Production and Action of Ethylene in Senescing Leaf Discs

EFFECT OF INDOLEACETIC ACID, KINETIN, SILVER ION, AND CARBON DIOXIDE

NEHEMIA AHARONI, JAMES D. ANDERSON, AND MORRIS LIEBERMAN
Post Harvest Plant Physiology Laboratory, Beltsville Agricultural Research Center, United States Department of Agriculture, Beltsville, Maryland 20705

ABSTRACT
Supraoptimal concentrations of indoleacetic acid (IAA) stimulated ethylene production, which in turn appeared to oppose the senescence-retarding effect of IAA in tobacco leaf discs. Kinetic acted synergistically with IAA in stimulating ethylene production, but it inhibited senescence. Silver ion and CO2, which are believed to block ethylene binding to its receptor sites, delayed senescence in terms of chlorophyll loss and stimulated ethylene production. Both effects of Ag+ were considerably greater than those of CO2, IAA, kinetin, CO3, and Ag3, combined, acted to increase ethylene production further. Although this combination increased ethylene production about 160-fold over that of the control, it inhibited senescence. Treatment with 25 μl of ethylene in the presence of IAA enhanced chlorophyll loss in leaf discs and inhibited by about 90% the conversion of L-[3,4-14C]methionine to 14C2H4, suggesting autoinhibition of ethylene production.

The results suggest that ethylene biosynthesis in leaves is controlled by hormones, especially auxin, and possibly the rate of ethylene production depends, via a feedback control system, on the rates of ethylene binding at its receptor sites.

Leaf discs 1 cm in diameter were excised from leaves which were surface-sterilized with sodium hypochlorite as previously described (3). In experiments with L-[3,4-14C]methionine, leaves were sterilized for 20 s with 70% ethanol instead of sodium hypochlorite because of the possibility that NaOCl might react with methionine (1). Subsequent handling of the tissue involved sterile techniques.

Leaf discs were pretreated by floating them under cool-white fluorescent light for about 3 h in open Petri dishes containing 20 ml of either H2O, GA3, kinetin, IAA, or AVG. In tests involving treatment with Ag+, leaf discs were initially floated on a solution of AgNO3, or water, for 30 to 40 min depending on the experiment. The leaf discs were then transferred to 25-ml (8 discs) or 50-ml (12 discs) Erlenmeyer flasks containing filter paper moistened with 1 or 2 ml, respectively, of test solution which contained no Ag+. Weights of 8 or 12 leaf discs of tobacco were about 120 and 180 mg, respectively.

To prevent bacterial contamination, penicillin and streptomycin were added (3). The flasks were sealed with rubber serum caps and incubated in darkness at 28 C. When required, appropriate amounts of ethylene or CO2 were injected into the flasks. Accumulation of CO2 evolved by leaf disc was avoided by absorption in KOH (2).

Gas samples were withdrawn with a hypodermic syringe for determination of ethylene, as previously described (3). After sampling, the flasks were flushed with sterile fresh air and, when required, ethylene and CO2 were reintroduced.

For tracer studies, 0.75 or 1.50 μCi of L-[3,4-14C]methionine (53 mCi/mmol) was added to 10-ml Erlenmeyer flasks, each containing 1 ml of test solution and six leaf discs. The labeled ethylene from methionine was transferred via an argyl extension tube into an evacuated 70-ml jar (12) containing the bottom two-thirds of a plastic miniscintillation vial. Ethylene was trapped in 2 ml of 0.1 M mercuric acetate in methanol contained in the plastic vial. By this method, during a 3-h period, over 95% of the ethylene in the incubation flasks was transferred to mercuric acetate. After transfer the plastic vial was placed in a glass scintillation vial to which 10 ml of Aquasol scintillation fluid was added. In experiments where exogenous C2H4 was added (e.g. Table III), measurements were made to confirm total absorption of C2H4 in mercuric acetate before the jars were opened.

Chl was extracted from leaf discs with dimethylformamide (3) and determined spectrophotometrically at 665 nm. Concentrations are expressed in optical density (O.D.) units.

Treatments within each experiment were tested in triplicate flasks. The standard errors for both ethylene production and Chl content were generally in the range of 5 to 10% of the means.

MATERIALS AND METHODS

Experiments were performed with fully expanded leaves of 8- to 10-week-old tobacco (Nicotiana tabacum L. cv. Xanthi). The plants were grown in a greenhouse under natural lighting at temperatures ranging between 20 and 30 C.

Received for publication January 4, 1979 and in revised form June 11, 1979

1 This work was supported by the United States Department of Agriculture, Science and Education Administration, Agricultural Research, and the University of Maryland in Cooperative Agreement No. 12-14-1001-1201.

