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Deferral of Leaf Senescence with Calcium[1]

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ABSTRACT

In view of the possibility that senescence may be a consequence of the deterioration of membrane compartments in the cells of leaves, calcium was studied as a possible agent which might defer senescence. The senescence of corn leaf discs was deferred by added calcium, and the effect was additive to the cytokinin deferral of senescence. Likewise, the senescence of Rumex leaf discs was deferred by added calcium, and the effect was additive to the gibberellin deferral of senescence. Detailed experiments with corn leaf discs established that the increase in apparent free space associated with senescence was completely prevented by calcium. An increase in hydraulic permeability during senescence was likewise demonstrated, and this increase was deferred by calcium; calcium plus benzyladenine was even more effective. Each of the measured functions of leaf senescence (chlorophyll content, protein decrease, apparent free space increase, and hydraulic permeability increase) was suppressed by calcium, and the interpretation is offered that the effects are a consequence of the calcium function in maintaining cellular membranes.

Since the discovery by Richmond and Lang (17) that cytokinins could defer leaf senescence, it has become generally evident that each of the five known plant hormones is capable of altering senescence; three of the known plant hormones are each capable of deferring leaf senescence (8, 15, 17), and the other two are stimulators of leaf senescence (1, 4). A general concept of endogenous regulation of senescence by hormones is, however, made less attractive by the difficulties of bringing about major alterations in senescence development through applications of hormones to intact plants. Recent work in our laboratory has shown that calcium ions can alter appreciably some aspects of senescence development in petiole explants (16); this finding together with the earlier theory that senescence may relate to deterioration of membrane integrity (18) and the evidence that calcium may sustain membrane integrity (13) leads us to examine the possible effectiveness of calcium in deferring leaf senescence.

MATERIALS AND METHODS

Corn seeds (*Zea mays* L., WF 9 MST × B 37 hybrid from Ag. Alumni Seed Improvement Assn., West Lafayette, Ind.) were germinated in plastic flats containing vermiculite in a growth chamber (2000 ft-c, 16 hr daily) at a temperature close to 22 C. At 8 to 10 days, leaf discs measuring 1 cm in size were taken from the primary leaf with the aid of a cork borer. The discs were floated in 10 ml of test solution, 10 discs per Petri dish in darkness at 25 C for 3 to 5 days.

For the Rumex assay (*Rumex obtusifolius L.*), following the procedure of Whyte and Luckwill (27), old leaves which were uniformly green were selected from matured plants in the greenhouse, and they were allowed to stand overnight at 25 C in the dark with petiole in water. Leaf discs (1 cm) were cut with a cork borer and randomized, and 10 discs were floated in each Petri dish containing 10 ml of solution to be tested in the darkroom at 25 C for 4 or 5 days. Chlorophyll was extracted in ethanol. Ten leaf discs were transferred to 10 ml of ethanol in a closed vial and allowed to stand overnight, and absorbance was read at 665 nm. Experiments were repeated 4 to 6 times.

For protein measurement 20 leaf discs were ground in a mortar and pestle with phosphate buffer at pH 6.5 and centrifuged at 800*g* to discard cellular debris. Protein was precipitated from the supernatant with 10% trichloroacetic acid and centrifuged at 10,000*g* for 10 min. The precipitate was dissolved in 5 ml of 0.1 N NaOH, and a 0.1-ml aliquot was used for protein analysis by the method of Lowry *et al.* (12).

Apparent free space was determined by the method of Epstein (5). Twelve leaf discs were blotted on a paper towel to remove solution adhering to the surface. To initiate the absorption, the leaf discs were transferred to a salt solution (1 mM KCl) containing a radioactively labeled ion (Rb-86). At the end of the absorption period (90 min), the solution containing the radioactively labeled ion was decanted, and the leaf discs were blotted on a paper towel. Some leaf discs were used to determine the initial uptake of radioactive rubidium, and the remainder were transferred to 100 ml of nonradioactive salt (1 mM KCl) for 30 min. The solutions were shaken gently, during both the absorption and desorption periods. Following the desorption period, the leaf discs were assayed for radioactivity. The apparent free space was calculated from the proportion of radioactive nuclide eluted out to the amount taken up.

