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Relationship between Ethylene Evolution and Senescence in Morning-Glory Flower Tissue[1]

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ABSTRACT

An excised tissue system consisting of corolla rib segments was developed to study the relationship between senescence and ethylene production in morning-glory flowers *(Ipomoea tricolor)*. Such segments, isolated 1 or 2 days (day −1 or day −2) before flower opening (day 0) passed through the same developmental phases as did the corresponding tissues of the intact organ. When excised on day −1 and incubated overnight, the rib segments turned from purple to blue and changed from a slightly curled to a flat configuration. On day 0, these segments rolled up during the afternoon and turned purple again, as did the ribs of an intact corolla; the rolling up coincided with an increased rate of ethylene production. Premature rolling up and associated ethylene evolution were induced by ethylene or propylene treatment. When segments were excised on day −2 and incubated overnight, there were no changes in color or shape; during day −1, no spontaneous rolling up and little ethylene evolution occurred. Application of ethylene or propylene to these immature segments elicited rolling up but did not stimulate endogenous ethylene production.

Overnight treatment of segments cut on day −1 with 10^{-6} M benzyladenine markedly retarded spontaneous rolling up and ethylene evolution, although the response to applied ethylene was only slightly slowed. Overnight treatment of segments cut on day −1 with the ethoxy analog of rhizobitoxine (10^{-5} to 10^{-4} M) resulted in almost complete (>99%) inhibition of both spontaneous and propylene-induced ethylene evolution. Although spontaneous rolling up was delayed, it was not abolished, and ethylene-induced rolling up was almost unaffected.

These data indicate that an ethylene-generating system develops as an integral part of the aging process in flower tissue. Ethylene hastens aging of the flower, but may not play an obligatory role in flower senescence.

Intact flowers of the morning-glory *(Ipomoea tricolor)* open at 5:00 to 6:00 AM and begin to fade at about 1:00 PM of the same day. During the fading process, the corolla rolls inwards driven by curling up of the ribs, and a sharp increase in the rate of ethylene evolution occurs; the rolling up and ethylene evolution can be induced prematurely by the application of exogenous ethylene (7). Rolling up of the corolla is accompanied by increases in the activities of several hydrolases and by a decrease in vacuolar pH that causes a color change from blue to purple (9). Since the above changes all take place within a single day, the senescence of morning-glory flowers provides a useful model for investigation of the role of ethylene in plant senescence, especially the phenomenon of ethylene-induced ethylene synthesis that is common to both flower senescence (3, 7, 11) and the ripening of climacteric fruits (12).

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Kende and Baumgartner (7) have proposed a model based on compartmentation changes to account for ethylene-induced ethylene synthesis; by increasing the permeability of the tonoplast, ethylene could enhance the flow of a precursor of ethylene from the vacuole to an ethylene-generating system located in the cytoplasm. Experimental support for ethylene-induced changes in cellular compartmentation was presented by Hanson and Kende (5). Using ribs excised from buds and flowers, we showed that ethylene enhanced the efflux of $^{36}Cl^-$, $^{86}Rb^+$, organic acids and sucrose; compartmental analysis indicated that the $^{36}Cl^-$ and $^{86}Rb^+$ which were lost from the tissue were of vacuolar origin. In subsequent experiments with excised rib segments we have found a close parallel between their behavior and that of the intact flower as regards both rolling up and ethylene evolution. Here, we describe these investigations and present data bearing on the role of ethylene evolution in the regulation of senescence.

MATERIALS AND METHODS

Plant Material. Seeds of *Ipomoea tricolor* Cav. (cv. Heavenly Blue) were purchased from Agway Inc. (Syracuse, N. Y.). Plants were grown as described previously (5) in an environmental chamber with a 16-hr photoperiod (from 5:00 AM to 9:00 PM). Buds were harvested between 4:00 PM and 7:00 PM 1 or 2 days before flower opening. On the day before flower opening, buds were approximately 5 cm long, were loosely coiled, and had lost all Chl from the corolla, whereas 2 days before flower opening, buds were approximately 3 cm long, tightly coiled, and quite green. In the text, we have used the following abbreviations to describe the age of flower tissues: day 0, day of flower opening and fading; day −1, 1 day before flower opening; day −2, 2 days before flower opening.

