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Inhibition by Calcium of Senescence of Detached Cucumber Cotyledons

EFFECT ON ETHYLENE AND HYDROPEROXIDE PRODUCTION

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ABSTRACT

The effect of Ca on senescence was followed in detached cucumber (*Cucumis sativus* L.) cotyledons floating on various solutions in the dark. Compared with those in water, cotyledons in 10^{-4} molar $CaCl_2$ exhibited reduced chlorophyll loss and H_2O_2 production, reduced and delayed ethylene production, and did not undergo a burst in CO_2 production. In contrast, Mg had little effect on cotyledon senescence, whereas K stimulated chlorophyll loss but did not increase H_2O_2 accumulation of ethylene and CO_2 production. This reduction in the rate of senescence by Ca could also be achieved by increasing the endogenous levels of Ca in the cotyledons before excision, although the reduction was less than that with Ca in the external solution. The addition of H_2O_2 to the solutions on which cotyledons were floated stimulated chlorophyll breakdown, but effects on ethylene and CO_2 were not consistent.

Ca is known to delay senescence of plant tissue. This is especially evident in fruit, where respiration rates (and thus rates of ripening) are reduced in tissue having high Ca concentrations (4). Other parameters of fruit ripening such as solute leakage can be reduced by Ca as well (9). Ca also lowers the rate of Chl and protein degradation in leaf discs of corn and *Rumex* (29), indicating an effect on vegetative senescence.

In senescing leaf discs, respiration and ethylene production both undergo a lag period followed by stimulation similar to that of the climacteric rise in ripening fruit (1, 3, 16, 31). This burst of activity is concomitant with the period of most rapid Chl breakdown. Recently, ethylene has been strongly implicated as a dominant hormone in the regulation of leaf senescence (1–3, 14), although the exact nature of control of the process is still unknown. However, if Ca is to have an effect on leaf senescence, it should be manifest in a change in the pattern of ethylene production.

There is strong evidence that oxidative metabolism also plays a role in senescence of both fruit and leaf tissue. Frenkel and coworkers (5, 6, 13) have demonstrated an association between the ripening of pears and tomatoes, and production of H_2O_2 and H_2O_2-producing enzyme systems. Increased levels of endogenous H_2O_2 or inhibition of catalase activity also enhanced fruit ripening. Other workers have demonstrated that exogenous H_2O_2 enhances leaf senescence (26, 30), and the activity of glycolate oxidase, a major peroxide-generating enzyme, has been found to increase during senescence of sunflower leaves (30), and may have allowed H_2O_2 to accumulate. It is therefore possible that there is an involvement of H_2O_2 in leaf senescence similar to that proposed for ripening fruit (5, 6).

We have used detached cucumber cotyledons to examine the effects of Ca on senescence. Such cotyledons undergo senescence in a manner similar to that of excised leaves, both in terms of Chl and protein breakdown (21) and in a decline in catalase activity (28). We show here that Ca has a substantial inhibitory effect on ethylene production, respiration, Chl breakdown, and H_2O_2 accumulation in this tissue.

MATERIALS AND METHODS

Plant Material. Cucumber (*Cucumis sativus* L. var Heinz Pickling) seedlings were germinated and grown without any nutrient supplement, in vermiculite, in a growth room (25 ± 1°C; 16-h photoperiod; light intensity, 300 μE m^{-2} s^{-1}).

Experimental Procedure. Cotyledons were excised from seedlings and floated in 20 ml of the appropriate solution in Petri dishes, eight cotyledons per dish in the dark at 25 ± 1°C. The Petri dish lids were left slightly off to prevent gas accumulation and reduce solution loss. All solutions contained streptomycin (25 μg/l) and were changed every 24 h.

Analyses. Chl was measured on four replicates of four cotyledons (0.5 g fresh weight) per sampling time by extracting the material in 5-ml volumes of cold acetone until all pigment was removed. The extract was adjusted to a volume of 50 ml at a final strength of 80% (v/v) acetone and then the absorbance of each extract was measured at 645, 652, and 663 nm. Chl content was calculated according to the method of Bruinsma (7).

