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Relation between senescence and stomatal opening: Senescence in darkness*

(abscisic acid/cycloheximide/cytokinins/diffusion resistance/dipyridyl/fusicoccin/phenazine methosulfate/spermidine)

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ABSTRACT **The senescence (proteolysis and loss of chlorophyll) of isolated leaves of oat seedlings in the dark is inhibited or delayed by compounds of six different types: phenazine methosulfate, fusicoccin, α,α'-dipyridyl, cycloheximide, spermidine, and two cytokinins. In every case but the last, these compounds in optimum concentration caused the stomata to open and remain partly or completely open throughout the 72- or 96-hr experimental period. The cytokinins caused only a partial opening, which is ascribed to their exerting two different effects. Taken together with the previous report that five different treatments that accelerated or promoted senescence in the light caused stomatal closure or occlusion, these data establish a general parallel between stomatal aperture and senescence, with strong indication that the stomatal aperture is the causal factor. A possible explanation of the relationship is proposed.**

The processes constituting senescence in leaves can be approached from either the molecular or the anatomical viewpoint. Up to now the molecular approach has been largely followed, changes in DNA and RNA having first been emphasized, then subsequently changes in respiration and metabolism (see refs. 1 and 2).

In the preceding paper, however (3), experiments were presented that indicated that anatomical changes were of major importance, at least in the seedling oat leaves which have been under continuous study for some time. Specifically, it was shown that the delay of leaf senescence brought about by white light was accompanied by opening of the stomata and that treatments that increased that opening increased the delaying effect of light on senescence, while several agents that caused the closing of stomata prevented this effect of light. Even artificial occlusion of the stomatal aperture by lipid films, or closure caused by simple immersion in media of high osmotic value, diminished or prevented the delaying effect of light. In all cases, chlorophyll degradation and proteolysis responded to very similar extents, indicating that the entire syndrome of senescence was being affected.

The work to be reported here carries the parallelism between senescence and stomatal aperture further, in that senescence in darkness is shown to be strongly delayed by treatments that cause stomata to open in the dark.

MATERIALS AND METHODS

The use of 7-day-old seedling oat leaves (*Avena sativa* cv. Victory) has been described (2). The 3-cm subapical segments were floated on test solutions, and the content of chlorophyll and free amino acids was followed in parallel with measurements of the diffusion resistance for water vapor, by the porometer of Kanemasu *et al.* (4) (Lambda Instruments, Lincoln, NE) with a 4-mm aperture. The *r* values were determined from a calculated calibration curve (3). Diffusion resistance and gain in leaf fresh weight were mutually confirmatory (see Table 2 and Fig. 4).

RESULTS

Phenazine Methosulfate (PMS). One of the most powerful agents that we have discovered for this function is the dye PMS. Widely used in photosynthesis researches, its actions in darkness have been little studied, though it does maintain phosphorylation in spinach chloroplasts stored in the dark (5). In the presence of 50 mM KCl this compound, at 30 μM, causes slow but eventually complete stomatal opening in the dark. Fig. 1*A* shows that even on day 3 there is no tendency to close. KCl alone has a slight effect, not remarkable in view of the known role of K^+ ions in the development of guard cell turgor. What is notable, however, as shown in Fig. 1*B*, is that the stomatal opening induced by PMS is acccompanied by complete maintenance of chlorophyll, the value at 30 μM being not significantly different from that of control leaves in light (it is actually slightly higher). Fig. 1*B* shows that the normal proteolysis is also inhibited almost as completely. However, the KCl exerts a partial inhibition of this last process itself, so that the zero value for amino nitrogen in the figure is well below that for salt-free controls.

The fact that chlorophyll is maintained and proteolysis is prevented is evidence that the total syndrome of senescence is inhibited by PMS. Comparison with the partial effects of lower concentrations of PMS on all three functions shows that the parallel with stomatal aperture is well maintained.

Fusicoccin. This compound, which has some of the effects of auxin but an entirely different structure, has been reported to cause stomatal opening for short times in several plant species (6). For the present experiments a much longer time span was needed. Fortunately, fusicoccin does indeed have a long-term effect on floating oat leaf segments. Due to its limited solubility, it was necessary to make solutions in 50% ethanol and dilute to a final concentration of 1% ethanol. This concentration of ethanol by itself has a real effect in temporarily lowering diffusion resistance; accordingly, the controls for this and the following series of experiments were also floated on 1% ethanol.

