 h. The ultrastructure of chloroplasts from 0.1 mM spermidine treatment was virtually identical to that for spermine. Bars are 1  $\mu$ m.

Table I. Retention of Chl and Soluble Protein in Barley Leaf Discs Floated on Polyamine Solutions in the Dark

The values are the averages of three independent experiments and the error of estimate was less than  $\pm 10\%$ .

Treatment	Chl retention (%) <sup>a</sup>				Protein retention (%) <sup>a</sup>			
	h <sup>b</sup>				h <sup>b</sup>			
	24	48	72	96	24	48	72	96
Control (H <sub>2</sub> O)	80	49	25	16	76	46	32	22
Putrescine (mM)								
0.1	79	44	25	18	76	48	33	21
0.5	104	98	97	89	91	84	63	42
1.0	108	101	99	94	91	75	38	26
Spermidine (mM)								
0.01	80	45	23	17	78	48	31	22
0.1	101	101	97	90	75	50	28	23
0.5	99	95	95	94	76	49	23	12
Spermine (mM)								
0.0005	86	53	28	18	79	53	32	22
0.05	102	98	97	89	61	31	21	13
0.5	102	96	89	91	58	24	14	9

<sup>a</sup> Expressed as per cent of value at initiation of experiment, i.e. 0 h.

<sup>b</sup> Duration following excision of discs from leaves.

Table II. PSII and PSI Activities of Chloroplast Preparations Isolated from Barley Leaf Discs Floated on Polyamine Solutions in the Dark

Treatment	PSII Activity ( $\mu\text{mol O}_2$ evolution/mg Chl · h)				PSI Activity ( $\mu\text{mol O}_2$ consumption/mg Chl · h)			
	h <sup>a</sup>				h <sup>a</sup>			
	6	24	48	96	6	24	48	96
Control (H <sub>2</sub> O)	168	130	74	0	1300	1223	784	348
Putrescine (0.5 mM)	120	70	34	0	735	636	372	86
Spermidine (0.1 mM)	99	0	0	0	471	156	72	75
Spermine (0.05 mM)								
	108	0	0	0	522	121	89	85

<sup>a</sup> Duration following excision of discs from leaves.

concentrations of the polyamines, there was a decrease in the rate of Chl loss giving a retention of about 90% even after 96 h.

There was a similar decline in soluble protein in the water control and in the lowest polyamine concentration. However, the intermediate concentration of putrescine (0.5 mM) showed a marked reduction of protein loss while in the highest spermidine concentration tested and the two effective spermine levels, protein retention in the tissue was lower than in the control (Table I).

**Photochemical Activity and Ultrastructure.** After only 6 h of incubation with polyamines there was a reduction in photochemical activity compared to the water control (Table II). At this time PSII was reduced by 30 to 40% and PSI was reduced by 40 to 60%. The envelopes of the chloroplasts of the water control at 48 h were still present, the grana stacks had many fused thylakoids, and there were a number of osmiophilic bodies present (Fig. 1A). However, PSII and PSI activities had decreased by about 50% compared to the 6-h treatment (Table II). After 96 h the envelope was still present but the grana that remained were scattered and single thylakoids were distributed throughout the stroma. The most typical feature was the presence of many large osmiophilic bodies (Fig. 1B). At this time PSII activity was completely lacking while PSI activity was reduced to approximately 25% of that at 6 h (Table II).

Chloroplasts from the 48-h putrescine treatment lacked envelopes but retained many large grana stacks (Fig. 1C). Compared to the 6-h treatment the PSII and PSI activities were reduced by 70 and 50%, respectively (Table II). Even after 96 h grana stacks were still present and there were only a few relatively small osmiophilic bodies (Fig. 1D). PSII activity was absent and only

10% of the PSI activity shown at 6 h remained (Table II).

Spermidine and spermine had similar effects on the ultrastructure and also on the photochemical activities of the chloroplasts. For both treatments, at 48-h chloroplast envelopes were absent, the grana stacks were distended, and there were no osmiophilic bodies present (Fig. 1E). By 96 h the grana were dilated, the thylakoids were extended to fill the intermembrane space with a series of parallel membranes, and there was evidence of a few small osmiophilic bodies (Fig. 1F). PSII activities had already disappeared by 24 h and PSI activities were markedly reduced (Table II). By 48 h PSI activity had decreased for both treatments to approximately 15% of the 6-h levels. Thus, while in comparison to the control the polyamines preserved the thylakoid membranes, they also caused a more rapid decline in photochemical activity.

## DISCUSSION

Senescence induced by dark stress of excised barley leaf discs incubated in water resulted in a decline in Chl and soluble protein. Similar results were reported previously for whole leaves of *Avena* (11). However, in contrast with the results of Choe and Thimann (11), the PSII activity declined more rapidly in the barley leaf discs than PSI activity (Table II).

Polyamine treatment prevented Chl loss and preserved thylakoid membrane structure. Similar effects have been shown with kinetin (2, 7, 15). The maintenance of structural integrity accompanied by a loss of function of the thylakoid membranes might be explained by the occurrence of differential proteolysis of the thylakoid membrane proteins required for Chl stability and function respectively. This type of proposal has also been put forward in the explanation of the naturally occurring nonyellowing foliar senescence of a mutant genotype of meadow fescue (33), a system exhibiting structural and functional characteristics analogous to those demonstrated in our present study on polyamine effects. Increases in proteinase activity have been found during leaf senescence (22, 26), and such activity could have occurred in the barley leaf discs since the data on protein retention did show a general loss of soluble protein.

Alternatively, the loss of photosynthetic activity while thylakoid membranes were retained may have been due to the alteration of membrane lipids (21, 34). Since polyamine treatment resulted in a more rapid loss of the chloroplast envelope, it may even be possible that an accompanying loss of some endogenous factor from the thylakoid membranes accounted for their loss in activity. The finding that the addition of an artificial electron donor (diphenylcarbazide) could restore the Hill reaction activity of barley chloroplasts isolated from senescing leaf tissue (data not shown and ref. 7) supports this possibility.

Since inorganic cations affect the functional and structural integrity of the chloroplast membrane system (3, 5, 24, 29) and there is evidence for anionic binding sites on thylakoid membranes (25, 28), it seems that the strongly cationic polyamines may be exerting their capabilities in preserving thylakoid morphology and Chl through interaction with the negatively charged loci on the membranes. Since polyamines are cations which can be actively synthesized within plant cells (refs. in 12), regulation of their intracellular distribution may have an as yet unassumed role *in vivo* related to various structural and functional characteristics of chloroplasts.

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