Ethylene and CO_2 were measured on five replicates of four cotyledons (~0.5 g fresh weight) per sampling time. The cotyledons were placed in 50-ml Erlenmeyer flasks containing 5 ml of experimental solution. The flasks were then stoppered with rubber septa and the gases allowed to accumulate for 24 h. Ethylene was measured in 1-ml gas samples by GC with photoionization detection; CO_2 in similar 1-ml samples by GC with thermal conductivity detection. Experiments which included a KOH well in the flask to absorb CO_2 showed no effect on ethylene production of CO_2 accumulation over 24 h. Flasks set up as blanks without tissue produced insignificant amounts of ethylene.

H_2O_2 was measured by a modification of the method of Brennan and Frenkel (5). Four replicates of eight cotyledons (approximately 1 g fresh weight) were homogenized in 5-ml cold acetone in a Kondes grinder. The extract and washings were centrifuged (1,250*g*) and then 0.5 ml $TiCl_4$ in HCl (20% v/v $TiCL_4$ in concentrated HCl) was added to the supernatant. The solution was set shaking, and 3.5 ml of one-fifth strength NH_4OH was added dropwise with thorough mixing. The samples were then centrifuged (1,250*g*) and the precipitates washed repeatedly with 5-ml volumes of acetone until the supernatant was colorless. The precipitates were solubilized in 10 to 15 ml 2 N H_2SO_4, made up to 20

or 25 ml with H_2SO_4 and filtered prior to measurement of A at 415 nm against a blank which had been carried through the same procedure. Standards in the range of 0.15 to 0.75 mmol H_2O_2 ml^{-1} were also reacted with $TiCl_4$ and carried through the procedure.

Calcium Uptake and Mineral Analysis. Where uptake of ^{45}Ca was to be studied, solutions in the Petri dishes contained 10^{-4} M $^{45}CaCl_2$ (37 kBq $nmol^{-1}$ Ca). At sampling, four replicates of four cotyledons per sample were washed for 30 s in ice water, blotted dry and weighed, digested in $HNO_3/HClO_4$, and made up to 10 ml. Aliquots (0.5 ml) were assayed for ^{45}Ca by liquid scintillation spectrophotometry.

Ca, Mg, and K were measured in $HNO_3/HClO_4$ digestates of three replicates of eight cotyledons per sample time. Ca and Mg were measured by atomic absorption spectrophotometry and K by flame emission.

RESULTS

Senescence in Water. Typical data for cotyledons senescing on water are shown in Figure 1. Chl breakdown occurred steadily after a lag period of about 2 d. This period of Chl loss coincided with a rapid increase in ethylene production from less than 0.1 nl 4 cotyledon^{-1} h^{-1} at day 2 to more than 1.3 nl 4 cotyledon^{-1} h^{-1} at day 10. A burst of CO_2 production starting at day 3 reached a maximum at day 7. Peroxide content more than doubled over the 10-d period.