2 On leave from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

3 Abbreviation: AVG: aminoethoxyvinylglycine or 1,2-amino-4-(2-aminoethyl)-trans-3-butenic acid.

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RESULTS

Effects of Growth Hormones on Ethylene Production and Chl Retention. The effects of increasing concentrations of IAA, kinetin, and GA3 on ethylene production after 2 days and retention of Chl after 5 days were examined with tobacco leaf discs held in darkness at 28 °C. Of the IAA concentrations tested, only the lowest (10^-8 M) retarded Chl loss; also, at that concentration stimulation of ethylene production was minimal (Fig. 1). IAA concentrations above 10^-7 M stimulated ethylene production and enhanced Chl loss. GA3 did not induce ethylene production and was somewhat effective in retarding Chl loss. Kinetin was the most effective growth regulator in retarding Chl loss, despite its slight stimulation of ethylene production.

Ag⁺ is known to oppose the action of ethylene in many physiological processes (5–7), but its anti-aging effect on senescing leaf discs was associated with its stimulation of ethylene production during the first days of aging (2). Therefore, we examined more closely the interaction between Ag⁺, IAA, and kinetin in relation to ethylene production and tobacco leaf senescence. Ethylene production by untreated leaf discs decreased during the first days of senescence and thereafter increased somewhat (Fig. 2), as previously described (2, 3). Treatments with Ag⁺, kinetin, or IAA, especially when combined, resulted in stimulations of ethylene production by day 2. The rates plotted in Figure 2 are averages usually based on the production of ethylene over 18 to 24 h. The figure thus shows that the rates of production were maximum between the 1st and 2nd days of treatment. After reaching these maximum rates of ethylene production, the rates sharply decreased.

Maximum ethylene production was relatively low when stimulated by Ag⁺ or kinetin alone, but was higher when these inducers were allowed to act together. Ethylene production was highest when stimulated by Ag⁺ plus IAA, and the leaf discs continued to produce ethylene at a relatively high level throughout the rest of the 9-day period of the experiment.

The antiethylene action of CO₂ (8, 10) in leaf aging, like Ag⁺, was also found to be associated with stimulation of ethylene production and Chl retention. The effects of increasing concentrations of IAA, kinetin, and GA3 on ethylene production and Chl retention by tobacco leaf discs. Leaf discs were allowed to senesce for 96 h in 50-ml flasks. Ethylene production was measured after the first 48 h of incubation, and the average hourly rate was calculated. Chl was extracted after additional 72 h of incubation in sealed flasks which were ventilated daily. Chl content at zero time was 0.647 O.D. units.

FIG. 2. Time course of average daily ethylene production rates by senescing tobacco leaf discs treated with IAA, kinetin, and Ag⁺, and their combinations. In treatments that included Ag⁺, leaf discs were pretreated by floating for 30 min on AgNO₃ (10 mg/l) solution; thereafter, they were allowed to senesce for 9 days in 50-ml flasks containing the test solutions, which contained no Ag⁺. In other treatments, leaf discs were pretreated with water. IAA and kinetin at 0.01 M were applied continuously from day zero.

FIG. 1. Effects of increasing concentrations of IAA, kinetin, and GA3 on ethylene production and Chl retention by tobacco leaf discs. Leaf discs were allowed to senesce for 96 h in 50-ml flasks. Ethylene production was measured after the first 48 h of incubation, and the average hourly rate was calculated. Chl was extracted after additional 72 h of incubation in sealed flasks which were ventilated daily. Chl content at zero time was 0.647 O.D. units.
production (2). Effects of increasing concentrations of CO₂ on ethylene production by leaf discs treated with Ag⁺, IAA, kinetin, and their combinations are shown in Figure 3. The ethylene response was relatively moderate in leaf discs treated with IAA, kinetin, or IAA plus kinetin, but it was very high, suggesting synergistic effects, when Ag⁺ was included in those treatments, especially with IAA plus kinetin. In each treatment except that of kinetin plus Ag⁺, ethylene production increased in response to increasing CO₂. In the discs treated with kinetin plus Ag⁺, ethylene production was maximum when the concentration of CO₂ was 5%. The highest rate of ethylene production, about 90 nL/g·h (average for 3 days of incubation), was recorded for leaf discs treated with a combination of IAA, kinetin, 15% CO₂, and Ag⁺. This rate is greater by about 160-fold than that of the control without CO₂. The effect of CO₂ on retention of Chl was much lower than that of either kinetin or Ag⁺ but greater than that of IAA (Fig. 4). The most effective levels of CO₂ for Chl retention were between 5 and 10%.