Hydraulic permeability was studied by incubating 12 leaf discs in 5 ml of [^{3}H]water (1 μc/ml) in a closed tube for 90 min (11, 23). The leaf discs were shaken gently during incubation. At the end of the 90-min absorption period, leaf discs were blotted on a paper towel and transferred to 10 ml of tritium-free water, and, at appropriate intervals, 0.1-ml aliquots were removed, and radioactivity was determined by liquid scintillation. The half-time for equilibrium with the external medium and the total time for equilibrium of [^{3}H]water were used as measures of water permeability.

RESULTS

In order to examine the effectiveness of calcium salts in deferring leaf senescence, corn leaf discs were placed in serial

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concentrations of $CaCl_2$ and held in darkness for 4 days, after which changes in chlorophyll were taken as measures of senescence. Parallel experiments were carried out in the absence and presence of optimal concentrations of the cytokinin benzyladenine. Results of such an experiment are shown in Figure 1, where higher chlorophyll levels were maintained with calcium concentrations between 10^{-4} and 10^{-1} M. Calcium is approximately as effective in deferring senescence in the presence of the cytokinin as in its absence.

The senescence of Rumex leaf discs is not sensitive to cytokinin but is sensitive to gibberellin (27); the calcium effects were similarly studied in this plant material. Calcium defers the senescence of Rumex discs in a manner roughly equivalent to the effect on corn discs (Fig. 2).

If the calcium effect is truly a deferral of senescence, one would expect a better maintenance of the protein content of the leaf discs along with the improvement of chlorophyll content. Measurements of the protein levels in corn leaf discs after 4 days in darkness (Fig. 3) show that calcium alone does maintain a higher protein level at 10^{-3} and 10^{-2} M concentrations.

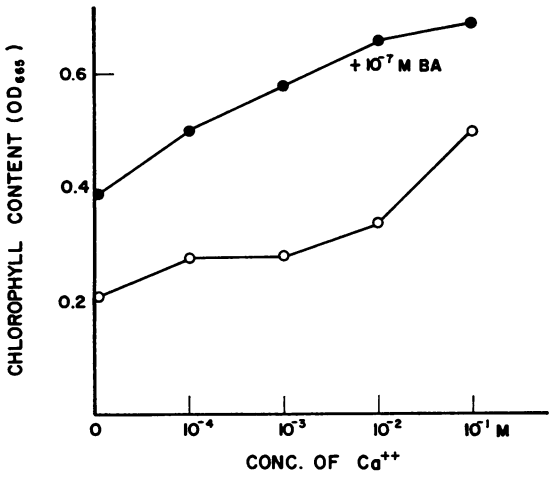

FIG. 1. Effects of calcium chloride solutions on the chlorophyll content of corn leaf discs, alone and in the presence of 10^{-7}M benzyladenine. All calcium treatments were significantly different from the controls (99% probability, Student's *t* test).

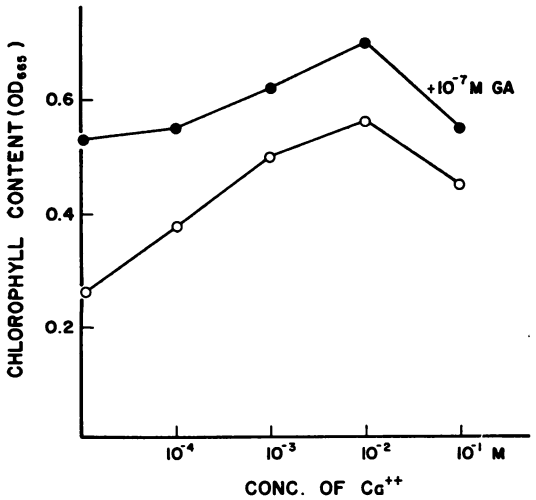

FIG. 2. Effects of calcium chloride on the chlorophyll content of Rumex leaf discs, alone and in the presence of 10^{-7}M gibberellic acid. All calcium treatments were significantly different from controls except GA+10^{-4}M $CaCl_2$ (99% probability, Student's *t* test).

