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# Production and Action of Ethylene in Senescing Leaf Discs

## EFFECT OF INDOLEACETIC ACID, KINETIN, SILVER ION, AND CARBON DIOXIDE[1]

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### 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. Kinetin acted synergistically with IAA in stimulating ethylene production, but it inhibited senescence. Silver ion and $CO_2$, 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 $CO_2$. IAA, kinetin, $CO_2$, and $Ag^+$, 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/l of ethylene in the presence of IAA enhanced chlorophyll loss in leaf discs and inhibited by about 90% the conversion of L-[3,4-$^{14}$C]methionine to $^{14}C_2H_4$ 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.**

Ethylene plays a considerable role in regulation of fruit ripening (21) and senescence of flowers (15) and leaves (2). Generally, the senescence-enhancing effect of ethylene is opposed by effects of other growth hormones—auxins, cytokinins, and gibberellins. These hormones, alone or in combination, can, in several instances, induce ethylene production (18).

$CO_2$ and $Ag^+$ oppose the effect of ethylene, presumably by blocking ethylene action at its receptor sites (6–8, 10). A previous study showed that the senescence-retarding effect of $Ag^+$ and $CO_2$ was associated with stimulation of ethylene production during early stages of senescence (2). In the present study we probed interactions between $Ag^+$, $CO_2$, kinetin, and IAA, as they relate to ethylene biosynthesis and action in senescing tobacco leaf discs.

### 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.

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-$^{14}$C]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 $H_2O$, $GA_3$, kinetin, IAA, or AVG.[3] In tests involving treatment with $Ag^+$, leaf discs were initially floated on a solution of $AgNO_3$, 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 $CO_2$ were injected into the flasks. Accumulation of $CO_2$ evolved by leaf discs 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 $CO_2$ were reintroduced.

For tracer studies 0.75 or 1.50 μCi of L-[3,4-$^{14}$C]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 $C_2H_4$ was added (*e.g.* Table III), measurements were made to confirm total absorption of $C_2H_4$ 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.

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[3] Abbreviation: AVG: aminoethoxy vinyl glycine or L-2-amino-4-(2-aminoethoxy)-*trans*-3-butenoic acid.

## RESULTS

**Effects of Growth Hormones on Ethylene Production and Chl Retention.** The effects of increasing concentrations of IAA, kinetin, and $GA_3$ 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. $GA_3$ 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 $CO_2$ (8, 10) in leaf aging, like $Ag^+$, was also found to be associated with stimulation of ethylene

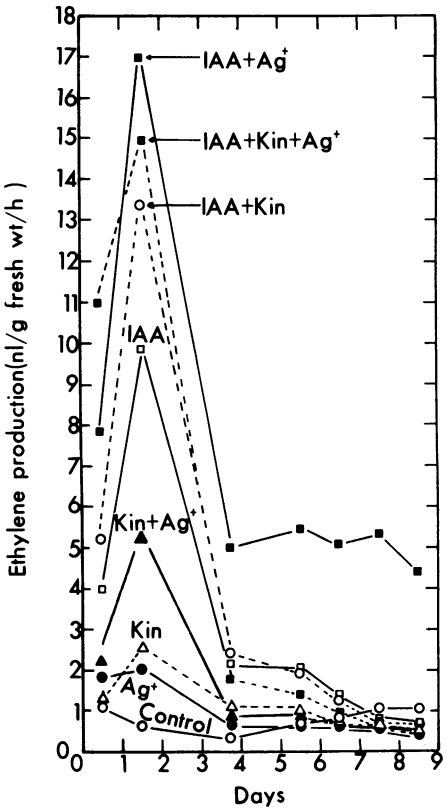


FIG. 2. Time course of average daily ethylene production rates by senescing tobacco leaf discs treated with IAA, kinetin, and $Ag^+$, and their combinations. In treatments that included $Ag^+$, leaf discs were pretreated by floating for 30 min on $AgNO_3$ (10 mg/l) solution; thereafter, they were allowed 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 mM were applied continuously from day zero.