Table 1 and Fig. 2 show that fusicoccin causes the diffusion resistance to remain low for 2 days and, although the closure begins thereafter, the resistance remains much lower than in the controls. As predicted, the chlorophyll content was correspondingly well maintained, the higher concentration, 0.1 mM, giving nearly as complete chlorophyll retention as the light.

α,α'-Dipyridyl. We earlier reported that this substance and its congener, α,α',α''-tripyridyl, the only ones of several chelating agents tested, powerfully delayed the senescence of oat

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Abbreviation: PMS, phenazine methosulfate.

* This paper is no. 2 in a series. Paper no. 1 is ref. 3.

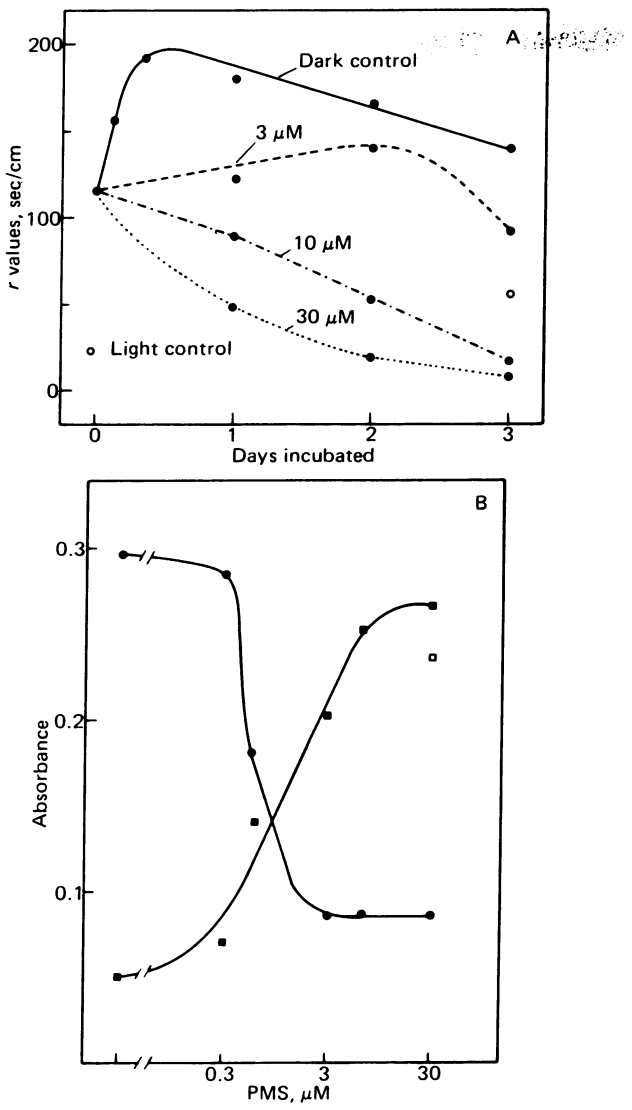

FIG. 1. (*A*) Time course of the stomatal diffusion resistance, *r*, of control leaves on water and of leaves in darkness on three concentrations of PMS. Open circle shows the *r* value for controls in light. (*B*) Chlorophyll concentration and free α-amino nitrogen after 3 days in darkness as a function of PMS concentration. ■, Chlorophyll ($A_{660\ nm}$); ●, α-amino nitrogen ($A_{570\ nm}$); □, chlorophyll (light control).

leaves in the dark (7). The optimum concentration was 1 mM. At the time, the action was ascribed to the chelation of a metal atom in an enzyme. However, the present data offer a quite different interpretation. Because of the presence of 1% ethanol, the controls of Fig. 3 show the temporary opening and subsequent steadily increasing closure seen in Fig. 2. However, the

Table 1. Effect of fusicoccin (FC) on stomatal opening and loss of chlorophyll in the dark

Day	Water	1% ethanol	50 μM FC	100 μM FC
		Diffusion resistance, *r*		
1	181.2	58.4	3.0	2.9
2	167.6	79.2	5.2	4.0
3	141.0	101.0	30.1	23.2
		Chlorophyll content, A_{660}		
4	0.032	0.097	0.170	0.212