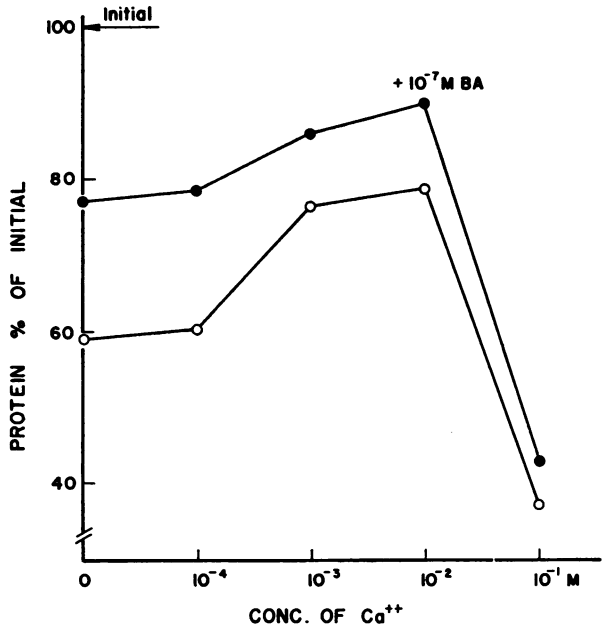

FIG. 3. Effects of calcium chloride on the protein content of corn leaf discs, alone and in the presence of 10^{-7}M benzyladenine. Protein content of fresh corn leaves (initial) was 11.07 mg/g fresh wt.

Table I. *Effects of Various Cations on Senescence of Corn Leaf Discs as Evidenced by Chlorophyll Content after 4 Days in Darkness*

Treatment (1 mM salt)	Chlorophyll Content	
	A_{665}	Percentage of control
O	0.37	100
$Ca(NO_3)_2$	0.58	156.7
$CaCl_2$	0.53	143.2
$LaCl_3$	0.54	145.9
$MgCl_2$	0.46	124.3
KCl	0.42	113.5
NaCl	0.36	97.2

In the presence of an optimal cytokinin concentration, there was a further gain in protein levels which was comparable to the effect of the calcium alone. At the highest calcium concentration, 10^{-1} M, protein fell off to levels below those of the water controls, indicating that tissue damage was obtained at this concentration of $CaCl_2$.

To examine the specificity of the effect, corn leaf discs were treated with two different salts of calcium and equimolar concentrations of four other cations; the results (Table I) show that the chloride and the nitrate of calcium were similarly effective in deferring the decline of chlorophyll, and that magnesium was somewhat less effective, with a lesser effect for potassium and no effect for sodium. Lanthanum, which is referred to in numerous biological situations as a "super-calcium," was as effective as calcium in the deferral of corn leaf disc senescence.

Mention has been made of the theory of Sacher (18) that a basic component of senescence was a deterioration of cellular membranes; if the effectiveness of calcium in deferring senescence involves the maintenance of cellular membranes, then one might expect that the calcium effect should be reflected in a lowering of the apparent free space. To examine this parameter, corn leaf discs were equilibrated with rubidium,

and the freely exchangeable radioactive ions were measured as the amount elutable with potassium; the apparent free space was calculated from the proportion of radioactive nuclides elutable out of the amount taken up. Experimental data for this type of experiment are shown in Figure 4, where it is evident that concentrations of $CaCl_2$ ranging from 10^{-4} to 10^{-1} M were very effective in maintaining the apparent free space at a low level. In the presence of optimal concentrations of benzyladenine, the same range of calcium was effective in holding the apparent free space down.