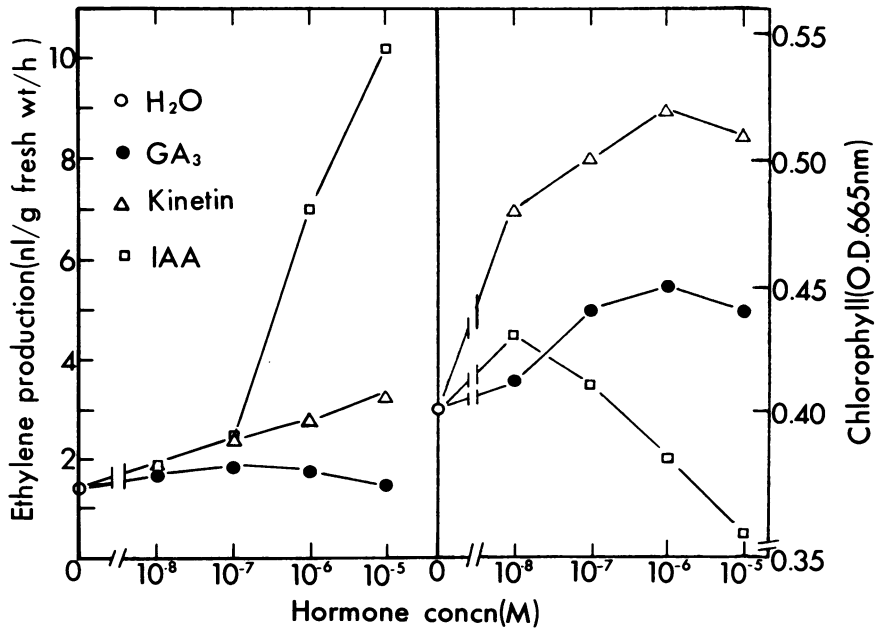


FIG. 1. Effects of increasing concentrations of IAA, kinetin, and $GA_3$ on ethylene production and Chl retention by tobacco leaf discs. Leaf discs were allowed to senesce for 96 h in 50-ml flasks. Ethylene production was measured after the first 48 h of incubation, and the average hourly rate was calculated. 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 $CO_2$ 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 $CO_2$. In the discs treated with kinetin plus $Ag^+$, ethylene production was maximum when the concentration of $CO_2$ 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% $CO_2$, and $Ag^+$. This rate is greater by about 160-fold than that of the control without $CO_2$. The effect of $CO_2$ 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 $CO_2$ for Chl retention were between 5 and 10%.

**Tracer Experiments with L-[3,4-$^{14}$C]Methionine.** Stimulatory effects of kinetin, $Ag^+$, and $CO_2$ on ethylene production and on conversion of L-[3,4-$^{14}$C]methionine to $^{14}C_2H_4$ 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 $^{14}C_2H_4$ formed from L-[3,4-$^{14}$C]methionine, during the first 26 h after $Ag^+$ or $CO_2$ treatment (Table I). $Ag^+$ suppressed ethylene production during the first 3 h of incubation. AVG inhibited almost completely the production of both $C_2H_4$ and

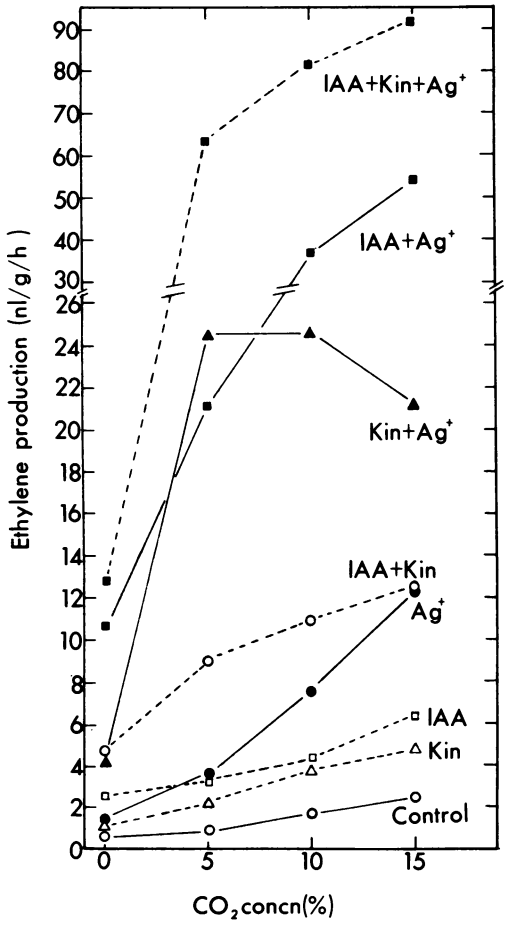


FIG. 3. Effect of increasing concentrations of $CO_2$ on average ethylene production rates by tobacco leaf discs treated with IAA, kinetin, and $Ag^+$, and their combinations. Treatment with hormones and pretreatment with $Ag^+$ were as described in Figure 2. Ethylene was allowed to accumulate in 25-ml flasks for 72 h.