Effect of Calcium. There was a strong influence of 10^{-4} M $CaCl_2$

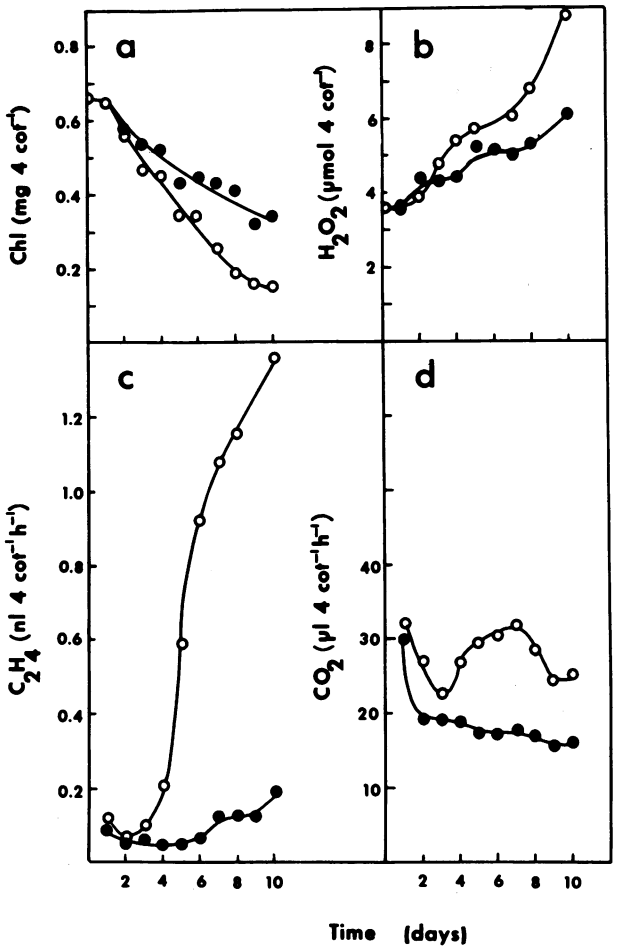

FIG. 1. Effect of 10^{-4} M $CaCl_2$ on senescence of cotyledons. Effect on (a) Chl content, (b) H_2O_2 content, (c) ethylene production, (d) CO_2 production. Sixteen-d-old cotyledons were detached and kept in the dark in water (○) or 10^{-4} M $CaCl_2$ (●).

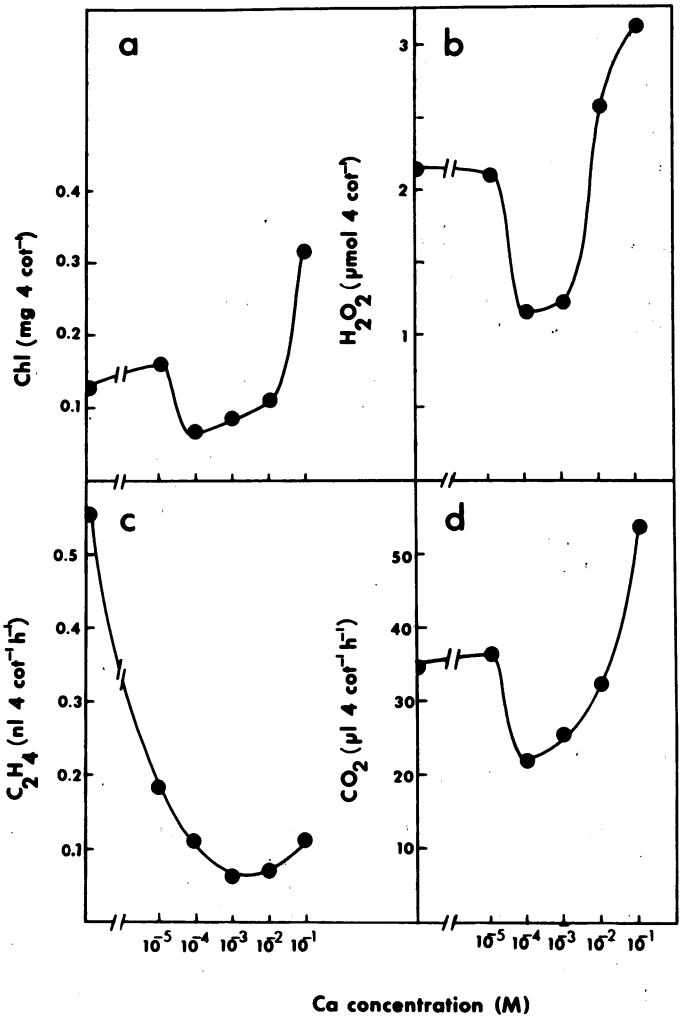

FIG. 2. Effect of different Ca concentrations on senescence of cotyledons. Effect on (a) Chl content, (b) H_2O_2 content, (c) ethylene production, (d) CO_2 production. Sixteen-d-old cucumber cotyledons were detached and kept in the dark. Measurements were made 6 d from excision.