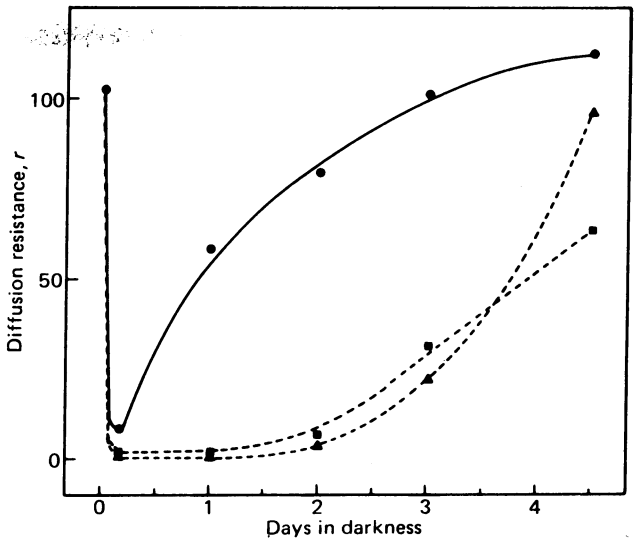

FIG. 2. Time course of stomatal diffusion resistance, *r*, of leaves in darkness on 1% ethanol (●) and on 50 μM (■) and 100 μM (▲) fusicoccin (also in 1% ethanol).

broken line of Fig. 3 shows that this stomatal closure is prevented by the dipyridyl, and the opening steadily increases to reach a value comparable with that of light controls by day 4. Correspondingly, the decrease of chlorophyll in the dark is largely prevented by 1 mM dipyridyl, as previously reported (though by day 4 the maintenance is somewhat less complete in Fig. 3 than in the experiment of Table 2, which is otherwise comparable). Table 2 shows that the gains in fresh weight are consistent with the *r* values and also that 1 mM dipyridyl completely prevents the normal proteolysis. The contrast between treated and control leaves is at least as great in stomatal aperture as in chlorophyll and amino nitrogen.

Cycloheximide. It has been shown (8) that cycloheximide prevents senescence of oat leaves in the dark, and figure 9 of ref. 8 showed that the concentrations effective on senescence also inhibit the incorporation of [^{14}C]leucine into protein. It has been pointed out, however (9, 10), that the latter action is not highly specific, the formation of RNA and DNA being inter-

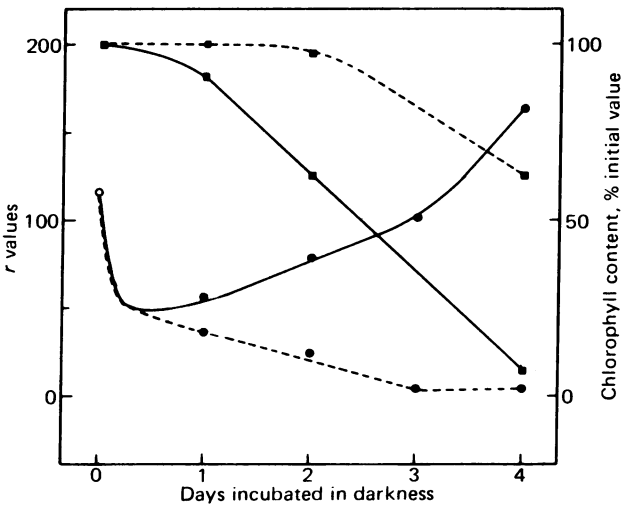

FIG. 3. Time course of stomatal diffusion resistance, *r*, and of chlorophyll content of leaves on 1% ethanol and on 1 mM α,α′-dipyridyl in darkness (in 1% ethanol). ■, Chlorophyll; ●, *r*; —, control (1% ethanol); ---, 1 mM α,α′-dipyridyl.

Table 2. Effects of a series of concentrations of α,α'-dipyridyl on senescence in the dark*

Concentration, mM	Gain in weight,† mg	Chlorophyll, A_{660}	Free amino nitrogen, A_{570}
0.0 (water)	35.0	0.042	0.779
0.03	26.9	0.068	0.794
0.1	11.3	0.145	0.576
0.3	3.2	0.280	0.560
1.0	1.0	0.283	0.190

* Values after 96 hr.

† These values were corrected for slightly differing weights of the initial leaf segments, being referred to 160 mg initial weight (eight leaves).

fered with and even respiratory rate being influenced. Nevertheless, it was surprising to find that cycloheximide in the same range of concentrations as those which inhibit protein synthesis caused stomatal opening in the dark. Fig. 4 shows the chlorophyll content and the resistance values after 72 hr in the dark. The agreement with the prevention of proteolysis is perfect; that with the chlorophyll is at least highly suggestive. At 100 mM, at which protein synthesis is maximally inhibited, the chlorophyll maintenance is about maximal and the stomatal aperture nearly so.