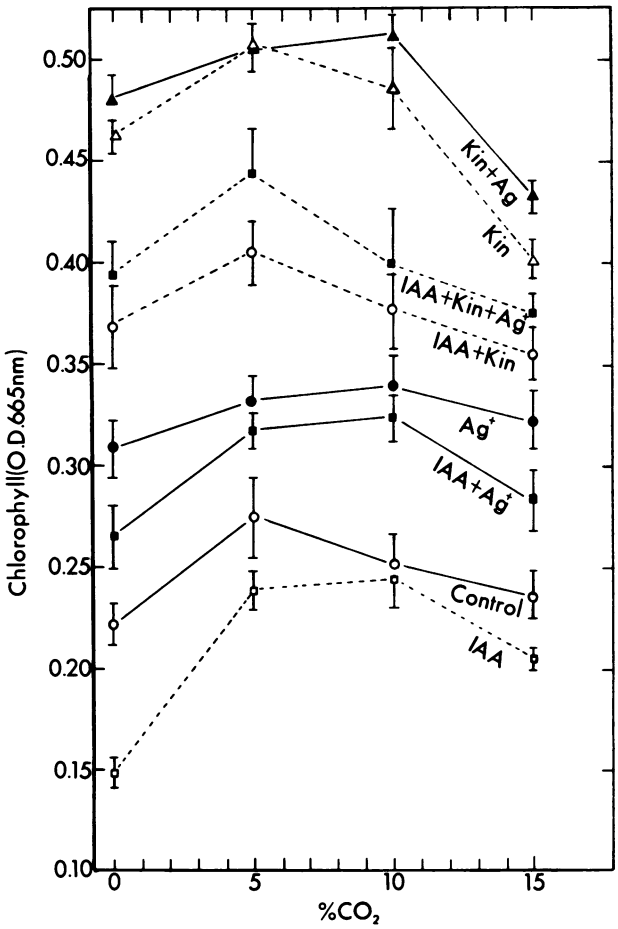


FIG. 4. Retention of Chl by tobacco leaf discs treated by IAA, kinetin, and $Ag^+$, and their combinations. Treatment with hormones and pretreatment with $Ag^+$ were as described in Figure 2. Flasks (25 ml) were ventilated after the first 72 h of incubation and thereafter $CO_2$ was reintroduced to flasks. Extraction of Chl was performed after 5 days of incubation. Chl content at zero time was 0.620 O.D. units.

$^{14}C_2H_4$. The rate of [$^{14}$C]ethylene 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 $CO_2$ 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 $^{14}C_2H_4$ production were obtained when both $CO_2$ and $Ag^+$ or kinetin and $Ag^+$ were combined with IAA. Highest production of both total and labeled ethylene was obtained when kinetin, $CO_2$ 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 [$^{14}$C]methionine to $^{14}C_2H_4$ (Table II). $CO_2$ alone or in combination with $Ag^+$ also enhanced production of $^{14}C_2H_4$. 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 [$^{14}$C]methionine 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 [$^{14}$C]methionine to $^{14}C_2H_4$, and the effect was maximum during hours 21 to 28 of incubation. During this period

$^{14}C_2H_4$ synthesis was inhibited by about 80 and 90% in untreated and $Ag^+$-treated leaf discs, respectively. In another experiment, a lower concentration of ethylene (10 μl/l) was used, and the maximum inhibition of $^{14}C_2H_4$ synthesis was 46%.

## DISCUSSION

Kinetin, $GA_3$, and IAA, at low concentrations, delayed senescence in tobacco leaf discs. These hormones, except for $GA_3$, 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 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. $CO_2$ was also shown to oppose ethylene action (8), and more recently Beyer (7) suggested that $CO_2$ inhibited the metabolism of labeled ethylene to labeled $CO_2$ without affecting tissue incorporation of label from ethylene. Consequently, the $Ag^+$ and $CO_2$ 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 $CO_2$ (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 production of ethylene 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 $CO_2$, IAA, and kinetin,

Table I. *Rate of $C_2H_4$ Production and Conversion of L-[3,4-$^{14}$C]-Methionine to $^{14}C_2H_4$ in Tobacco Leaf Discs Treated with 0.05 mM Kinetin, 0.1 mM AVG, and 10% $CO_2$*

All treatments contained 0.05 mM IAA and 0.5 μCi/ml L-[3,4-$^{14}$C]methionine. Pretreatment with 10 mg/l $Ag^+$ was as described in Figure 2.