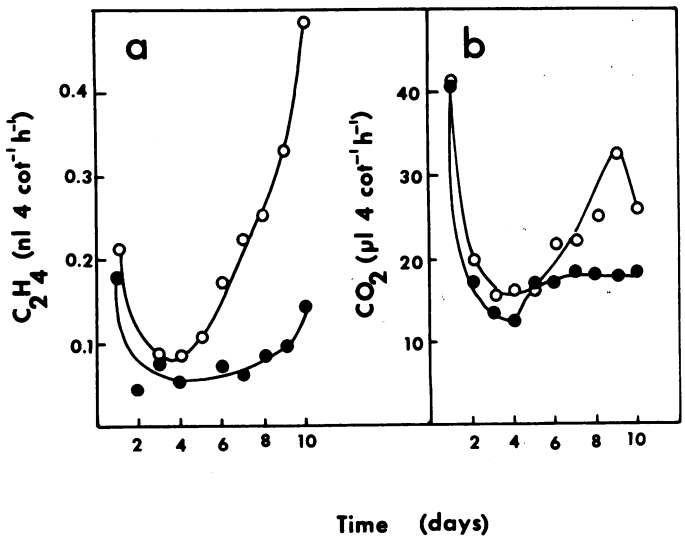

FIG. 3. Effect of 10^{-4} M $CaCl_2$ on senescence of cotyledons. Effect on (a) ethylene production, (b) CO_2 production. Eight-d-old cotyledons were detached and kept in the dark on water (○) or 10^{-4} M $CaCl_2$ (●).

on cotyledon senescence (Fig. 1). The rate of Chl breakdown was approximately halved, the rise in ethylene production delayed and reduced, the CO_2 burst eliminated and H_2O_2 accumulation reduced. Ca-treated cotyledons produced 0.37 nl 4 cotyledon^{-1} h^{-1} of ethylene after 11 d, indicating that a rise in production was occurring, although the tissue was showing signs of breakdown at this stage. A reduction in H_2O_2 accumulation due to Ca was not substantial until after about 6 d, when there was a 54% increase in peroxide level, compared with a 95% increase in water alone.

In a separate experiment where all cotyledons were floated on water, addition of 10^{-4} M $CaCl_2$ only for the 24-h period of ethylene accumulation resulted also in reduced ethylene production. Two d from excision, ethylene production in water was 0.22 nl 4 cotyledon^{-1} h^{-1} and in $CaCl_2$, 0.12 nl 4 cotyledon^{-1} h^{-1}. After 8 d, it was 0.52 and 0.26 nl 4 cotyledon^{-1} h^{-1} in water and $CaCl_2$, respectively.

A Ca concentration series from 10^{-6} to 10^{-1} M showed that concentrations of 10^{-3} and 10^{-4} M were the most effective in reducing all four parameters (Fig. 2). Ethylene production was reduced by all Ca concentrations relative to the water control, but Chl breakdown, CO_2 production, and H_2O_2 accumulation were either unaffected by or actually increased with Ca concentrations other than 10^{-3} and 10^{-4} M.

Effect of Cotyledon Age. Cotyledons excised at 8 d underwent a slower rate of senescence than those excised at 16 d. There was a longer lag time (5–6 d) before the burst in production of both ethylene and CO_2. However, the Ca treatment still resulted in delayed and depressed gas production (Fig. 3). As with older cotyledons, Ca reduced H_2O_2 accumulation (50% less than that in water after 9 d).

Calcium Uptake. Uptake of Ca by the detached cotyledons (from 10^{-4} M $CaCl_2$) did not reach maximal levels until 6 to 7 d (Fig. 4). In both water and $CaCl_2$, there was substantial loss of Ca from the tissue during the first 2 d. Although this loss leveled off in the Ca-treated cotyledons, the internal Ca concentration never returned to more than about 70% of the original concentration.

Effect of Other Cations. A comparison was made of the effects of Ca Mg, or K on cotyledon senescence (Table I). Chl breakdown was stimulated by K, whereas Mg had no effect and Ca delayed it. Despite the influence of K on Chl, the levels of ethylene, CO_2, and H_2O_2 were not stimulated above those of the water treatment. K depressed peroxide levels below that of the water and Mg treatments, although Ca generally had greater depressant effects than the other cations tested.

