Ethylene as a Regulator of Senescence in Tobacco Leaf Discs

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ABSTRACT

The regulatory role of ethylene in leaf senescence was studied with excised tobacco leaf discs which were allowed to senesce in darkness. Exogenous ethylene, applied during the first 24 hours of senescence, enhanced chlorophyll loss without accelerating the climacteric-like pattern of rise in both ethylene and CO2, which occurred in the advanced stage of leaf senescence. Rates of both ethylene and CO2 evolution increased in the ethylene-treated leaf discs, especially during the first 3 days of senescence. The rhizobitoxine analog, aminoethoxy vinyl glycine, markedly inhibited ethylene production and reduced respiration and chlorophyll loss. Pretreatment of leaf discs with Ag+ or enrichment of the atmosphere with 5 to 10% CO2 reduced chlorophyll loss, reduced rate of respiration, and delayed the climacteric-like rise in both ethylene and respiration. Ag+ was much more effective than CO2 in retarding leaf senescence. Despite their senescence-retarding effect, Ag+ and CO2, which are known to block ethylene action, stimulated ethylene production by the leaf discs during the first 3 days of the senescing period; Ag+ was more effective than CO2. The results suggest that although ethylene production decreases prior to the climacteric-like rise during the later stages of senescence, endogenous ethylene plays a considerable role throughout the senescence process, presumably by interacting with other hormones participating in leaf senescence.

The involvement of ethylene in induction of ripening and senescence of climacteric fruits (13, 28) and many flowers (19, 22, 24) has been established. In these systems a rise in ethylene production correlates with the onset of ripening or other symptoms of senescence. In the nonclimacteric orange fruit, exogenous ethylene enhanced Chl loss; since reducing ethylene levels by subatmospheric pressure or blocking ethylene action by CO2 did not inhibit Chl destruction, it was concluded that endogenous ethylene may not be the primary inducer of the natural color change in detached orange fruit (8).

Much evidence showing the involvement of ethylene in leaf abscission has been reported (2), but this phenomenon occurs late in leaf senescence and may follow initiation of senescence of the leaf blade. Although exogenous ethylene could induce Chl loss in leaves (12, 28) a regulatory role of endogenous ethylene in senescence of leaf blades is still obscure, since it is not clear whether its influence precedes or follows onset of senescence. In leaves both respiration and ethylene production increased in a climacteric-like pattern (6, 23), but this occurred in an advanced stage of the blade senescence (6).

We undertook to examine leaf senescence with respect to: (a) endogenous ethylene production and Chl loss; (b) response to exogenous ethylene; (c) presence of AVG (21, 26) which inhibits ethylene synthesis; and (d) reduced ethylene action by Ag+ (9-11) and by CO2 (1, 14). Experiments were conducted with discs of tobacco leaf, previously found to be an appropriate model system for studies on the role of ethylene in leaf senescence (6).

MATERIALS AND METHODS

All experiments were performed with fully expanded mature leaves of 8- to 10-week-old tobacco plants (Nicotiana tabacum L. cv. Xanthi) grown in a greenhouse under natural lighting at temperatures between 20 and 30 C. Discs, 1 cm in diameter, were cut from leaves which were surface-sterilized with sodium hypochlorite as previously described (6). Subsequent handling of the tissue involved sterile techniques.

Leaf discs were pretreated by floating under cool-white fluorescent light for about 3 h in open Petri dishes containing either 20 ml of H2O or 0.1 mM of the rhizobitoxine analog AVG. This allowed wound ethylene to subside. In all tests with Ag+, leaf discs were pretreated by floating on AgNO3 solutions. The concentrations of solutions and durations of the pretreatments are described in legends of the table and figures. After the pretreatment, leaf discs were transferred to 25-ml (8 discs) or 50-ml (12 discs) Erlenmeyer flasks containing filter paper moistened with 1 ml or 2 ml, respectively, of H2O or AVG solution. Weights of 8 or 12 leaf discs were about 120 and 180 mg, respectively. To prevent bacterial contamination, penicillin and streptomycin were added (6). The flasks were sealed with rubber serum caps and incubated in darkness at 28 C. When required, ethylene or CO2 was injected to give desired concentrations. In experiments with exogenous CO2, control flasks contained small vials with 1 ml of 10% KOH to absorb CO2 evolved.

At appropriate time intervals gas samples were withdrawn with a hypodermic syringe for determination of ethylene and CO2 as previously described (6). After sampling, flasks were flushed with sterile fresh air, and when required, ethylene or CO2 was reintroduced.

Chl was extracted from leaf discs with dimethylformamide (6), determined spectrophotometrically at 665 nm, and expressed in O.D. units.

Number of replicates, leaf discs per replicate, and repeat experiments were as detailed previously (6).

