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Cell Surfaces in Plant-Microorganism Interactions[1]

III. *IN VIVO* EFFECT OF ETHYLENE ON HYDROXYPROLINE-RICH GLYCOPROTEIN ACCUMULATION IN THE CELL WALL OF DISEASED PLANTS

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ABSTRACT

Ethylene production and cell wall hydroxyproline-rich glycoprotein (HRGP) biosynthesis are greatly enhanced in melon (*Cucumin melo* cv. Cantaloup charentais) seedlings infected with *Colletotrichum lagenarium*. Short-term experiments performed in the presence of specific inhibitors of the ethylene pathway from methionine, namely L-canaline and aminoethoxyvinylglycine, indicate that under non-toxic conditions, both ethylene and [^{14}C]hydroxyproline deposition in the cell wall of infected tissues are significantly lowered. On the contrary, treatment of healthy tissues with 1-aminocyclopropane 1-carboxylic acid, a natural precursor of ethylene, stimulates both the production of the hormone and the incorporation of [^{14}C]hydroxyproline into cell wall proteins.

The data provide the first evidence of the *in vivo* effect of ethylene on the cell wall hydroxyproline-rich glycoprotein biosynthesis in plants.

It has been reported in previous papers (10, 11) that melon seedlings respond to a fungal attack by the accumulation of HRGP[2] in their cell walls. Special attention has been paid to this modification of the cell surface for it involves glycoproteins, a class of components often mediating cell to cell interactions (5). Availability of plants with higher or lower than normal amounts of cell wall hydroxyproline allowed us to ascertain that the enrichment of diseased plants in HRGP is closely associated to their defense against microorganisms (9). Indirect evidence suggests a role for ethylene in the regulation of this mechanism. When supplied exogenously to intact plants, this hormone promotes an enrichment of the cell wall in hydroxyproline (24) corresponding to an enrichment in HRGP (9). A similar hydroxyproline response occurs after wounding of plant tissues (7), a process otherwise well-known to stimulate the production of ethylene (13). Inasmuch as large amounts of this hormone are often released by infected plants (1, 22), the aim of this work was to establish whether ethylene is involved in the regulation of *in vivo* cell wall HRGP biosynthesis in melon seedlings infected by *Colletotrichum lagenarium*. The amounts of ethylene produced by these plants and the incorporation of hydroxyproline into their cell walls measured in the presence of inhibitors or of a precursor of ethylene synthesis, are subsequently reported.

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[2] Abbreviations: HRGP, hydroxyproline-rich glycoprotein; L-Can, L-canaline; BITC, benzylisothiocyanate; AVG, aminoethoxyvinylglycine; ACC, aminocyclopropane carboxylic acid.

MATERIALS AND METHODS

Biological Material. Melon seedlings (*Cucumis melo*, cv. Cantaloup charentais) healthy and inoculated with *Colletotrichum lagenarium*, were obtained as previously described (10). In some experiments, *C. lagenarium* was grown on a liquid medium containing sugars, salts, and supplemented either with ammonium nitrate (8) or L-methionine.

Chemicals. L-Can (Sigma), benzylisothiocyanate (Fluka), and aminoethoxyvinylglycine (Hoffman-La Roche) were used as inhibitors (17, 20, 21) of ethylene synthesis, and 1-aminocyclopropane-1-carboxylic acid (Sigma) as a precursor of ethylene (4). L-Can was prepared from L-Can dipicrate before each experiment by filtration through a column (5 × 1 cm) of Dowex AG 2 × 8, 200-400 mesh, Cl^- form, in water as reported by Murr and Yang (20). The recovered L-Can was measured according to Yemm and Cocking (29) and adjusted to a suitable concentration with H_2O. A saturated solution of BITC (0.92×10^{-3} M) was prepared in H_2O according to Patil and Tang (21), and then diluted to appropriate concentrations with 50 mM phosphate buffer (pH 6.0).

AVG and ACC were each dissolved and adjusted to 1×10^{-3} M in 50 mM phosphate buffer (pH 6.0). Appropriate dilutions were then made with the same buffer.