Effect of Endogenous Calcium. To investigate the influence of increased endogenous Ca, the vermiculite containing the seedlings was watered with 10^{-1} M $CaCl_2$ 24 h before excision. This treatment increased the Ca concentration of the cotyledons by about 68% (Table II). During senescence on water, Chl breakdown and CO_2 and ethylene production were reduced relative to the cotyledons with lower Ca concentrations (Table II). These differences were greater after 6 d than after 3 d. However, H_2O_2 concentrations were unaffected by the high internal Ca concentrations. A further sample after 9 d still showed no differences in H_2O_2 levels (data not given).

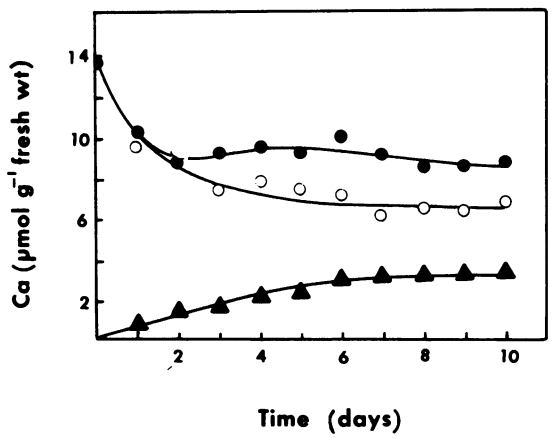

FIG. 4. Ca uptake by senescing cotyledons from 10^{-4} M $CaCl_2$ (▲), and Ca content of cotyledons senescing in the dark either in water (○) or in 10^{-4} M $CaCl_2$ (●).

Table I. *Effect of Ca, Mg, and K on Senescing 16-Day-Old Cotyledons Detached and Floated in the Dark on Water or 10^{-4} M $CaCl_2$, $MgCl_2$, or KCl*

Samples were taken at 0 and 6 d. Data are means ± SE.

Treatment	Chl	H_2O_2	Ethylene	CO_2
	mg 4 cot^{-1}	*μmol 4 cot^{-1}*	*nl 4 cot^{-1} h^{-1}*	*μl 4 cot^{-1} h^{-1}*
Initial content	0.58 ± 0.04	3.87 ± 0.38		
Water	0.30 ± 0.07	7.45 ± 0.79	1.99 ± 1.08	66.56 ± 4.34
$CaCl_2$	0.40 ± 0.05	4.45 ± 0.46	0.01 ± 0.01	38.11 ± 8.51
$MgCl_2$	0.29 ± 0.02	7.01 ± 0.56	0.53 ± 0.22	54.95 ± 8.66
KCl	0.22 ± 0.03	6.00 ± 0.54	1.01 ± 0.66	54.31 ± 9.13

Effect of H_2O_2 on Senescence. Senescence of cotyledons was followed in water, or H_2O_2 solutions of 10^{-3}, 10^{-2}, and 5×10^{-2} M concentration, with and without 10^{-4} M $CaCl_2$, for 4 d. H_2O_2 had a rapid and marked effect on Chl loss, increasing it by as much as 50% at the highest concentration (Table III). The effect of H_2O_2 on ethylene and CO_2 production, however, did not parallel the effect on Chl. Ethylene production at 10^{-3} and 10^{-2} M H_2O_2 was lower than in the water control even though peroxide-treated cotyledons had lower Chl levels. H_2O_2 at 10^{-3} M slightly reduced CO_2 production but actually halved ethylene production relative to that in water. A difference between Chl breakdown and CO_2 or ethylene production can also be seen if gas production rates at equivalent Chl levels shown in Figure 1 are compared with data in Table III. Where Chl was reduced to similar levels, ethylene and CO_2 production was lower in peroxide-treated tissue (Table III) than in tissue senescing in the absence of external peroxide (Fig. 1).

There was negligible influence of Ca on Chl loss or ethylene production induced by peroxide (Table III), although a reduction in CO_2 production was found in all treatments.

