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Enhancement by Ethylene of Cellulysin-Induced Ethylene Production by Tobacco Leaf Discs[1]

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EDO CHALUTZ[2], AUTAR K. MATTOO*, THEO SOLOMOS, AND JAMES D. ANDERSON
Plant Hormone Laboratory, PPHI, Beltsville Agricultural Research Center-West, United States Department of Agriculture, Beltsville, Maryland 20705 (E. C., A. K. M., J. D. A.); and the University of Maryland, College Park, Maryland 20742 (E. C., A. K. M., T. S.)

ABSTRACT

Cellulysin-induced ethylene production in tobacco (*Nicotiana tabacum* L.) leaf discs was enhanced several-fold by prior exposure of the leaf tissue to ethylene. This enhancement in the response of the tissue to Cellulysin increased rapidly during 4 and 8 hours of pretreatment with ethylene and resulted from greater conversion of methionine to ethylene. On treatment with Cellulysin, the content of 1-aminocyclopropane-1-carboxylic acid (ACC) in leaf discs not pretreated with ethylene markedly increased while that of the ethylene-pretreated tissue was only slightly higher than in the tissue incubated in the absence of Cellulysin. Ethylene-treated tissue, however, converted ACC to ethylene at a faster rate than air controls. These data indicate that ethylene stimulates Cellulysin-induced ethylene production by stimulating the conversion of ACC to ethylene. Data are also presented on a possible relation of this phenomenon to ethylene produced by the tobacco leaf upon interaction with its pathogen, *Alternaria alternata*.

Ethylene is produced by higher plants as well as microorganisms and its role as a plant hormone is well established (1, 15, 16). In most diseased plants, ethylene production is stimulated and the involvement of ethylene in pathogenesis has been suggested (6, 21, 25). Ethylene may be involved in disease resistance by induction of enzymes or by formation of antifungal compounds (8, 10, 11, 21, 28), while it may also promote sensitivity of higher plants to external stimuli by accelerating senescence (1). Little is known about the mechanism of production and roles of ethylene during the interaction of host and parasite. There is also no clear knowledge of the contribution of host or pathogen to ethylene produced during disease or of the site of ethylene production (21). However, an early event in the interaction between the host and its pathogen is the secretion, by the pathogen, of cell-wall degrading enzymes (5) including cellulase (26). It was therefore of considerable interest to us that a fungal cell wall digesting preparation, 'Cellulysin,' induced ethylene biosynthesis in tobacco leaves (4) by causing a rapid formation of ACC[3], the immediate precursor of ethylene. We report here on further studies of this phenomenon, its enhancement in tissues pretreated with ethylene, and its possible relationship to ethylene produced by the tobacco leaf upon interaction with its pathogen, *Alternaria alternata*.

MATERIALS AND METHODS

Three cultivars of tobacco (*Nicotiana tabacum* L.) plants were used, viz. Burly Mammoth, Maryland 609, and Xanthi. Materials and methods used for the preparation of leaf discs and ethylene determination were described earlier (4).

Tobacco leaves were pretreated in air or ethylene in 3.8-L desiccators. Each leaf was divided in half by cutting along its mid-rib and each half was placed on filter paper, moistened with water, in individual desiccators. A vial containing filter paper soaked with 2 ml of 0.25 M mercuric perchlorate was placed in the 'air control' desiccator to absorb traces of ethylene. Similarly, in some experiments, whole, potted tobacco plants were pretreated with ethylene. Four plants were placed inside a 2-ply (3 mil), 45-L polyethylene bag for 16 h with the desired ethylene concentration. Unless otherwise indicated, three leaf discs (1 cm in diameter, weighing 50 mg) were incubated in 25-ml Erlenmeyer flasks with 0.5 ml of the basal medium containing 700 mM sorbitol, 10 mM Mes (pH 6.0), 10 mM $CaCl_2$, 50 μg/ml streptomycin sulfate, and 50 units/ml penicillin G in the absence or presence of Cellulysin (Calbiochem) (4). Cellulysin was desalted before use by ultrafiltration with an Amicon PM-10 membrane (3). Ethylene was allowed to accumulate for 1 h and quantified by GC (17). Between each sampling, flasks were flushed with sterile fresh air.