In the preparation of rib segments, the top 1 to 2 mm of each bud was discarded, and the remaining upper 15 to 25 mm of the corolla was cut from the flower base. This coiled corolla was unrolled gently, and 12- to 15-mm long rib segments were excised with a razor blade from the upper portion, leaving 1 to 2 mm of colored corolla tissue along each long edge. The segments were then trimmed to 10 mm and floated at once on the incubation medium (Fig. 1). The whole procedure was performed as rapidly and gently as possible to minimize injury to the rib tissue.

In the 3- to 4-hr period after excision, copious "wound ethylene" production occurred in segments on day −1 and day −2. This burst of ethylene was allowed to escape during overnight incubation, during which time the evolution of ethylene subsided (Table I).

Incubation of Rib Segments. Excised segments were usually floated in lots of 15 to 50 on 2 to 5 ml of incubation medium in 6-cm plastic Petri plates (Fig. 1). Control incubation medium contained 5 mM KCl with a pH of 5.5 to 6.2; when additions were made to the control medium, the pH was readjusted to this range with dilute HCl or KOH. The Petri plates were enclosed in transparent plastic boxes containing moistened filter paper and

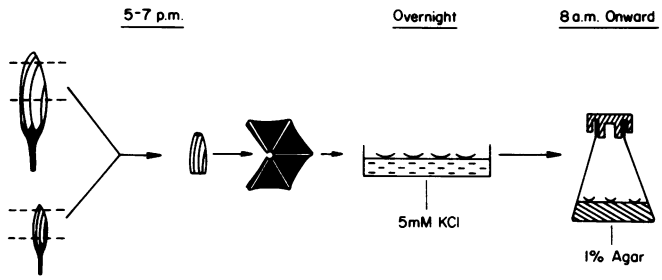

FIG. 1. Diagram of preparation methods and incubation procedures for rib segments.

Table I. *Rate of Ethylene Evolution by Rib Segments at Various Times After Excision*

Segments were excised on day -1 and day -2 between 5:00 PM and 6:00 PM, and were incubated continuously on agar medium for up to 26 hr. Rates of ethylene evolution were measured during 2- to 3-hr periods by temporarily sealing the incubation flasks with serum caps.

Time of Segment Excision	Time of C_2H_4 Evolution Assay	C_2H_4 Evolution	Rolling Up
		nl/hr·30 segments	*degrees*
Day -2	7:00-9:10 PM (day -2)	6.21	223
	6:55-8:55 AM (day -1)	0.96	240
	12:00AM-2:00 PM (day -1)	0.22	227
	4:00-6:00 PM (day -1)	0.31	220
Day -1	7:45-9:45 PM (day -1)	1.69	222
	6:15-8:15 AM (day 0)	0.09	186
	12:00 AM-2:35 PM (day 0)	0.89	260
	6:05-8:05 PM (day 0)	4.58	484

were then returned for overnight incubation to the growth chamber. The rib segments were usually removed from the growth chamber at 7:30 to 8:30 AM, blotted dry, and transferred to 25-ml Erlenmeyer flasks containing 5 ml of sterile agar medium (5 mM KCl with 1% w/v agar). Each flask contained 15 to 25 segments. If floated segments were kept on liquid and not transferred to agar on the morning of the experiment, their ethylene production was markedly reduced. In experiments in which 4% CO_2 was used to inhibit ethylene action and where acidification of the medium was a problem, a single layer of cheesecloth was interposed between the rib segments and the agar. The Erlenmeyer flasks were flushed for 2 min with ethylene-free air, closed with serum caps and incubated in darkness at 27 C (Fig. 1). When required, ethylene or propylene was injected into the flasks to give final concentrations of 10 and 100 μl/l, respectively. When production of endogenous ethylene was measured after an application of ethylene, the segments were transferred following ethylene treatment to a fresh flask containing 5 ml of agar medium.