DISCUSSION

The pattern of senescence of whole, detached cucumber cotyledons on water in darkness, as followed by Chl breakdown, is typical of that found during senescence of attached and detached leaves, cotyledons, and leaf discs (21, 24, 30). Associated with this Chl loss in cucumber cotyledons is a burst of CO_2 production, an increase in ethylene production, and accumulation of H_2O_2. Both the CO_2 burst and the ethylene increase have been demonstrated by other workers using many leaf systems (1–3, 14, 16, 31), but there appears to be no previous report of an increase in H_2O_2 concentration in senescing leaf or cotyledonary tissue.

There are three major aspects to our work that we wish to highlight.

Ethylene Production. It is not yet certain whether ethylene is the primary regulator of senescence. Exogenous ethylene can enhance Chl breakdown in leaf discs (2), and inhibitors which both inhibit ethylene biosynthesis prior to 1-aminocyclopropane-1-carboxylic acid in the methionine to ethylene pathway, and block ethylene action, retard senescence in leaf material (1–3, 14). Our results suggest that ethylene may be important in regulating cotyledon senescence, since the most rapid period of production coincides with the period of most rapid Chl loss and CO_2 production, and because of the sensitive response of ethylene to factors

Table II. *Effect of Internal Ca Concentration on Senescence of 16-Day-Old Cotyledons*

Two populations of cotyledons were used. Cotyledons of low Ca status were treated with water during growth; those of high Ca status were pretreated with 10^{-1} M $CaCl_2$ 24 h before excision. Senescence was in the dark on water, and samples were taken at 0, 3, and 6 d. Data are means ± SE.

Sampling Time	Ca Status	Chl	H_2O_2	Ethylene	CO_2
	μmol g^{-1} fresh wt	*mg 4 cot^{-1}*	*μmol 4 cot^{-1}*	*nl 4 cot^{-1} h^{-1}*	*μl 4 cot^{-1} h^{-1}*
Day 0	11.77 ± 1.90	0.58 ± 0.02	3.41 ± 0.43		
	19.71 ± 1.55				
Day 3	8.72 ± 1.33	0.49 ± 0.06	4.99 ± 0.13	0.19 ± 0.08	39.52 ± 11.27
	13.93 ± 1.60	0.54 ± 0.07	4.62 ± 0.22	0.15 ± 0.02	37.80 ± 6.36
Day 6	8.30 ± 0.57	0.42 ± 0.05	5.91 ± 0.85	0.37 ± 0.04	34.09 ± 6.41
	12.23 ± 1.47	0.48 ± 0.04	5.81 ± 0.62	0.21 ± 0.07	23.35 ± 3.56

Table III. *Effect of Externally Applied H_2O_2 on Senescence of 16-Day-Old Detached Cotyledons in the Dark*

Samples were 4 d after senescence on the appropriate solutions. Chl content before treatments was 0.52 ± 0.03 mg 4 cot^{-1}.

Treatment	Chl	Ethylene	CO_2
	mg 4 cot^{-1}	*nl 4 cot^{-1} h^{-1}*	*μl 4 cot^{-1} h^{-1}*
Water	0.46 ± 0.05	0.49 ± 0.30	44.34 ± 1.11
H_2O_2			
10^{-3} M	0.41 ± 0.05	0.20 ± 0.06	38.11 ± 7.98
10^{-2} M	0.31 ± 0.05	0.22 ± 0.10	53.07 ± 6.55
5×10^{-2} M	0.23 ± 0.03	0.53 ± 0.06	57.35 ± 8.19
H_2O_2 + 10^{-4} M $CaCl_2$			
10^{-3} M	0.43 ± 0.03	0.17 ± 0.06	35.12 ± 6.93
10^{-2} M	0.31 ± 0.04	0.25 ± 0.09	46.29 ± 9.56
5×10^{-2} M	0.23 ± 0.02	0.55 ± 0.23	49.13 ± 3.69

such as Ca and exogenous H_2O_2, which retard or stimulate senescence. We are currently investigating further the relationship between Ca and ethylene synthesis.