Radioactive ethylene produced by the leaf discs upon incubation with [3,4-^{14}C]methionine was accumulated for 1 h in the incubation flask. A 12-ml gas sample from the atmosphere above the discs was then injected (9) into a 485-ml jar which contained a filter paper wetted with 0.6 ml of 0.25 M mercuric perchlorate in a scintillation vial. After 2 h incubation on ice, 10 ml of a toluene-based scintillation cocktail (25% Triton X-100 in Liquifluor, New England Nuclear) was added to each vial. Radioactive ethylene absorbed by the filter paper was counted in a liquid scintillation counter (9). Uptake of [^{14}C]ACC by the leaf discs was checked by incubating the leaf discs for 15, 60, or 120 min with 0.5 ml of [2,3-^{14}C]ACC (3754 dpm/nmol). Aliquots of the incubation solution were sampled at the indicated times and counted to estimate the residual radioactivity. After each incubation, the discs were removed from the incubating solution, washed, and further incubated in water for 2 min to release the

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[2] On leave from Division of Fruit and Vegetable Storage, ARO, The Volcani Center, Bet Dagan, Israel.

[3] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; SAM, *S*-adenosylmethionine.

Table I. *Effect of Different Pretreatments on the Cellulysin-Induced Ethylene Production by Tobacco Leaf Discs*

Tobacco (cv Burly Mammoth) leaf tissue was pretreated in ethylene (60 μl/l) or in air (less than 8 nl/l of ethylene) for 16 h at 25°C at high (95%) RH under cool-white fluorescent light (1750 lux). Ethylene production was measured during the 3rd h of incubation after the addition of Cellulysin (1.2%, w/v). Discs without Cellulysin addition produced ethylene at rates lower than 45 nl/g fresh wt·h.

Treatment	Ethylene Production
	nl/g·h
Freshly cut (no pretreatment)	160
Pretreated as leaf (halves)	
Air	185
C_2H_4	550
Pretreated as discs	
Air	495
C_2H_4	760

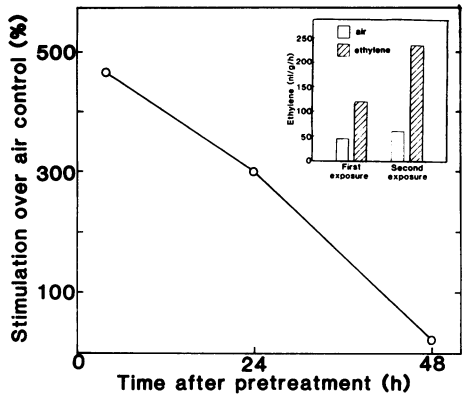

FIG. 1. Effect of the time elapsing between pretreatment in ethylene and Cellulysin applications on the Cellulysin-induced ethylene production by tobacco (cv Xanthi) leaf discs. Potted plants were pretreated in 60 μl/l ethylene for 16 h. Cellulysin was then applied to leaf discs cut from the treated plants at the times indicated. Absolute values of air controls, ranged from 100 to 150 μl C_2H_4/g fresh wt·h. Inset shows the response of leaf discs to Cellulysin after a second, 16-h exposure of the leaf to ethylene, 48 h after the first exposure, as compared to the response after the first exposure.

Table II. *Production of Ethylene by Cellulysin-Treated Leaf Discs of Several Tobacco Cultivars*

Procedures were similar to those outlined in Table I. Control discs incubated without Cellulysin produced ethylene at less than 25 nl/g·h for Burley Mammoth and less than 6.5 nl/g·h for all other cultivars.