Benzyladenine Treatments. In experiments with rib segments cut on day −1, benzyladenine (6-benzylaminopurine, Nutritional Biochemicals Corp., Cleveland, Ohio) was included in the overnight incubation medium at concentrations of 10^{-6} or 3×10^{-6} M. In some experiments, 10^{-6} M BA was also added to the agar medium to which the segments were transferred.

Pretreatments with Rhizobitoxine Analog. Segments cut on day −1 were floated overnight on solutions of the rhizobitoxine analog L-2-amino-4-(2′-aminoethoxy)-*trans*-3-butenoic acid, at concentrations of 10^{-5} to 10^{-4} M. This analog was a gift of Dr. M. Lieberman, Agricultural Research Center, Beltsville, Md. In order to determine as precisely as possible the effectiveness of this compound in inhibiting ethylene production, the incubation conditions were modified to increase the amount of rib segment tissue per unit volume. On day −1, two lots of 100 rib segments were prepared and floated overnight on a solution containing 5 mM KCl and 3×10^{-5} M rhizobitoxine analog. Between 5:30 and 6:30 AM, each lot of 100 segments was packed gently into a 10-ml disposable syringe equipped with a side arm containing 20 μl of 10% (w/v) KOH to absorb CO_2. Filter paper moistened with 5 mM KCl was placed in the syringes to ensure high relative humidity. The syringes were closed with serum caps and incubated in darkness at 27 C.

Measurement of Rolling Up of Rib Segments. Angular measurements (α) were taken during the rolling up process by aligning the sides of the segments in the flask or syringe alongside a protractor (5). When segments were flat and unrolled, α was scored as 180°; as rolling up occurred, α increased to >360°, with the segment ends overlapping.

Ethylene Determination. One-ml samples were taken at intervals through the serum caps of flasks, and the samples withdrawn were replaced with 1 ml of ethylene-free air. Ethylene in the samples was determined with a gas chromatograph (Varian Aerograph Series 1700 or 1400), equipped with a flame ionization detector. A column (45 × 0.32 cm) of 60 to 80 mesh Al_2O_3 was used, operating at 60 or 80 C; under these conditions the retention times of ethylene and propylene were approximately 30 sec and 2 min, respectively. An ethylene concentration of 10 nl/l was readily measurable in a 1-ml sample. In calculating the amount of ethylene evolved in an experiment, a correction was applied for the repeated removal of samples and their replacement with ethylene-free air.

RESULTS

Behavior of Segments Excised on Day −1 and Day −2. When rib segments were excised in the afternoon of day −1, they were purple in color and had a slight curvature (α = 220-230°). One margin of the remaining corolla was folded over the inner face of the rib. During overnight incubation on KCl solution, such segments underwent changes that resembled the changes which occur in intact buds during flower opening: their color changed from purple to blue, their curvature was lost (α = 180°), and the corolla margins unfolded and adopted a position in which they projected straight out from the rib. In the course of subsequent incubation on agar, these segments again behaved similarly to intact flowers. Segments began to roll up spontaneously at about mid-day; the rate of endogenous ethylene production rose as rolling up began, and did not reach a maximum until rolling was almost complete (Fig. 2A). Expressed on a fresh weight basis, the quantity of ethylene evolved by the rib segments was at least 5-fold greater than that evolved by intact senescing flowers (7). Exposure of the segments to ethylene (10 μl/l, 3 hr) caused premature rolling up and ethylene evolution (Fig. 2A).

When rib segments were excised in the afternoon of day −2, they were purple and showed curvatures (α = 220-230°) and folded corolla margins similar to those of segments cut on day −1. After overnight incubation these immature segments had elongated but showed no change in color or shape. During subsequent incubation on agar on day −1 they displayed no tendency to roll up spontaneously and produced only small amounts of ethylene, mainly during the morning hours (Fig. 2B). Application of exogenous ethylene to these juvenile segments caused rolling up, although the time required for this response to develop was longer than in segments on day 0 (3 hr as against 1.5 hr). Ethylene treatment did not stimulate endogenous ethylene production in the immature segments.