Another means of examining the possible role of membrane integrity in senescence is through the measurement of the permeability of tissues to water. Thimann and Samuel (23) used tritiated water as a means of measuring the hydraulic permeability of potato slices; using similar types of experiments on corn leaf discs, we can observe striking differences in the elution of the labeled water out of fresh and out of

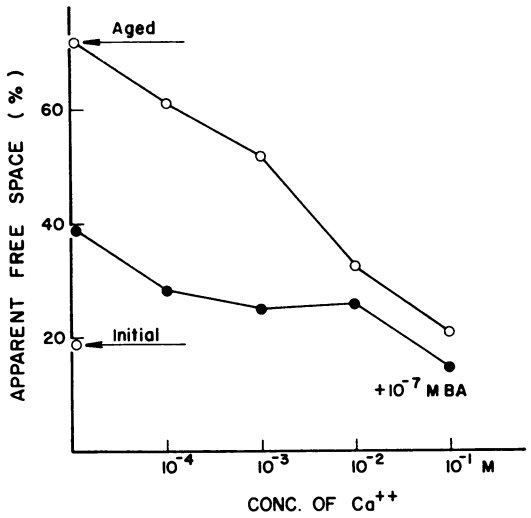

FIG. 4. Changes in the apparent free space in corn leaf discs as influenced by calcium chloride solutions, alone and in the presence of 10^{-7}M benzyladenine. The apparent free space for leaves before aging in the dark is indicated by initial arrow.

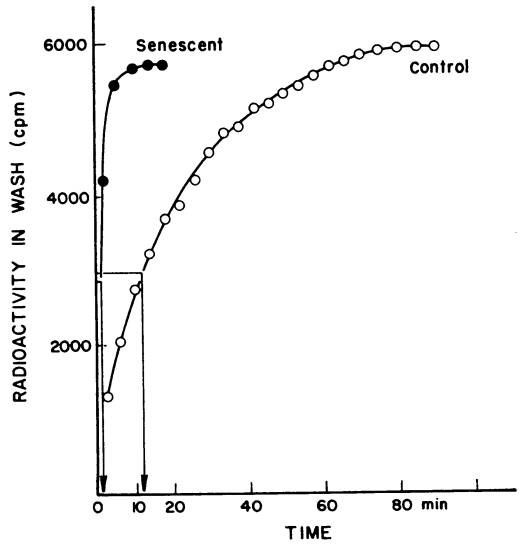

FIG. 5. The time course of elution of tritiated water from corn leaf discs without aging in the dark (control) and after 4 days of aging in the dark (senescent). The ordinate is radioactivity per 0.1 ml of ambient solution. Arrows indicate elution half-time.

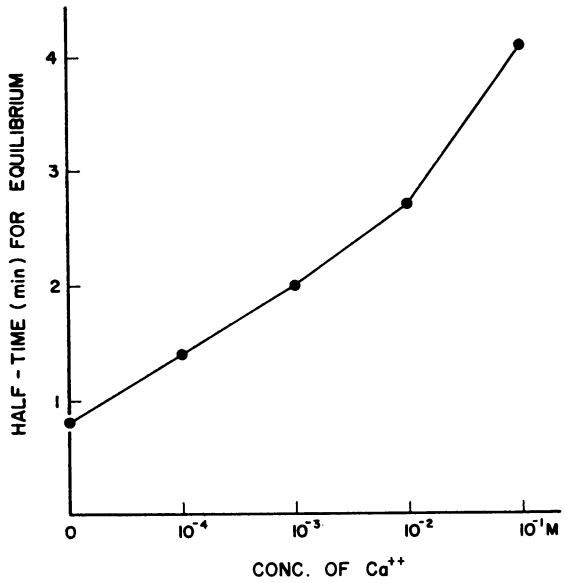

FIG. 6. Effects of calcium chloride solutions on the half-time for equilibration of tritiated water out of corn leaf discs. Half-time for unaged leaf discs was 11.2 min, as in Table II.