Tracer Experiments with [L-3,4-14C]Methionine. Stimulatory effects of kinetin, Ag⁺, and CO₂ on ethylene production and on conversion of L-3,4-14Cmethionine to 14C₂H₄ were tested in the presence of IAA (Table I). The change in rate of ethylene production by treated leaf discs was generally similar to the change in rate of 14C₂H₄ formed from L-3,4-14Cmethionine, during the first 26 h after Ag⁺ or CO₂ treatment (Table I). Ag⁺ suppressed ethylene production during the first 3 h of incubation. AVG inhibited almost completely the production of both C₂H₄ and 14C₂H₄. The rate of 14Cethylene synthesis increased during the second incubation period but decreased in the third incubation period, probably due to dilution and loss of the label to other pathways after 26 h. In the 44-h period of the tracer experiment, kinetin or CO₂ did not much influence IAA-induced ethylene production (Table I). However, Ag⁺ did increase IAA-induced ethylene production considerably after a 3-h incubation period. Additional increases in IAA-induced 14C₂H₄ production were obtained when both CO₂ and Ag⁺ or kinetin and Ag⁺ were added to the IAA-induced tobacco discs system.

Increasing the concentration of Ag⁺ from 0 to 10 mg/l in the presence of 0.05 mM IAA increased conversion of [14C]methionine to 14C₂H₄ (Table II). CO₂ alone or in combination with Ag⁺ also enhanced production of 14C₂H₄. An additional increase in the concentration of Ag⁺ to 25 mg/l resulted in a great decline in ethylene synthesis, perhaps due to decreased uptake of either 14Cmethionine or IAA.

The possibility that high levels of endogenous ethylene can cause autoinhibition of ethylene synthesis (23) was studied in a test with exogenous ethylene added at 25 µl/l to the atmosphere of aging leaf discs (Table III). High rates of ethylene synthesis were induced by 0.05 mM IAA. Exogenous ethylene decreased conversion of [14C]methionine to 14C₂H₄, and the effect was maximum during hours 21 to 28 of incubation. During this period

![Figure 3](https://example.com/image3.png)

**Figure 3.** Effect of increasing concentrations of CO₂ on average ethylene production rates by tobacco leaf discs treated with IAA, kinetin, and Ag⁺, and their combinations. Treatment with hormones and pretreatment with Ag⁺ were as described in Figure 2. Ethylene was allowed to accumulate in 25-ml flasks for 72 h.

![Figure 4](https://example.com/image4.png)

**Figure 4.** Retention of Chl by tobacco leaf discs treated by IAA, kinetin, and Ag⁺, and their combinations. Treatment with hormones and pretreatment with Ag⁺ were as described in Figure 2. Flasks (25 ml) were ventilated after the first 72 h of incubation and thereafter CO₂ was reintroduced to flasks. Extraction of Chl was performed after 5 days of incubation. Chl content at zero time was 0.620 O.D. units.
in the presence of high levels of ethylene (Figs. 2 and 4).

Like kinetin, Ag⁺ also retarded Chl loss and was shown by Beyer (5) to oppose ethylene action in a number of physiological processes. Possibly, Ag⁺ binds to a site which normally binds ethylene and thereby blocks ethylene action. CO₂ was also shown to oppose ethylene action (8), and more recently Beyer (7) suggested that CO₂ inhibited the metabolism of labeled ethylene to labeled CO₂ without affecting tissue incorporation of label from ethylene. Consequently, the Ag⁺ and CO₂ reaction sites were suggested as the binding sites for ethylene which are involved in its metabolism.

In our present experiments, and those previously reported (2), we found that treating leaf discs with Ag⁺ not only prevented Chl loss but also increased ethylene production (Fig. 2). Very large increases in ethylene production occurred when Ag⁺-treated discs were also given IAA or IAA and kinetin. Even greater increases in ethylene production were obtained from IAA-kinetin-Ag⁺-treated leaf discs in the presence of CO₂ (Fig. 3).