Cultivar	Ethylene Production		
	Freshly Cut	Pretreatment	
		Air	Ethylene
	nl/g·h		
Burly Mammoth	160	200	575
Maryland 609	30	55	275
Xanthi	23	50	130
Yellow Mammoth	34	40	73
TI 102	35	38	36

label from intracellular spaces. Thereafter, the tissue was frozen and homogenized with 0.8 ml of 80% ethanol. Aliquots of the homogenate were then sampled and counted. Total ACC and [^{14}C]ACC in the tissue after incubation with [3,4-^{14}C]methionine were determined in extracts homogenized with 80% ethanol as

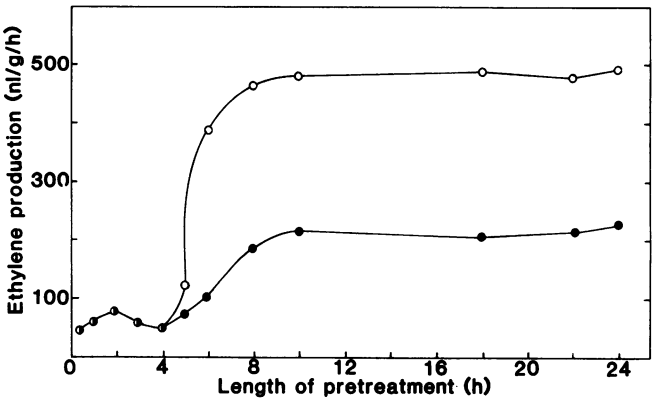

FIG. 2. Effect of the length of the pretreatment period on the Cellulysin-induced ethylene production in tobacco (cv Burly Mammoth) leaf discs. (●), Half leaf pretreated in air; (○), half leaf pretreated in ethylene (60 μl/l). Values are for the 3rd h of incubation after the addition of Cellulysin.

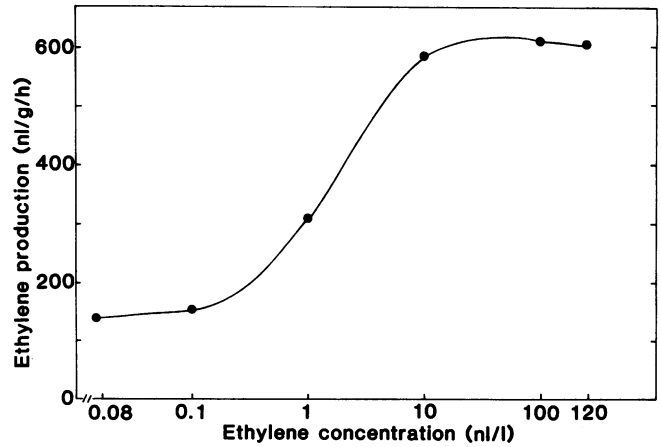

FIG. 3. Effect of the concentrations of ethylene during pretreatment on the Cellulysin-induced ethylene production in tobacco (cv Burly Mammoth) leaf discs. Procedures were similar to those outlined in Table I.

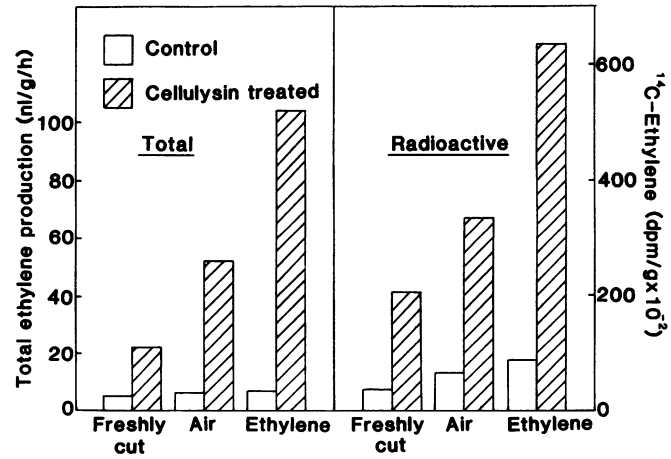

FIG. 4. Comparison between rates of total and labeled ethylene production from [3,4-^{14}C]methionine by tobacco (cv Xanthi) leaf discs. Leaves were pretreated in air or ethylene, then discs were cut and treated with Cellulysin. The values represent average rates of ethylene production during the first 2 h of incubation after the addition of Cellulysin.