Cycloheximide has no detectable effect in light.

Spermidine. Our earlier observation (11) that the action of L-serine in accelerating senescence is antagonized by L-arginine has led to a study of other polyamines. Of those found to modify senescence, spermidine was given further study. Table 3 shows that at 30 mM, the optimum concentration, spermidine in the dark maintains the chlorophyll about 60% as well as does light. Surprisingly, it lowers both the free amino nitrogen and the diffusion resistance even more than does the light. (PMS at 10 and 30 μM has the latter effect also, as seen in Fig. 1.) In any event, the three effects are again modified in rough parallel.

Cytokinin. It is ironical that the one substance with which the parallel between senescence and stomatal aperture is not clear is kinetin, the only widely accepted inhibitor of senescence. Cytokinins have been reported several times to promote stomatal opening, though those experiments have lasted only up to 24 hr (e.g., ref. 12 and literature there cited). In the present work kinetin has indeed been found to cause stomatal opening of detached oat leaves in darkness. However, the effect

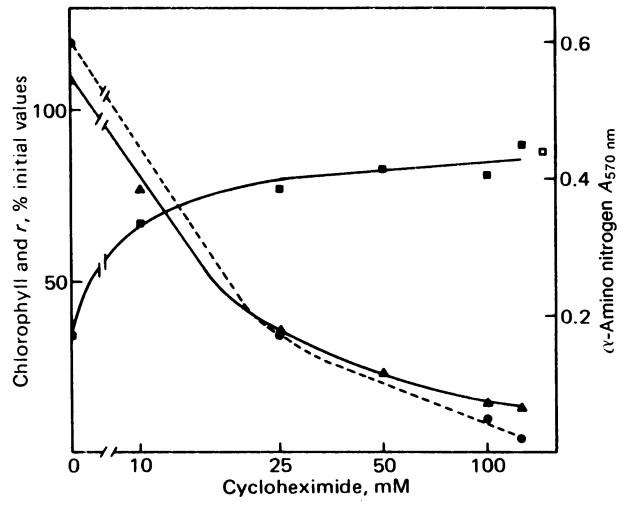

FIG. 4. Chlorophyll (■), α-amino nitrogen (▲), and diffusion resistance (r) (●) after 72 hr in darkness as a function of cycloheximide concentration. □, Chlorophyll (light control).

Table 3. Effect of spermidine on senescence in the dark

Datum measured	Dark control	Spermidine, 30 mM	Light control
Chlorophyll content at 96 hr (A_{660})	0.045	0.136	0.222
Amino nitrogen at 96 hr	0.760	0.245	0.354
Diffusion resistance (r) at 72 hr	144	10.4	54

is only partial, as shown in Fig. 5. The concentration used in Fig. 5, 3 mg/liter, is that which causes maximum delay of senescence (2). The stomatal opening is clearly less than that of most of the reagents listed above. Another cytokinin, 6-benzylaminopurine, has quantitatively about the same activity on senescence as kinetin (2), yet Fig. 5 shows that its action on the stomata is also far from complete.

DISCUSSION

Of the total of 11 different treatments in this and the preceding paper (3), 6 in the dark and 5 in the light, 10 have shown unequivocal parallelism, stomatal closure accompanying accelerated senescence and stomatal opening accompanying delay or inhibition of senescence. The parallels are in some cases close, in others only rough; some treatments tend to be more effective on chlorophyll maintenance than on proteolysis and vice versa. However, these 10 treatments made use of 10 different and chemically unrelated compounds. It is improbable that the mechanism of their action on the stomata could be the same in all cases, which makes the connection all the more significant. The remaining treatment, that with cytokinins in the dark, shows at least a partial parallel. The most probable explanation of this last is that the action of the cytokinins may be dual, part resulting in stomatal opening and part in some other processes which also, independently, lead to the control of senescence. Effects on RNase, on protein synthesis, and on respiration have all been reported many times and could doubtless participate. With this proposal all the remaining facts would fall into line.