The Ag⁺-increased ethylene production was not due to silver toxicity, which might have induced wound ethylene production, since even very low levels of Ag⁺ (2-5 µl/l) increased labeled ethylene production (Table II). Also, no physical damage to the discs was evident at the Ag⁺ concentrations used. Ethylene production increased because the conversion of methionine to ethylene was stimulated. This was shown by virtual complete inhibition of ethylene production by AVG (Table I), which inhibits ethylene production from methionine (20). It is possible that Ag⁺ stimulates ethylene production by preservation of IAA in the leaf tissue, since the rate of ethylene production in vegetative tissue is controlled mainly by IAA (9, 13, 16). This suggestion is supported by experiments (unpublished data) in which Ag⁺ did not increase ethylene production when applied to bean leaf discs incubated in 2,4-D, which is more stable in plant tissues (14). Alternatively or additionally, the increase in ethylene production in the presence of Ag⁺, especially when combined with CO₂, IAA, and kinetin,

**DISCUSSION**

Kinetin, GA₃, and IAA, at low concentrations, delayed senescence in tobacco leaf discs. These hormones, except for GA₃, stimulated ethylene production in aging discs. The marked stimulatory effects on ethylene production of IAA and IAA plus kinetin are similar to those induced in pea and mung bean seedlings (9, 11, 13, 16). Increased IAA-induced ethylene production in leaf discs was directly associated with loss of Chl, except when kinetin was present. IAA plus kinetin stimulated ethylene production more than IAA alone, but with less Chl loss. Retention of Chl was greatest in leaf discs treated only with kinetin, and this was associated with lowered ethylene levels. The special effect of kinetin in promoting Chl retention has long been noted (22). These experiments show that the kinetin-antagonized loss of Chl is related to the action of ethylene. Kinetin may have a complex role in senescence, probably acting on different systems. First, kinetin preserves IAA levels in the tissue by preventing its loss due to IAA conjugation (16). However, a high level of IAA contributes to stimulation of ethylene production which accelerates Chl loss. Additionally, kinetin is known to antagonize ethylene action in accelerating senescence, presumably by maintaining protein synthesis (22) and suppressing RNase (4). This maintenance of protein synthesis might explain the preservation of Chl.

**Table I. Rate of CO₂, Production and Conversion of L-[3,4,14C]-Methionine to 14C₂H₄ in Tobacco Leaf Discs Treated with 0.05 mM Kinetin, 0.1 mM AVG, and 10% CO₂**

All treatments contained 0.05 mM IAA and 0.5 µCi/ml L-[3,4,14C]methionine. Pretreatment with 10 µg/l Ag⁺ was as described in Figure 1.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Total CO₂</th>
<th>14C₂H₄</th>
<th>CO₂/h</th>
<th>14C₂H₄/h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-3 h</td>
<td>3-26 h</td>
<td>26-44 h</td>
<td>0-3 h</td>
</tr>
<tr>
<td>IAA</td>
<td>14.40</td>
<td>10.96</td>
<td>25.50</td>
<td>173</td>
</tr>
<tr>
<td>IAA + AVG</td>
<td>0</td>
<td>0</td>
<td>0.16</td>
<td>1</td>
</tr>
<tr>
<td>IAA + CO₂</td>
<td>21.40</td>
<td>11.18</td>
<td>16.49</td>
<td>178</td>
</tr>
<tr>
<td>IAA + CO₂ + AVG</td>
<td>0</td>
<td>0.37</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>IAA + kinetin</td>
<td>11.52</td>
<td>12.08</td>
<td>19.17</td>
<td>147</td>
</tr>
<tr>
<td>IAA + kinetin + CO₂</td>
<td>21.40</td>
<td>23.26</td>
<td>31.82</td>
<td>181</td>
</tr>
<tr>
<td>IAA + Ag⁺</td>
<td>11.32</td>
<td>63.74</td>
<td>103.52</td>
<td>71</td>
</tr>
<tr>
<td>IAA + Ag⁺ + Ag⁺</td>
<td>19.34</td>
<td>69.45</td>
<td>97.77</td>
<td>81</td>
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<tr>
<td>IAA + CO₂ + Ag⁺</td>
<td>0</td>
<td>0.17</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table II. Effect of Increasing Concentrations of Ag⁺ on Conversion of L-[3,4,14C]-Methionine to 14C₂H₄ by Tobacco Leaf Discs in the Presence or Absence of CO₂**

All treatments contained 0.05 mM IAA and 1 µCi/ml methionine. Ag⁺ was applied in a pretreatment by which leaf discs were floated for 40 min on AgNO₃ solution. CO₂ concentrations = 10% CO₂ was reintroduced after the measurement at hour 20.