| Treatments | Total $C_2H_4$ | | | $^{14}C_2H_4$ | | |
|---|---|---|---|---|---|---|
| | 0–3 h | 3–26 h | 26–44 h | 0–3 h | 3–26 h | 26–44 h |
| | *nl/g fresh wt·h* | | | *dpm/six leaf discs·h* | | |
| IAA | 14.40 | 10.96 | 25.50 | 173 | 230 | 171 |
| IAA + AVG | 0 | 0 | 0.16 | 1 | 1 | 1 |
| IAA + $CO_2$ | 21.40 | 11.18 | 16.49 | 178 | 244 | 136 |
| IAA + $CO_2$ + AVG | 0 | 0 | 0.37 | 4 | 1 | 0 |
| IAA + kinetin | 11.52 | 12.08 | 19.17 | 147 | 230 | 146 |
| IAA + kinetin + $CO_2$ | 21.40 | 23.26 | 31.82 | 181 | 365 | 223 |
| IAA + $Ag^+$ | 11.32 | 63.74 | 103.52 | 71 | 925 | 685 |
| IAA + AVG + $Ag^+$ | 0 | 0 | 0.35 | 1 | 1 | 1 |
| IAA + $CO_2$ + $Ag^+$ | 19.34 | 69.45 | 97.77 | 81 | 1,242 | 1,195 |
| IAA + $CO_2$ + AVG + $Ag^+$ | 0 | 0 | 0.17 | 1 | 1 | 1 |
| IAA + kinetin + $Ag^+$ | 13.17 | 74.92 | 93.74 | 167 | 1,031 | 720 |
| IAA + kinetin + $CO_2$ + $Ag^+$ | 16.05 | 122.11 | 162.18 | 170 | 1,410 | 1,010 |

Table II. *Effect of Increasing Concentrations of $Ag^+$ on Conversion of L-[3,4-$^{14}$C]Methionine to $^{14}C_2H_4$ by Tobacco Leaf Discs in the Presence or Absence of $CO_2$*

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 $AgNO_3$ solution. $CO_2$ concentrations = 10%. $CO_2$ was reintroduced after the measurement at hour 20.

| $Ag^+$ Concn. | $^{14}C_2H_4$ | | | |
|---|---|---|---|---|
| | 0–20 h | | 20–44 h | |
| | $-CO_2$ | $+CO_2$ | $-CO_2$ | $+CO_2$ |
| *mg/l* | *dpm/eight leaf discs·h* | | | |
| 0 | 514 | 908 | 287 | 666 |
| 2.5 | 1,798 | 2,131 | 1,592 | 1,887 |
| 5.0 | 2,745 | 3,301 | 1,810 | 2,856 |
| 10.0 | 3,200 | 4,103 | 2,411 | 3,270 |
| 25.0 | 1,895 | 3,081 | 1,745 | 2,357 |

Table III. *Effect of Exogenous Ethylene on the Conversion of L-[3,4-$^{14}$C]Methionine to $^{14}C_2H_4$ 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-$^{14}$C]methionine. Tissue was pretreated with $Ag^+$ by floating leaf discs for 30 min on $AgNO_3$ solution. $C_2H_4$ concentration was 25 μl/l. $C_2H_4$ was reintroduced after each measurement. Uptake of [$^{14}$C]methionine measured after 45 h of incubation was 96 to 97% in all treatments.

| Treatments | Rate of Formation of $^{14}C_2H_4$ and Percentage Inhibition | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0–2 h | | 2–4 h | | 4–21 h | | 21–28 h | | 28–45 h | |
| | A[1] | B[2] | A | B | A | B | A | B | A | B |
| IAA | 436 | | 992 | | 850 | | 610 | | 337 | |
| IAA + $C_2H_4$ | 386 | 11.5 | 689 | 30.6 | 218 | 74.4 | 112 | 81.6 | 77 | 77.2 |
| IAA + $Ag^+$ | 348 | | 885 | | 1387 | | 1303 | | 652 | |
| IAA + $Ag^+$ + $C_2H_4$ | 284 | 18.4 | 502 | 43.3 | 178 | 87.2 | 143 | 89.0 | 220 | 66.3 |

[1] A is dpm/six leaf discs·h.

[2] B is per cent inhibition.

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 $CO_2$ 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 $Ag^+$, $CO_2$, or $Ag^+$ plus $CO_2$ treatment. It should be noted that the stimulatory effect of $Ag^+$ and $CO_2$ 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) has been reported.

Table III shows that when cold ethylene at 25 $\mu$l/l was added to mature green tobacco leaf discs, conversion of [$^{14}$C]methionine to $^{14}C_2H_4$ 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 $CO_2$, on ethylene production are most obvious in the presence of IAA, which directly effects ethylene production.

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