Table II. *Changes in Hydraulic Permeability of Corn Leaf Discs With Senescence and as Influenced by Benzyladenine (10^{-7} M) and Calcium (10^{-2} M)*

Efflux of radioactivity in minutes after 90-min incubation in ^3H_2O.

Time	Time to Reach Equilibrium			Half-time to Equilibrium		
	Control	BA	BA plus Ca	Control	BA	BA plus Ca
	min			*min*		
Initial	71			11.2		
4 days in dark	6	32	58	0.8	3.1	8.0
5 days in dark		17	39		2.4	5.6

senescent tissues, as shown in Figures 5 and 6. Thimann and Samuel used two parameters of water movement as measurements of permeability: the time for equilibrium of the label moving out of the tissue, and the time for exit of half of the equilibrium amount (the half-time for equilibration). In the experiment in Figure 5, the equilibrium time is approximately 75 min for control and 8 min for senescent leaf disc tissues. Half-times are approximately 11 and 1.0 min, respectively. In experiments in which the effects of senescence-deferring treatments were included (Table II), it can be seen that cytokinin treatment keeps the equilibrium time at a 5-fold higher level, and the further addition of calcium keeps it 10-fold higher than controls. The half-times again show 4-fold higher values with cytokinin and 10-fold with cytokinin plus calcium. A concentration curve for the effects of calcium on the half-times is shown in Figure 6, and the effective range of calcium in decreasing hydraulic permeability can be seen to be similar to the range for maintaining chlorophyll content (Fig. 1).

DISCUSSION

The experiments reported here indicate that the senescence of leaf discs can be deferred by calcium application; the calcium response is evident in terms of improved maintenance of chlorophyll levels and the maintenance of protein levels, and

it applies both to cytokinin-regulated leaves of corn and to gibberellin-regulated leaves of Rumex. Associated with the deferral of senescence, the calcium treatments prevent the rise in apparent free space associated with senescence and similarly prevent the increase in the ease of water flow out of the leaf tissues—an increase shown to be associated with senescence development in corn leaf discs.

The concept that membrane failure may be involved in leaf senescence was first suggested by Sacher (18). Eilam (3) showed that senescence of bean leaves was associated with a leakage of potassium from the leaves and an associated increase in apparent free space. Das (2) observed an increase in leakage of total electrolytes from senescing bean leaves. Measurements of hydraulic permeability have not been used in studies of leaf senescence prior to the present experiments, but stem tissues have been reported to show little or only small changes in hydraulic permeability in response to metabolic inhibitors (9, 14) or to growth regulators (11, 21). Pea stems were found to decrease in hydraulic permeability in response to calcium applications (11).

The possible participation of calcium in regulatory systems in plants may be inferred from the fact that calcium is widely known to play a major role in membrane structure and function (10). Its importance in the regulation of ion transport is well known (6, 26). Its effects on the maintenance of RNA and of protein levels in Lemna have been described by Trewavas (24, 25), and these components are considered central indices of senescence. Calcium shows a singular lack of mobility in plants (19, 20). Since one of the first symptoms of leaf senescence is a deterioration of the chloroplasts, it may be pertinent to note that the chloroplast is the location of more than half of the calcium content of leaves (22).

Each of the parameters of leaf senescence measured has been found to be markedly deferred or prevented by the addition of calcium ions. While the highest concentration utilized, 10^{-1} M, caused a sharp decrease in protein content suggesting that there might have been tissue damage at this level, still, each of the senescence deferral parameters measured showed deferral effects at 100 to 1000 times lower concentrations than those which might have caused injury. The possibility that changes in the calcium supply to leaves in the intact plant may participate in the endogenous regulation of senescence is being studied.

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