<table>
<thead>
<tr>
<th>Ag⁺ Conc.</th>
<th>0-20 h</th>
<th>0-24 h</th>
<th>20-24 h</th>
<th>20-44 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/l</td>
<td>CO₂</td>
<td>CO₂</td>
<td>CO₂</td>
<td>CO₂</td>
</tr>
<tr>
<td>dpm/eight leaf discs h</td>
<td>dpm/eight leaf discs h</td>
<td>dpm/eight leaf discs h</td>
<td>dpm/eight leaf discs h</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>514</td>
<td>908</td>
<td>287</td>
<td>666</td>
</tr>
<tr>
<td>2.5</td>
<td>1,798</td>
<td>2,131</td>
<td>1,592</td>
<td>1,887</td>
</tr>
<tr>
<td>5.0</td>
<td>2,745</td>
<td>3,301</td>
<td>1,810</td>
<td>2,856</td>
</tr>
<tr>
<td>10.0</td>
<td>3,200</td>
<td>4,103</td>
<td>2,411</td>
<td>3,270</td>
</tr>
<tr>
<td>25.0</td>
<td>1,895</td>
<td>3,081</td>
<td>1,745</td>
<td>2,357</td>
</tr>
</tbody>
</table>

**Table III. Effect of Exogenous Ethylene on the Conversion of L-[3,4,14C]-Methionine to 14C₂H₄ in Tobacco Leaf Discs in the Presence or Absence of Ag⁺**

All treatments contained 0.05 mM IAA and 1 µCi/ml L-[3,4,14C]methionine. Tissue was pretreated with Ag⁺ by floating leaf discs for 30 min on AgNO₃ solution. CO₂ concentration was 25 µl/l. C₂H₄ was reintroduced after each measurement. Uptake of [14C]methionine measured after 45 h of incubation was 96 to 97% in all treatments.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Rate of Formation of 14C₂H₄ and Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>0-2 h</td>
</tr>
<tr>
<td>IAA</td>
<td>436</td>
</tr>
<tr>
<td>IAA + C₂H₄</td>
<td>386</td>
</tr>
<tr>
<td>IAA + Ag⁺</td>
<td>348</td>
</tr>
<tr>
<td>IAA + Ag⁺ + C₂H₄</td>
<td>284</td>
</tr>
</tbody>
</table>

1 A is dpm/six leaf discs h.
2 B is per cent inhibition.

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may be due to the loss of feedback control which modulates the rate of ethylene production. The feedback mechanism we envisage depends on interaction of ethylene with its binding sites, such that a feedback signal would serve to modulate or diminish ethylene production. As a result of Ag⁺ and/or CO₂ binding to and blocking the receptor sites, no negative feedback signal is produced and ethylene production would continue unabated. This hypothesis is supported by data shown in Figures 2 and 3 and Tables I and II, in which large increases in ethylene production due to IAA and IAA plus kinetin are further increased after Ag⁺, CO₂, or Ag⁺ plus CO₂ treatment. It should be noted that the stimulatory effect of Ag⁺ and CO₂ on ethylene production by tobacco leaf discs was found in the early stage of their senescence. However, our previous work (3) showed that in later stages of senescence these agents delayed the appearance of the peak in the climacteric-like rise of ethylene in leaf discs and Ag⁺ also markedly lowered it. This was associated with decreased Chl loss and respiration. Similar effects of Ag⁺ on suppression of the onset of the climacteric production of ethylene in fruit (23) and flowers (24) have been reported.

Table III shows that when cold ethylene at 25 μL/L was added to mature green tobacco leaf discs, conversion of [14C]methionine to [14C]2H₄ was inhibited in both the IAA and IAA-Ag⁺ treatments. These results agree with reports of Vendrell and McGlasson (25) and McMurchie et al. (19), who found that ethylene or propylene inhibited ethylene production in ripening fruit. These results differ from those observed in fruit wherein exogenous ethylene stimulated ethylene production (8).

The autoinhibition effect of ethylene may also be related to a feedback control system according to which a relatively high ethylene concentration could affect auxin concentration levels and consequently auxin-induced ethylene biosynthesis (17). Thus, systems which control the rate of ethylene production might be controlled both at the induction level and the level of utilization. While Ag⁺ is known to block ethylene action, presumably at an ethylene-binding site, Ag⁺ might also preserve IAA in tissues or accelerate the mechanisms by which IAA or kinetin, or their combination, stimulate ethylene biosynthesis.

The results of this study suggest that ethylene biosynthesis in leaves is controlled by hormones, particularly auxin. Also, some evidence is presented which suggest a hypothesis for a feedback control system relating ethylene production to its binding. This hypothesis does not exclude the possibility that other factors, such as IAA maintenance, which are directly associated with ethylene biosynthesis, are involved in controlling ethylene production. In fact, the influences of the receptor site blocking agents, Ag⁺ and CO₂, on ethylene production are most obvious in the presence of IAA, which directly affects ethylene production.

Acknowledgments—We thank W. Meudt for providing tobacco and bean leaves for these studies, T. Johnson for technical assistance, and I. Newman for preparing illustrations. We also thank A. Stempel of the Research Division, Hoffman LaRoche, for a gift of AVG.

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