Effect of Benzyladenine on Rolling Up and Ethylene Evolution. Because cytokinins retard senescence of detached leaves and leaf disks of many species, the effect of BA on the rolling up and ethylene production of rib segments was investigated. When segments excised on day −1 were floated overnight on 10^{-6} M or 3×10^{-6} M BA, the onset of both rolling up and ethylene evolution was markedly delayed (Fig. 3). Extending the length of BA treatment by inclusion of BA in the agar medium did not additionally delay senescence. Although spontaneous rolling up

was delayed by BA treatment, rolling up in response to ethylene treatment was almost unaffected (Fig. 4).

Effect of Rhizobitoxine Analog on Rolling Up and Ethylene Evolution. Rhizobitoxine and its ethoxy analog at concentrations of 10^{-5} to 10^{-4} M are potent inhibitors of ethylene production in apple tissue (8). Rib segments excised on day −1 and treated overnight with 10^{-5} or 10^{-4} M rhizobitoxine analog underwent the normal changes in color and shape, but their capacity to produce ethylene either spontaneously or in response to propylene treatment was severely reduced (Fig. 5). Although ethylene evolution was inhibited almost completely at both inhibitor concentrations, spontaneous rolling up was delayed only by about 2 hr at 10^{-5} M inhibitor and by about 5 hr at 10^{-4} M. It is possible that this longer delay of spontaneous rolling up at the higher inhibitor concentration was at least in part due to secondary effects of the analog not related to the inhibition of ethylene evolution. Rhizobitoxine has been reported to inhibit pyridoxal phosphate-dependent reactions in spinach (4) and might, especially at high concentrations, affect several metabolic pathways in addition to ethylene biosynthesis. The data of Figure 5B lend some support to this suggestion; while segments treated with 10^{-5} M inhibitor rolled up as rapidly as control segments in response to applied propylene, those exposed to 10^{-4} M inhibitor rolled up more slowly. This instance of retarded rolling cannot be ascribed to an effect on endogenous ethylene production.

To measure the inhibition of ethylene production more accurately, a larger number of segments was enclosed in a smaller gas space after overnight treatment with 3×10^{-5} M rhizobitoxine analog. In the experiment shown in Figure 6, inhibition of ethylene production was found to be greater than 99%; slow rolling up was again observed in the inhibitor-treated segments.

Although the rib segments are only 5 to 20 cells thick, and contain large intercellular air spaces (14), the ethylene concentration within the tissue might still be far higher than in the surrounding gas space. In this case, the low rate of ethylene production following inhibitor treatment (<1% of control value) could suffice to elicit the slow spontaneous rolling up response observed. To test this possibility, the effects on rolling up of a high CO_2 level (4% v/v) and of a mercuric perchlorate trap for ethylene (15) were examined, using both inhibitor-treated and untreated rib segments. Burg and Burg (2) have shown that CO_2 can act as a competitive inhibitor of ethylene action in pea stems