Table III. *Total and Labeled ACC Content and Ethylene Produced by Tobacco (cv Xanthi) Discs Incubated with [3,4-^{14}C]Methionine in Relation to Different Pretreatments*

Leaf discs, either freshly cut or after the indicated treatments given to whole leaves, were incubated with the basal medium (6 discs/ml) containing 1952 dpm/nmol methionine.

Leaf Tissue, Treatment	Cellulysin Addition	C_2H_4[a]		ACC[a]		[^{14}C]ACC/[^{14}C]ESF[b]
		nmol	*dpm* × 10^3	*nmol*	*dpm/nmol*[c]	× 10^3
Fresh	−	0.15	3.80	1.40	2714	1.45
	+	0.65	14.30	26.95	531	8.06
Pretreated in air; 16 h	−	0.20	3.50	1.05	3333	1.04
	+	1.50	7.30	30.10	243	6.24
Pretreated in C_2H_4; 16 h	−	0.20	3.45	1.05	3286	1.38
	+	3.25	2.85	7.70	370	1.46

[a] Values are for rate, g^{-1} h^{-1}.
[b] ESF ethanol-soluble fraction.
[c] Specific activity.

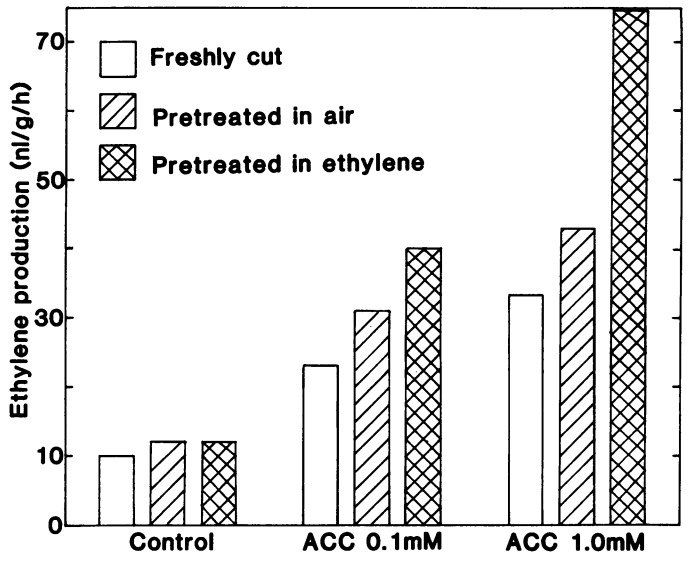

FIG. 5. Effect of pretreatment of tobacco (cv Xanthi) leaves in ethylene on their subsequent conversion of ACC to ethylene, in the absence of Cellulysin. The procedure was similar to that described in Table I. Values of ethylene production are for the 1st h of incubation following the addition of ACC, in the presence of 1 mM AVG.

described earlier (18).

Inoculation of tobacco leaves with *A. alternata* (Fr.) Keissl was carried out by the method of Spurr (24). Leaf discs, 1 cm in diameter, were inoculated by dipping them for 1 min in a spore suspension (10^3 spores/ml). Uninoculated control discs were dipped in water. Eight discs were then transferred into each 25-ml Erlenmeyer flask, placed on moist filter paper, and incubated at 25°C in the dark. Ethylene produced by the inoculated and control discs was measured by GC.

Three replicates were routinely used and experiments were repeated at least twice and gave similar pattern of results. However, due to the variability of the greenhouse-grown plants, results of typical experiments are presented.

RESULTS

As shown previously (4), tobacco leaf discs incubated in a medium containing Cellulysin respond by increased ethylene production (Table I). This response of leaf discs to Cellulysin was further enhanced several-fold by pretreating the detached leaf in ethylene (Table I). However, when leaf discs instead of whole leaves were pretreated in air, their subsequent response to Cellulysin was similar to that of the discs cut from ethylene-treated leaves. Ethylene-treated leaf discs produced ethylene in response to Cellulysin at a rate 1.5 times higher than discs treated in air (Table I). This response of ethylene-treated leaf discs or single leaves to Cellulysin was also evident when whole, potted plants were pretreated in ethylene. Under these conditions, the effect of the treatment was maintained for at least 24 h after the plants were removed from ethylene (Fig. 1). When leaves of the treated plants were detached and pretreated for a second time in ethylene, 48 h after the first treatment, the subsequent response of discs cut from these leaves to Cellulysin was 50% more than that of discs cut from leaves pretreated in ethylene for the first time (Fig. 1, inset).