RESULTS AND DISCUSSION

The rapid Chl breakdown phase in tobacco leaf discs senescing in darkness was accompanied by a rise and then a decline in both respiration and ethylene production (6). In fruit these phenomena characterize the climacteric stage and represent the onset of ripen-

1 Abbreviation: AVG: aminoethoxyvinyl glycine or L-2-amino-4-(2-aminoethoxy)-trans-3-butenolic acid.
ing (13, 28). Tests with leaf discs led us to conclude that these phenomena do not indicate the start of senescence in leaves but are associated with a stage rather late in the process. The aging process in morning glory flowers also commenced before ethylene production began (19). Applying exogenous ethylene to precocious fruit (27) or flowers (25) hastened the rise in ethylene production. However, treatment of tobacco leaf discs with 10 μl/l ethylene for the first 24 h did not significantly hasten the climacteric-like rise in ethylene production (Fig. 1). Ethylene evolution was somewhat greater in ethylene-treated than in control leaf discs, and could represent either increased synthesis and/or release of the exogenous absorbed ethylene. Continuous treatment with AVG markedly inhibited ethylene synthesis during senescence (Fig. 1). The inhibition indicates that methionine is the principal precursor of ethylene biosynthesis in leaves (3, 4, 21) as in fruits (21) and flowers (18). Effects of exogenous ethylene and AVG on rates of respiration and Chl loss are shown in Figures 2 and 3, respectively. AVG markedly decreased these rates after 3 days whereas exogenous ethylene increased them. These results suggest that ethylene is involved in the regulation of leaf senescence even at an early stage of senescence.

Further evidence of a regulatory role for ethylene in leaf senescence was obtained with Ag+, which has been reported to oppose effects of ethylene on growth, senescence, and abscission (9-11). Increasing concentrations of Ag+ from 0 to 20 mg/l completely nullified the ability of ethylene to enhance both Chl loss (Fig. 4) and rate of respiration (Fig. 5). The level of Ag+ that prevented Chl loss in ethylene-treated or ethylene and AVG-treated leaf discs was 20 mg/l or 10 mg/l, respectively. The senescence-retarding effect of AVG was not completely nullified by exogenous ethylene (Fig. 4), probably because treatment with AVG was continuous whereas ethylene was applied for the first 4

**Fig. 1.** Time course of average daily ethylene production rates by senescing tobacco leaf discs treated with ethylene and AVG. Discs were cut from fully expanded mature leaves. Ethylene at 10 μl/l was included in the 25-ml flasks for the first 24 h. AVG at 0.1 mM was applied continuously from day 0.

**Fig. 2.** Time course of respiration by senescing tobacco leaf discs treated with ethylene and AVG. Treatments were applied as described in Figure 1.

**Fig. 3.** Time course of Chl breakdown in senescing tobacco leaf discs treated with ethylene and AVG. Leaf discs senesced in Petri dishes containing H2O or 0.1 mM AVG. Ethylene at 10 μl/l was introduced to a 10-liter desiccator in which the Petri dishes were kept for the first 24 h.

**Fig. 4.** Effect of increasing concentration of Ag+ on Chl retention by tobacco leaf discs treated with ethylene and AVG. Chl content at zero time was at 0.520 O.D. units. Leaf discs were pretreated by floating for 30 min on AgNO3 solutions and thereafter were allowed to senesce for 5 days in 25-ml flasks. Ethylene at 5 μl/l was applied during the first 4 days. AVG at 0.1 mM was applied continuously from day 0. Measurements of Chl were made on the 5th day.
days only. The fact that increasing concentrations of Ag⁺ in the presence of AVG further delayed Chl loss suggests that a low level of endogenous ethylene, which may be bound to receptor sites, can also accelerate Chl loss. The ability of ethylene to antagonize senescence-retarding effects of either AVG (Fig. 4) or Ag⁺ (Figs. 4–6) indicates that AVG and Ag⁺ inhibit either ethylene synthesis (21) or action (9), as previously proposed. The capability of Ag⁺, in the concentrations used, to overcome some of the ethylene effects and *vice versa* (Fig. 6) may indicate a competitive interaction.

Increasing the concentration of Ag⁺ from 0 to 20 mg/l, which reduced Chl breakdown (Fig. 4), simultaneously increased the rate of ethylene production in a biphasic linear fashion (Fig. 7). AVG inhibited this Ag⁺-induced ethylene production by 90 to 95%. These data suggest that Ag⁺ may induce ethylene synthesis. Floating tobacco leaf discs for 30 min on AgNO₃ solution at concentrations of 10 mg/l or lower decreased their respiration rate (Fig. 5) without any visible toxic effect. Concentrations above 50 mg/l AgNO₃ markedly increased ethylene production and rates of respiration and yellowing; it also severely damaged the leaf discs, blackening the cut surface (data not shown). Apparently, induction of ethylene synthesis caused by relatively low concentrations of AgNO₃ and the decrease in respiration are evidence not of Ag⁺ toxicity but of the antiethylene property of Ag⁺ (5).

CO₂ is known to antagonize ethylene action in many physiological processes (1, 14). We examined effects of CO₂ on Chl content, ethylene production, and respiration in leaves during senescence. When applied at concentrations of 5 or 10%, CO₂ enhanced ethylene production during the first phase of senescence, delayed the appearance of the peak in the climacteric-like rise of ethylene (Fig. 8), decreased Chl loss (Table I), and decreased respiration, as measured by O₂ consumption (data not given). Ethylene pro-

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**FIG. 5.** Effect of increasing concentrations of Ag⁺ on rate of respiration of tobacco leaf discs in the presence or absence of ethylene. Details as in Figure 4.