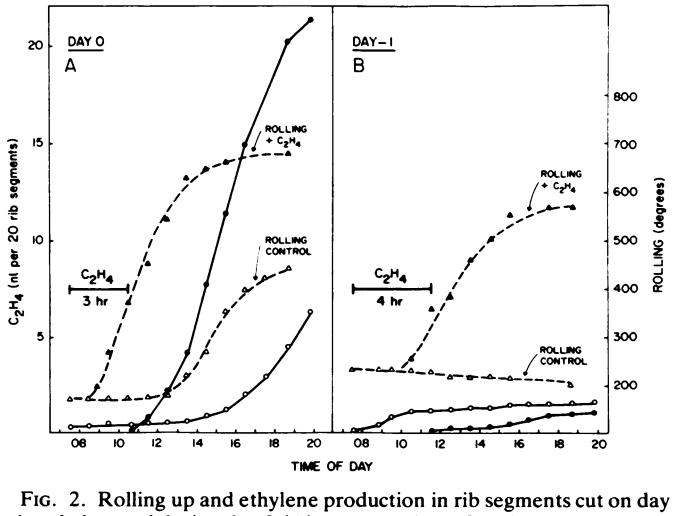

FIG. 2. Rolling up and ethylene production in rib segments cut on day −1 and observed during day 0 (A) or cut on day −2 and observed during day −1 (B). Segments were incubated overnight (7:00 PM to 7:30 AM) on 5 mM KCl; ethylene was added to certain flasks to give a final concentration of 10 μl/l. △-----△: rolling up of control segments; ▲-----▲: rolling up of ethylene-treated segments; ○——○: ethylene production by control segments; ●——●: ethylene production by ethylene-treated segments.

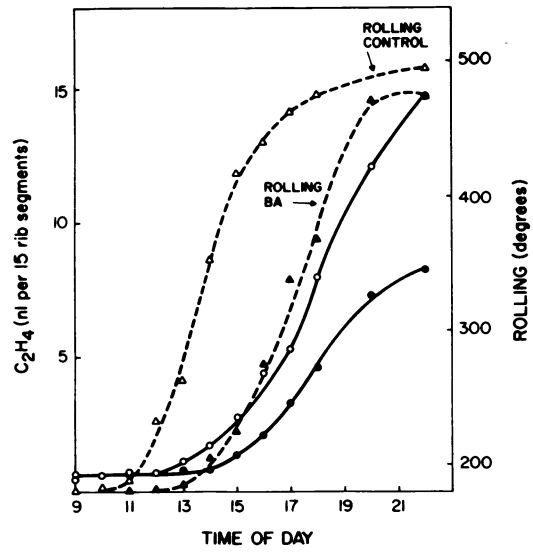

FIG. 3. Effect of BA on rolling up and ethylene production. Segments were excised on day −1 and incubated overnight (5:00 PM to 9:00 AM) on 5 mM KCl (control) or 5 mM KCl containing 3×10^{-6} M BA. △-----△: rolling up of control segments; ▲-----▲: rolling up of BA-treated segments; ○——○: ethylene production by control segments; ●——●: ethylene production by BA-treated segments.

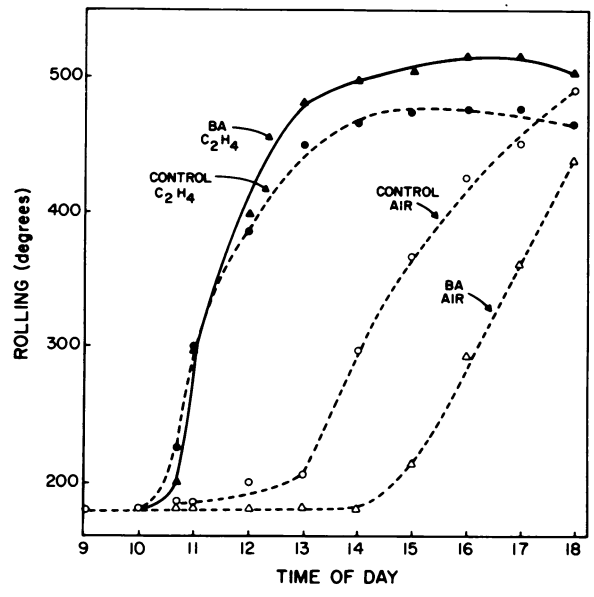

FIG. 4. Effect of BA on spontaneous and ethylene-induced rolling up. Segments were excised on day −1 and incubated overnight (5:00 PM to 9:00 AM) on 5 mM KCl (control) or 5 mM KCl containing 10^{-6} M BA. Ethylene was added to certain flasks to give a final concentration of 10 μl/l. ○-----○: rolling up of control segments in air; ●-----●: rolling up of control segments in ethylene; △-----△: rolling up of BA-treated segments in air; ▲——▲: rolling up of BA-treated segments in ethylene.