Of the tobacco cultivars studied, Burly Mammoth produced the highest rates of ethylene in response to Cellulysin; cultivar differences occurred whether freshly cut leaves or leaves pretreated in ethylene were used, although some cultivars (*i.e.* TI 102) did not respond to the ethylene pretreatment (Table II). The maximal response of the leaf to ethylene pretreatment was reached between 4 and 8 h of incubation. Periods longer than 10 h of incubation did not further increase the subsequent response of the discs to Cellulysin (Fig. 2). The optimal concentration of ethylene for maximal response was between 10 and 100 μl/l (Fig. 3).

To verify that the increased production of ethylene by the discs from ethylene-pretreated leaves in the presence of Cellulysin was due to *de novo* synthesis instead of release of absorbed or bound ethylene from the tissue, discs from leaves pretreated in ethylene were incubated with [3,4-^{14}C] methionine and Cellulysin. Total and labeled ethylene were then assayed. The results (Fig. 4) showed that Cellulysin-induced ethylene production in ethylene-treated tissue resulted from the conversion of methionine to ethylene. Furthermore, these data showed close similarities in the pattern of total and labeled ethylene produced and in the specific radioactivity of ethylene produced by Cellulysin-treated discs from freshly cut or ethylene-pretreated leaves suggesting a common biosynthetic pathway.

Cellulysin caused a marked accumulation of ACC in the treated tissue (Table III), a phenomenon observed earlier (4). However, this effect was pronounced in the freshly cut or air-pretreated discs and was relatively inconspicuous in discs pretreated in ethylene (Table III). The lower content of ACC in the ethylene-treated discs in the presence of Cellulysin could result from a higher rate of conversion of ACC to ethylene. To test this

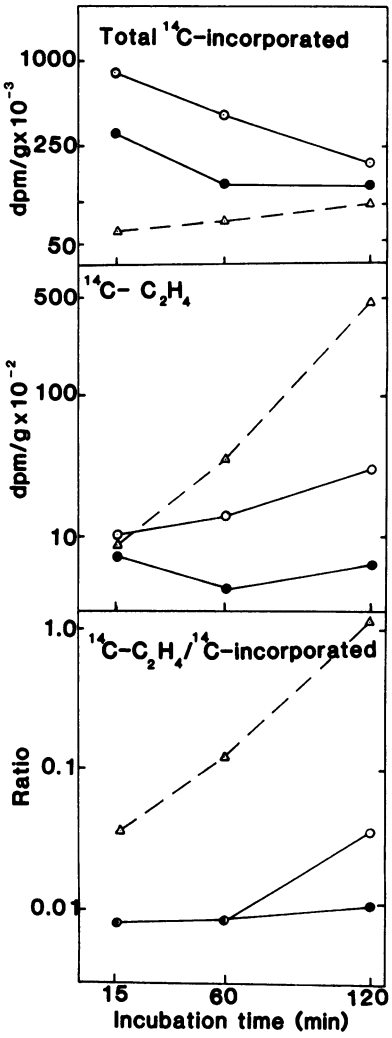

FIG. 6. Incorporation of [2,3-^{14}C]ACC and labeled ethylene production by tobacco (cv Burly Mammoth) leaf discs following pretreatment in air or ethylene, in the absence of Cellulysin. Pretreatment conditions were similar to those described in Table I. (●), Freshly cut discs; (○), discs cut from air-pretreated leaves; (△), discs cut from ethylene-pretreated leaves.

possibility, we compared the rates of ethylene production by freshly cut leaf discs as well as by discs cut from air- or ethylene-pretreated tissue in response to exogenously added ACC (without Cellulysin) while blocking the formation of endogenous ACC by including 1 mM AVG in the incubation medium. The results (Fig. 5) confirmed previous observations in *Nicotiana* (19) and other systems (14, 22) that aging of leaf tissue increases their capacity to produce ethylene from ACC. Our data further indicated that the conversion of exogenously added ACC to ethylene was twice as much in ethylene-treated than in air-treated leaf tissue while the uptake of labeled ACC by the ethylene-treated discs was lower than that by the air-treated or freshly cut leaf discs (Fig. 6).