**FIG. 6.** Effect of increasing concentrations of exogenous ethylene applied with or without Ag⁺ on Chl breakdown in tobacco leaf discs. Chl content at day 0 was 0.382 O.D. units. Leaf discs were pretreated by floating for 30 min on 20 mg/l AgNO₃ solution and then allowed to senesce for 4 days in 50-ml flasks. Ethylene was applied during the first 3 days. Final concentrations of ethylene in flasks after 3 days were (from left to right): 0.2, 9.3, 18.2, 0.7, 8.8, and 19.2 nl/l. After flushing with air the leaf discs were allowed to senesce for 1 more day in closed flasks. Measurements of the Chl were made on the 4th day.

**FIG. 7.** Effect of increasing concentrations of Ag⁺ on average ethylene production rates by tobacco leaf discs. Treatments were applied as described in Figure 4. Rate of ethylene production was determined after the first 24 h of incubation.

**FIG. 8.** Time course of ethylene production by senescing tobacco leaf discs treated with Ag⁺ or CO₂. Leaf discs were pretreated by floating for 45 min on 10 mg/l AgNO₃ solution or water and then allowed to senesce in 50 ml-flasks with or without 5% CO₂.
Table 1. Effects of AVG, Ag+, and CO2 on Ethylene Production and Chl Retention by Senescing Tobacco Leaf Discs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C2H4 Production between 0 and 18 h</th>
<th>Chl Content after 6 Days’ Senescence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/fresh weight ± 1 s.e.</td>
<td>O.D. 665 nm ± 1 s.e.</td>
</tr>
<tr>
<td>H2O</td>
<td>0.53 ± 0.08</td>
<td>0.071 ± 0.004</td>
</tr>
<tr>
<td>CO2</td>
<td>0.81 ± 0.08</td>
<td>0.106 ± 0.005</td>
</tr>
<tr>
<td>Ag+</td>
<td>1.83 ± 0.21</td>
<td>0.138 ± 0.014</td>
</tr>
<tr>
<td>CO2 + Ag+</td>
<td>4.51 ± 0.12</td>
<td>0.183 ± 0.014</td>
</tr>
<tr>
<td>AVG</td>
<td>0.08 ± 0.04</td>
<td>0.150 ± 0.012</td>
</tr>
<tr>
<td>AVG + CO2</td>
<td>0.04 ± 0.04</td>
<td>0.218 ± 0.020</td>
</tr>
<tr>
<td>AVG + Ag+</td>
<td>0.09 ± 0.04</td>
<td>0.274 ± 0.014</td>
</tr>
<tr>
<td>AVG + CO2 + Ag+</td>
<td>0.69 ± 0.17</td>
<td>0.382 ± 0.027</td>
</tr>
</tbody>
</table>

The conclusion that the climacteric-like rise of ethylene production in senescent leaf discs could not have triggered leaf senescence was derived from previous studies (4, 6, 7), which showed that this rise occurs considerably after the commencement of Chl loss. Actually, the first phase of Chl loss was accompanied by a decline in ethylene production. Nevertheless, results obtained with inhibitors of either ethylene synthesis (AVG) or action (Ag+ and CO2) clearly imply that the internal endogenous ethylene, which may never be released from the tissue, plays a considerable role in the regulation of leaf senescence. Levels of internal, endogenous ethylene in mature leaves of tobacco and other plants range generally between 0.1 and 0.2 µl/l (4, 6), and threshold values for an ethylene effect on leaf senescence were reported to be 0.01 to 0.1 µl/l (2). Therefore, the level of endogenous ethylene is sufficient to induce senescence, if other factors involved allow it. Endogenous levels of IAA (16), GA (17, 15), and cytokinins (25), which are antagonistic to ethylene action, decline before and with the onset of senescence symptoms. Generally, the rate of ethylene production is greatly affected by the level of auxin, which sometimes acts synergistically with cytokinins (17, 20). Therefore, the decrease of ethylene production during the slow Chl degradation phase could have been partly due to reductions in endogenous levels of auxin (17) and cytokinins (22). It is likely that the mechanisms inducing leaf senescence are associated with early depletion of growth hormones, and that this depletion in turn greatly influences actual levels of ABA and ethylene, the senescence-enhancing hormones. The onset of leaf senescence does not appear to be rapid and sudden or triggered by dynamic changes in the level of any one hormone; rather, it appears to be brought about gradually by the integrated actions of all of the hormones participating in the process.

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LITERATURE CITED

22. MAYAK S, AH HALEVY, M KATZ 1972 Correlative changes in phytochromes in relation to senescence processes in rose petals. Physiol Plant 27: 1–4
23. McCLAGAN WB, BW POOVASH, HC DOSTAL 1975 Ethylene production and respiration in aging leaf segments and in disks of fruit tissue of normal and mutant tomato. Plant Physiol 56: 547–549