and, in preliminary experiments, we found that 4% (v/v) CO_2 maximally inhibits rolling up of rib segments. Addition of a mercuric perchlorate trap would remove traces of ethylene from the atmosphere surrounding the segments and would increase the diffusion gradient of ethylene escaping from the tissue. Both CO_2 addition and ethylene removal would be expected to inhibit rolling up in segments treated with rhizobitoxine analog if this response is controlled by the level of endogenous ethylene. Figure 7 shows that both addition of CO_2 and trapping of ethylene retarded spontaneous rolling up in untreated rib segments. Both addition of CO_2 and removal of ethylene also

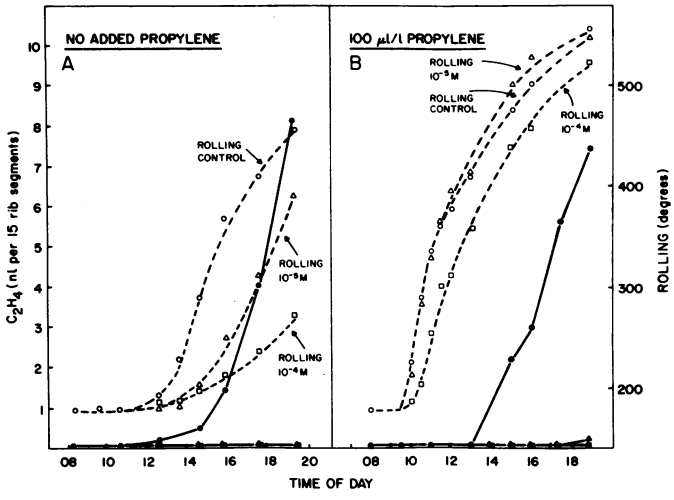

FIG. 5. Effect of rhizobitoxine analog on spontaneous rolling up and ethylene production (A) and on propylene-induced rolling up and ethylene production (B). Segments were excised on day −1 and incubated overnight (6:30 PM to 8:00 AM) on 5 mM KCl (control) or on 5 mM KCl containing 10^{-5} and 10^{-4} M rhizobitoxine analog. Propylene was added to certain flasks to give an initial concentration of 100 µl/l. The use of air alone to replace withdrawn samples caused a decline in propylene concentration during the experiment to a final value of about 75 µl/l. O-----O: rolling up of control segments; Δ-----Δ: rolling up of segments incubated on 10^{-5} M analog; □-----□: rolling up of segments incubated on 10^{-4} M analog; ●——●: ethylene production by control segments; ▲——▲: ethylene production by segments incubated on 10^{-5} M analog; ■——■: ethylene production by segments incubated on 10^{-4} M analog. Total amounts of ethylene produced per 15 inhibitor-treated segments were as follows: air, 10^{-5} M <0.05 nl; air, 10^{-4} M <0.05 nl; propylene, 10^{-5} M = 0.18 nl; propylene, 10^{-4} M = 0.09 nl.