Tobacco leaf discs inoculated with the pathogen, *A. alternata*, produced ethylene at higher rates than did uninoculated controls (Fig. 7). Moreover, inoculated discs cut from leaves pretreated in ethylene produced higher rates of ethylene than those cut from fresh tissue or from leaves pretreated in air.

DISCUSSION

Enhancement by ethylene of the Cellulysin-induced ethylene production in tobacco leaf discs as evidenced here exhibited

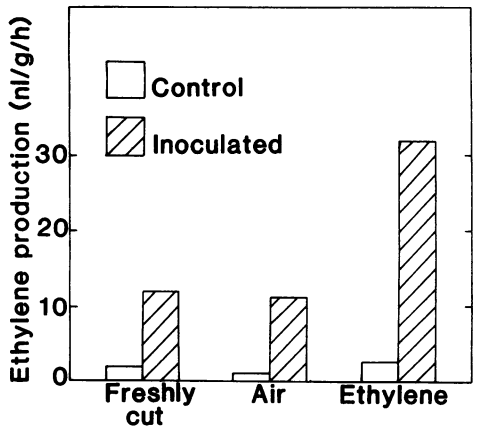

FIG. 7. Effect of pretreating tobacco (cv Burly Mammoth) leaves in air or ethylene on the subsequent ethylene production, 24 h after inoculation of leaf discs with *Alternaria alternata*. Freshly cut leaf discs were inoculated by dipping them for 1 min in a spore suspension of 10^3 spores/ml.

characteristics common to many other physiological effects of ethylene (1, 16), viz. concentration-dependence during pretreatment (Fig. 3), a several-hour lag period (Fig. 2), and a requirement for the continuous presence of ethylene for maximal response (Fig. 1). The greater response of tobacco leaf discs to Cellulysin following a second exposure of the leaves to ethylene (Fig. 1, inset) also seems to be a common effect of ethylene on plant tissues. Such a response was reported earlier (10) for the ethylene-induced formation of the phytoalexin, isocoumarin, in carrot roots, and recently (23) for the induction by ethylene of ethylene biosynthesis in citrus leaf discs. Such responses to ethylene could be observed only in tissues where an initial exposure of the tissue to ethylene does not evoke the autocatalytic production of ethylene common to climacteric-type fruits (1, 22). A possible explanation of such a phenomenon was offered earlier (10) based on the data obtained on the isocoumarin formation. There it was suggested that the ethylene induction may be a two-step process: the first, activation of the biosynthetic system through the induction of enzymes. This process is quantitatively dependent upon the length of the initial ethylene induction period. The second step involves the actual synthesis of the phytoalexin and also requires the presence of ethylene (10). The relatively slow rate of decline in the response of tobacco to Cellulysin (Fig. 1) following removal of ethylene may be due to a higher rate of ethylene production by ethylene-treated leaves than by those pretreated in air. Thus, ethylene may be present in or near these leaves at slightly higher concentrations than in or near control leaves. Our findings (Fig. 4, controls) support this suggestion as do those of Aharoni and Lieberman (2) who showed that tobacco leaf discs pretreated in ethylene for 24 h produced ethylene at a slightly higher rate for at least 1 to 3 d after the treatment compared to control discs.