Cycloheximide deserves special comment, since the maximal effects for chlorophyll maintenance, stomatal aperture, and the inhibition of protein synthesis are all attained at the same concentration (Fig. 4 and ref. 8). Yet, with cycloheximide the effect on senescence certainly cannot be attributed directly to the stomatal opening, because Makovetzki and Goldschmidt

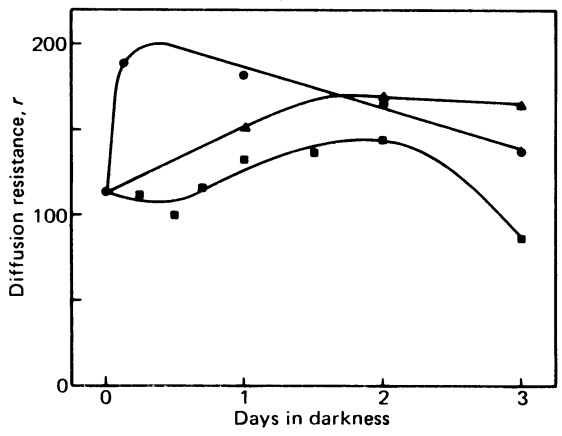

FIG. 5. Time course of stomatal diffusion resistance, r, of control leaves on water and on two cytokinins at concentrations optimal for chlorophyll maintenance. ●, Dark control; ■, kinetin, 3 ppm (average of 60 determinations); ▲, benzyladenine, 3 ppm.

have reported that the drug exerts powerful inhibition of senescence on *Elodea* (*Anacharis*) leaves (13). These leaves are normally completely submerged. This fact supports the idea of an interrelated *syndrome*, interference with any one constituent of which causes simultaneous effects on the other processes.

It is notable also that spermidine has recently been found to prolong life of isolated leaf protoplasts (14). This response also strongly suggests an interrelated group of processes in the leaf cells. It was earlier suggested that senescence could result from a failure of phosphorylation (15). Light, by this concept, would act by contributing cyclic photophosphorylation, and considerable evidence links stomatal opening with this process (16). The possibility of a three-part syndrome—senescence, phosphorylation, stomatal aperture—is indeed an intriguing one which is now being examined.

In the history of the action of cytokinins in preventing or delaying senescence, a recurring theme has been that of *directed transport*. Mothes (17) offered a molecular basis for this process, ascribing it to an increased capacity of the treated area to retain small molecules and inhibit their diffusion. Some indirect evidence does link the cytokinins with an action on membranes. Müller and Leopold (18) noted directed transport of ^{32}P but not of several other ions and envisaged the treated area as a suction pump, causing mass flow transport. In the light of the present data, it seems possible that stomatal opening localized around the cytokinin-treated area would cause transpirational flow to that area, carrying solutes along in the conducting units. Indeed, Kuraishi and Ishikawa (19) have shown that application of petroleum jelly to the treated area, on a dicotyledonous leaf, largely prevents the directional transport of amino acids to the area, which gives this explanation strong support.

A parallel alone does not establish cause and effect. Both senescence and stomatal aperture, and perhaps also cyclic photophosphorylation, might well be controlled by some additional factor which remains to be discovered. Against that, the experiments in which stomatal closure in light was brought about by physical and osmotic means point strongly to the stomatal aperture's being *in control*. Just what the link could be, whereby stomatal movement can control senescence, is far from clear. The ultimate effector in senescence might be a gas, whose concentration in the intercellular spaces is the determining factor. Yet, with a single exception (20), the most probable such gas, namely, ethylene, has not been found to control senescence in the leaves of any species, and our own (unpublished) efforts to implicate ethylene with oat leaves have been fruitless (see *Note Added in Proof*). There might be physiological or anatomical reasons why no effect of ethylene, endogenous or exogenous, could be disclosed, of course, and participation of another unknown gas cannot be excluded. CO_2 would evidently not fit the facts. But a quite different explanation is possible and will be tentatively presented here.

It is well known that wilting causes production of abscisic acid and that abscisic acid then causes stomatal closure (21). Suppose that the reverse were also true—namely, that stomatal closure, or even occlusion of the aperture, causes the production of abscisic acid. Stomatal opening, by endogenous causes or exogenous treatments, would not only prevent this, but might lead to the degradation of any abscisic acid already present. In other words, the effects of stomatal aperture on senescence would actually be mediated by the internal concentration of abscisic acid. If this were true, it would explain the peculiar case of cytokinins, since kinetin directly antagonizes abscisic acid in several responses and table 1 of the preceding paper (3) shows that it does so also for senescence. For the moment, this proposal must remain as no more than a suggestion, but we hope to test it in the near future.

Note Added in Proof. Ethylene does somewhat inhibit the light effect on senescence, but the effect is too small to be the basis for the stomatal control reported here.

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