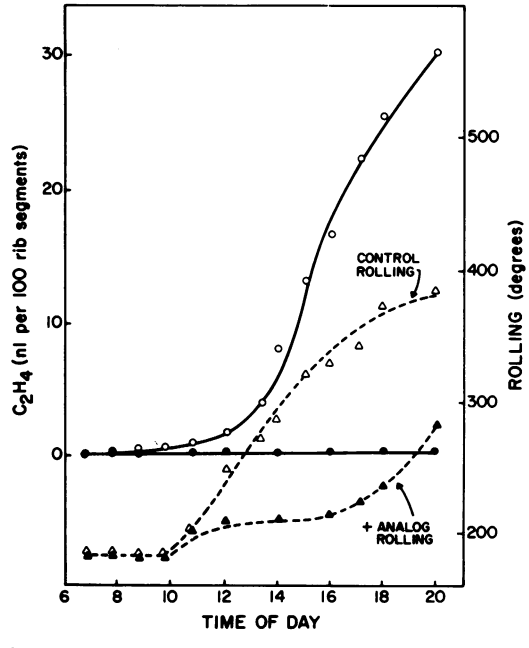

FIG. 6. Inhibition of spontaneous ethylene production and rolling up by rhizobitoxine analog. Batches of 100 segments were cut on day −1 and incubated overnight (5:00 PM to 5:30 AM) on 5 mM KCl (control) or 5 mM KCl containing 3 × 10^{-5} M rhizobitoxine analog. Each batch was enclosed in a 10-ml syringe for measurement of ethylene production. Δ-----Δ: rolling up of control segments; ▲-----▲: rolling up of inhibitor-treated segments; O——O: ethylene production by control segments; ●——●: ethylene production by inhibitor-treated segments. The total amount of ethylene evolved by the inhibitor-treated segments was 0.22 nl.

delayed the onset of rolling up in inhibitor-treated segments, but did not greatly reduce its final extent.

DISCUSSION

Because the morning-glory flower fades rapidly it is a useful system to study senescence in plants (7, 9, 14). Since investigations with intact flowers and buds are restricted by slow uptake and uneven distribution of metabolites and inhibitors, we turned to excised flower and bud rib segments for further experiments on permeability changes (5), methionine metabolism (6), and the role of ethylene evolution during aging. Using such segments, all compounds tested were readily absorbed and production of "wound ethylene" posed no problems as it had subsided by the following morning. Excised rib segments go through the same morphological changes and patterns of ethylene evolution as the respective tissue on the intact flower or bud.

Because the amount of ethylene produced by the senescing segments is greater, on a weight basis, than that produced by the whole flower, the rib tissue may represent the major source of ethylene in the intact corolla. In contrast to whole flowers (7), isolated rib segments do not show two distinct phases of ethylene evolution.

In rib segments of the morning-glory corolla, the following developmental stages leading to senescence can be distinguished. Two days before flower opening (day −2), the segments are juvenile and do not roll up in response to exogenous ethylene (results not shown). On the day before flower opening (day −1), rib segments roll up but do not evolve ethylene as a result of ethylene treatment. On the day of flower opening (day 0), rolling up and ethylene production can both be induced prematurely by exogenous ethylene. Hence, the sensitivity to ethylene and the capacity to produce ethylene "autocatalytically" develop sequentially over a 2-day period.

There is general agreement that ethylene hastens the ripening

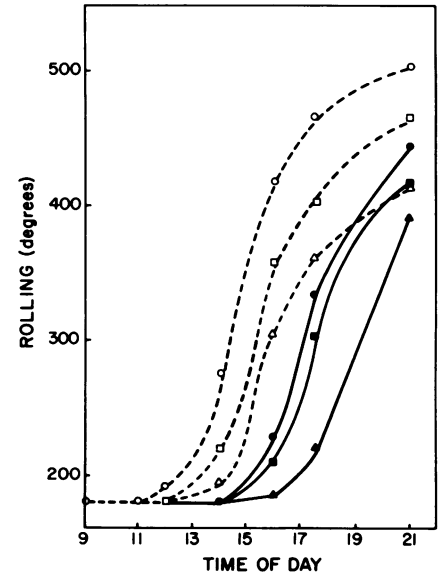

FIG. 7. Effect of CO_2 addition and ethylene removal on spontaneous rolling up. Segments were cut on day −1 and incubated overnight (5:00 PM to 9:00 AM) on 5 mM KCl or 5 mM KCl with 10^{-4} M rhizobitoxin analog. Segments were then transferred to flasks with agar medium with air only, with air to which 4% (v/v) CO_2 was added, or air from which ethylene was removed with a filter paper wick soaked in mercuric perchlorate solution. O-----O: rolling up of untreated segments in air; Δ-----Δ: rolling up of untreated segments in 4% CO_2; □-----□: rolling up of untreated segments in the presence of an ethylene trap; ●——●: rolling up of inhibitor-treated segments in air; ▲——▲: rolling up of inhibitor-treated segments in 4% CO_2; ■——■: rolling up of inhibitor-treated segments in the presence of an ethylene trap.