The mode of action of ethylene in enhancing the response of tobacco leaves to Cellulysin may be complex. The following findings suggest an explanation: (a) pretreatment of leaf discs in air could partially substitute the effect of pretreatment in ethylene of whole leaves (Table I); and (b) pretreatment of leaf tissue in air also slightly, but consistently, enhanced the response of the tissue to Cellulysin (Table I; Figs. 2 and 5) as compared to freshly cut leaves. On this basis, we suggest that ethylene could be involved in the enhancement of the tissues' response at extremely low concentrations (*i.e.* less than 8 nl/l). Alternatively, these findings may suggest that other factors, in addition to ethylene, may be involved in the process. Such a suggestion was offered by Geballe and Galston (13) who studied wound-induced resist-

ance to cellulase in oat leaves and reported that ethylene was a factor in this process (12). A similar phenomenon was observed earlier in prune tissue (27). However, since exogenously applied ethylene could only partially substitute for the wounding effect, Geballe and Galston suggested (12) that the induction of resistance may require a wound signal in addition to ethylene.

Whether or not ethylene is the direct factor in the enhancement of the Cellulysin-induced ethylene biosynthesis in tobacco leaves, the results presented here clearly indicate that higher rates of ethylene production are not merely the result of the release of ethylene from the tissue. Rather, it originates from enhanced biosynthesis from methionine (Fig. 4). Also, these data indicate that in all leaf treatments, *i.e.* freshly cut, air-, and ethylene-pretreated, a common precursor and biosynthetic pathway lead to the production of ethylene (Fig. 4). However, following incubation with Cellulysin, the ACC content was much lower in discs from ethylene-treated leaves than from freshly cut or air-pretreated discs (Fig. 6). We interpret these findings to suggest that while Cellulysin induces the formation of ACC, presumably due to increased activity of ACC synthase (4), ethylene treatment of leaves causes a higher rate of conversion of ACC to ethylene, thus increasing the utilization of newly formed ACC in the ethylene-treated tissue. This suggestion is supported by the findings that discs from ethylene-treated leaves converted exogenously applied ACC to ethylene at a higher rate than discs from freshly cut or air-pretreated leaves (Fig. 5), irrespective of the differences in the uptake of [^{14}C]ACC (Fig. 6).

In a recent report, Riov and Yang (23) reported that a 24-h exposure of the citrus leaf discs to ethylene induced ethylene production by the discs. The stimulatory effect of ethylene resulted, 36 to 48 h after exposure to ethylene, from an increased formation of ACC as well as conversion of ACC to ethylene. In some respects, this effect of ethylene, and a similar observation reported recently for preclimacteric cantaloupe (14) resembles the enhancement by ethylene of the Cellulysin-induced ethylene production reported here. However, unlike the requirement of 36 to 48 h by citrus leaf discs to exhibit increased ACC formation and ethylene production in response to ethylene, the enhanced response of tobacco leaf to ethylene as exhibited by measuring Cellulysin-induced ethylene production occurs within 4 to 8 h. Also, in our studies the faster response of the tissue to ethylene was on the ethylene-forming enzyme and not on ACC synthesis. This delineation was possible since ACC synthesis is induced by Cellulysin within 1 h of incubation (4) and thus is not rate limiting.

Since the ethylene-forming enzyme seems to be membrane associated (20, 29) and ethylene is known to cause changes in membrane permeability (1), it is possible that increased conversion of ACC to ethylene in ethylene-treated tissue may be mediated through a change in the membrane milieu of this enzyme.

Earlier it was suggested (4) that Cellulysin-induced ethylene production may be a hypersensitive response of the tissue normally elicited under conditions of stress or infection. Our preliminary findings (Fig. 7) indicate that the interaction between tobacco leaves and the fungal pathogen *Alternaria alternata* can cause increased ethylene production. An enhanced response of the leaf tissue to a cellulase, to other hydrolytic enzymes (7), or to another component of fungal origin, following exposure to ethylene, may thus create an autocatalytic-like process of ACC induction and ethylene production during fungal growth in the leaf. This may predispose healthy leaf tissue to the advancing fungal mycelium by promoting premature senescence. Alternatively, it may be a process by which the plant tissue maintains its ethylene-induced defense reactions aimed at combating infection. At the present time, we are examining the possibility that induction of ethylene by *A. alternata*, during its growth on tobacco leaf tissue (Fig. 7), may involve the action of a fungal elicitor.

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