H_2O_2 Accumulation. The major cause of H_2O_2 accumulation may be the reduction in activity of catalase, which is a feature of senescing leaf tissue (10, 18, 26–28, 30). The increase in peroxide levels that we found were often substantial within 2 d. This preceded measurable Chl degradation, and was related to the age of the cotyledons. Younger cotyledons had low levels of peroxide at the time of excision, which is consistent with the increase and subsequent decrease in catalase activity found in intact cucumber cotyledons during development and aging (27). The data of Trelease *et al.* (32) suggests that the high levels of catalase in 8-d-old cucumber cotyledons might be instrumental in maintaining comparatively low levels of peroxides, even when glycolate oxidase activity is high. The subsequent decline in catalase might result in accumulation of peroxides.

Accumulation of H_2O_2 during cotyledon senescence may be of regulatory significance. Although H_2O_2 is a relatively stable species, its breakdown mediated by catalase may provide a source of O_2 species active in processes of senescence such as lipid peroxidation (12). Thus, increased levels of peroxides in senescing tissue such as we have shown, may indicate an increased activity of free oxygen radicals. Peroxides, or associated free radical activity have been implicated in ethylene synthesis (22), auxin turnover (19), and lipid peroxidation, through reaction with superoxides (10), all in senescing tissues.

There was a close association between Chl loss and H_2O_2 in our experiments: regression analysis of Chl and endogenous H_2O_2 data collected from a number of experiments produced a regression coefficient of −0.966 (significant at the 1% level). Furthermore, the close association between exogenous peroxide and Chl loss that we found is similar to that found in *Helianthus* discs (30) and in excised rice leaves (26). It is possible that exogenous peroxide has a direct effect on cell membranes; Chia *et al.* (8) found that free radical activity induced by paraquat treatment was probably responsible for increased membrane lipid peroxidation. Matile (25) has also suggested that H_2O_2 can increase Chl breakdown, and our data, and that of Kar and Mishra (18) which showed a positive relationship between Chl content and catalase activity, tends to support this.

It should be noted, however, that there were exceptions to the close relationships between peroxide levels and the other parameters of senescence (even Chl loss). K increased Chl loss without increasing peroxide accumulation or ethylene production. Furthermore, although we could alter the rate of senescence by altering endogenous Ca levels, peroxide levels were unaffected by the subsequent increase in calcium content of the tissue. These results confirm that there is no evidence yet available suggesting a causal relationship between peroxide production and ethylene synthesis.

Influence of Calcium. Relatively low concentrations of Ca had a depressant effect on cotyledon senescence, even though a net loss of Ca occurred in the presence of exogenous $CaCl_2$. It has been commented that depressant effects of Ca on fruit tissue respiration can be demonstrated *in vitro* only with high Ca concentrations (11), although these effects are often indistinct from osmotic ones. This is not the case with cotyledon senescence. Concentrations of Ca greater than 10^{-3} M tended to increase ethylene and CO_2 production, H_2O_2 accumulation, and enhance Chl loss. High salt concentrations have been shown to lower catalase activity (17) and this might explain the increased H_2O_2 levels that we obtained.

There is some similarity between the Ca-induced delay in senescence and that resulting from hormone treatment or hormone inhibition. The nature of inhibition is similar to that of the inhibitor of ethylene synthesis, aminoethoxyvinylglycine, in its suppression of ethylene production (2), and of the suppression of respiration by cytokinins (15, 16, 31). This is distinct from the inhibition of senescence due to IAA, which results in an increase in ethylene production (1, 14). It is possible, however, that Ca can have a number of roles in senescing systems. We have also found (unpublished data) that Ca can enhance IAA-stimulated ethylene production from the same cotyledonary tissue in which Ca is retarding senescence, a stimulation similar to that found in hypocotyl tissue by Lau and Yang (20). This suggests that Ca may have a regulatory role in hormonal action in senescing tissues; recent data from Lieberman and Wang (23) would place this at the site of membrane-associated enzyme activity involved in ethylene synthesis.

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