Ethylene Measurement. The production of ethylene was measured from excised seedlings, petiole of the first leaf, and from *C. lagenarium*, as follows. Four excised seedlings (without roots) were enclosed for 24 h in 570-ml serum flasks stopped with serum caps, and containing 30 ml of the usual growth medium (28). Newly excised seedlings were used everyday. The petioles were cut into 5-mm segments as previously indicated (9), and divided into lots of 2 g. The segments of each lot were incubated for 4 h under the light in 13 ml serum vials, stopped with serum vaccine caps, containing 6 ml of a medium consisting of 2% sucrose in 50 mM K-phosphate buffer (pH 6.0). A CO_2 trap made of filter paper soaked with 200 μl of 1 N KOH was inserted into each vial.

Measurements of ethylene from *C. lagenarium* were performed on a 6-d-old culture in 1-L flasks containing 85 ml of the culture. They were sealed with two layers of a polyethylene film for 24 h prior to gas sampling. Corrections were made due to 30% losses of ethylene through this film under these conditions.

Ethylene was assayed in 1 ml of the internal atmosphere above the seedlings, the incubated petioles, and the fungus. The samples were withdrawn with a gas-tight syringe through the serum caps or films and immediately injected onto an alumina column (150 × 0.32 cm) of a gas chromatograph equipped with a flame ionization detector. The column was operated at 35°C, with nitrogen as the carrier gas. Under these conditions, ethylene was readily detected at concentrations of 50 nl/l in a 1-ml sample, with a retention time of 55 s.

All experiments were repeated at least three times. The results

presented were calculated relative to standards, and correspond to a typical experiment.

Ethylene and Cell Wall HRGP Synthesis in the Presence of Inhibitors and of a Precursor of Ethylene Synthesis. Petioles from healthy and infected seedlings of the same age were divided into lots of 2.0 g, surface sterilized in 4% (w/v) calcium hypochlorite for 15 min and rinsed with sterile H_2O. They were then cut into 5-mm segments and incubated in 13-ml vials containing 5 ml 50 mM phosphate buffer (pH 6.0) either made up to 10^{-6} to 10^{-3} M in L-Can, 0.0191 to 0.153 × 10^{-3} M in BITC, 10^{-8} to 10^{-3} M in AVG, or 10^{-6} and 10^{-5} M in ACC. [^{14}C]Proline (2 μCi, C.E.A. 119 on 220 mCi/mmol) was added to the vials either at the beginning of the incubation time in the case of L-Can, BITC, and ACC, or 1 h after, as in the case of AVG, and then stoppered with serum caps. Incubation in the presence of inhibitors and [^{14}C]proline lasted for 4 h, except in experiments with AVG where [^{14}C]proline was present for 5 h. At the end of the incubation time, the amount of ethylene produced in each vial was assessed by withdrawing 1 ml of the internal atmosphere above the petiole segments. The segments were then rinsed and their cell walls isolated and hydrolyzed as already described (9). From the hydrolysates, proline and hydroxyproline were then separated either by paper chromatography (9) in experiments involving L-Can and BITC, or by ion exchange chromatography according to Pope (23) in the experiments with AVG and ACC. In the two later cases, the hydrolysate corresponding to the cell wall of 1 g of petioles was applied to a column (0.8 × 12 cm) of Dowex 50 × 8, 100–200 mesh, H^+ form, which was eluted with 0.35 N HCl. Proline and hydroxyproline were separately eluted into liquid scintillation vials with simultaneous evaporation to dryness by a stream of air. The residues in the vials were then taken up in 1 ml of H_2O to which 10 ml of a scintillation liquid (Unisolve, Koch-Light, Colnbrook, U. K.) were added.

The radioactivity of proline and hydroxyproline was measured by liquid scintillation counting of either the corresponding chromatogram strips (9) or the column eluates. All data are expressed as a percentage of the absorbed radioactivity which was measured on an aliquot of the incubation medium. All experiments using radiolabeled proline were done two or three times independently. Results of typical experiments are reported.