of fruit and the senescence of certain flowers (1, 13). It is a matter of continued controversy whether ethylene acts as the actual trigger of aging, or whether its production is a consequence of earlier processes of senescence (10, 12). Our experiments illustrate the difficulties of differentiating between these two possibilities. The results with the rhizobitoxine analog indicate that the bulk, perhaps as much as 99%, of the ethylene produced by rib segments is not involved in the initiation of senescence. Gas chromatographic analysis shows that the ethylene concentration in the atmosphere surrounding the rib segments treated with the rhizobitoxine analog is far below the threshold level required to induce premature aging with exogenous ethylene. Three lines of evidence indicate that this residual trace of ethylene may be physiologically significant. *(a)* The rhizobitoxine analog at both 10^{-5} and 10^{-4} M inhibited ethylene production by over 95%, but the higher inhibitor concentration caused a longer delay in the rolling up of the rib segments. *(b)* Rib segments treated with the rhizobitoxine analog and kept in an atmosphere of 4% CO_2 rolled up later than those kept in air. *(c)* The rolling up of rib segments was delayed when a mercuric perchlorate trap was included in the incubation flasks. The first two lines of evidence mentioned above are open to the criticism that both agents, the rhizobitoxine analog at high concentration and CO_2, have secondary effects which are not related to the synthesis and action of ethylene. The third line of evidence, namely the effectiveness of an ethylene trap in retarding rolling up of rib segments treated with the rhizobitoxine analog, is a strong indication that trace amounts of ethylene are still physiologically active.

We nevertheless suggest, as a working hypothesis, that the aging process in morning-glory flowers commences before ethylene production begins. According to this view, ethylene does not trigger aging but accelerates it. The following observations support this hypothesis. (*a*) An increase in the rate of ethylene evolution was never detected before the onset of rolling up. The two processes usually began together and, in experiments where aging was induced by ethylene or propylene application, ethylene evolution lagged behind rolling up. Rolling up is a turgor-driven movement resulting from a change in the ability of the cells on the inner rib side to retain solutes (5). Rolling up is therefore a manifestation of irreversible cell degeneration which must have been preceded by more subtle changes; our data indicate that increased ethylene production was not one of these changes. (*b*) In the experiments where treatment with the rhizobitoxine analog was combined with ethylene trapping or exposure of segments to CO_2, rolling up still occurred at rates comparable to those in the control, albeit with some delay. It is possible that this rolling up was independent of ethylene. (*c*) Indirect evidence for the existence of early metabolic changes which lead to senescence and which take place before rolling up and ethylene evolution occur is provided by the retarding effect of BA applied during the night between day −1 and day 0. Benzyladenine presumably delays some early events in senescence, thus postponing the appearance of the subsequent sequence of symptoms. The effect of BA is probably not attributable to lowered sensitivity of the segments to ethylene, because the increase in ethylene production was retarded to the same extent as rolling up, and because the rolling-up response to applied ethylene was almost unaffected by BA.

The significance of ethylene in initiating senescence is more complex when viewed at the level of the whole organ rather than when viewed at the level of the cell. We propose that ethylene production is an integral but fairly late part of the aging process at the cellular level, and that ethylene does not initiate the degenerative events in the cell where it is produced. Rib segments consist of a relatively few layers of cells which are in fairly unrestricted contact with the surrounding atmosphere. Hence, the timing of ethylene evolution in rib segments may provide more information on senescence of individual cells than does the timing of ethylene production in intact fruits or flowers. Such organs comprise many types of cells that probably do not senesce synchronously. Because of its unique capacity to induce both senescence and its own synthesis, ethylene originating from a few cells which age precociously could trigger the remaining tissues in a fruit or flower to enter the senescent phase. In orchids, such a chain reaction due to diffusion of ethylene from the column and lip to the rest of the blossom appears to take place following pollination (3). Thus the view that ethylene production is a consequence of cellular aging can readily be reconciled with the concept that ethylene acts as the ripening or senescence factor in climacteric fruits and in many flowers.

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