Protein Synthesis in the Presence of Inhibitors of Ethylene Biosynthesis. Incorporation of [^{14}C]leucine into the total protein fraction was followed in the presence of L-Can and BITC. The experimental protocol was the same as above except that 0.25 μCi [1-^{14}C]leucine (Amersham, 56 mCi/mmol) was used instead of proline. After an incubation of 4 h, the petiole segments were carefully rinsed, frozen in liquid N_2 and ground in a mortar. The powder obtained from 2 g petioles was suspended in 30 ml of 0.2% unlabeled leucine solution in 10% TCA. The proteins of the homogenate were allowed to precipitate overnight at 0 to 4°C and were collected by centrifugation at 27,000*g* for 10 min. The pellet was successively washed and sedimented in 10 ml of 10% TCA and then in 5 ml of 96% ethanol. The amount of incorporated radioactivity was measured by liquid scintillation counting, after combustion of the pellet, with a Packard Tri-Carb Oxydizer.

RESULTS

Ethylene Production. Ethylene was measured every 24 h after inoculation, on plant material that had been enclosed in vessels 24 h only before gas sampling. The production of ethylene by excised seedlings (Fig. 1) and petioles (Fig. 2) was very low in healthy controls and highly enhanced upon infection by *C. lagenarium*. In both assays, this increase occurred about 2 d after inoculation, with a maximum production 4 d after inoculation in the case of seedlings and 3 d in the case of petioles. Ethylene was found only in trace amounts in *C. lagenarium* cultures when the fungus was grown on a glucose-ammonium nitrate medium. At

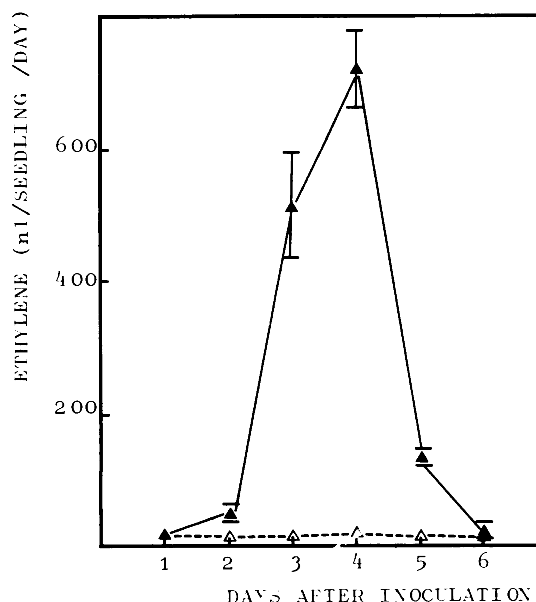

FIG. 1. Production of ethylene by seedlings inoculated with *C. lagenarium* (▲) and by healthy controls (△). Newly excised seedlings were enclosed in vessels everyday. Twenty-four h later, ethylene was measured by GC on 1 ml of the internal atmosphere of the vessels. Error bars represent individual values for two replicates.

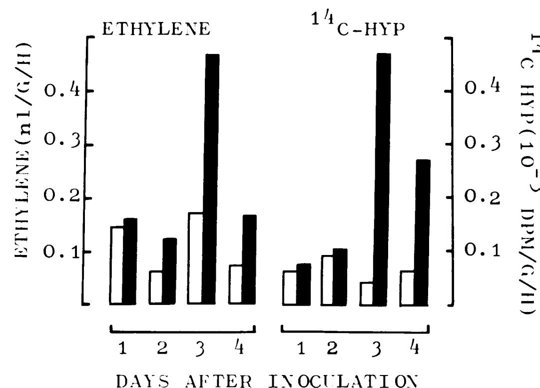

FIG. 2. Production of ethylene and [^{14}C]hydroxyproline (Hyp) deposition in the cell wall of petioles excised from infected (■) melon seedlings, from day 1 to 4 after inoculation, and in healthy controls (□).

high concentrations of L-methionine (30 mM), the mycelium of a 6-d-old culture was able to produce 0.013 nl of ethylene/mg dry weight of mycelium. Whether or not this production might contribute to the amount of ethylene produced by infected seedlings is difficult to assess from this assay because infected seedlings contain much less methionine than in the assay (28) and only 2.7 mg of mycelium per seedling (27).

The relatively smaller amounts of ethylene produced in the petiole assay, as compared to excised seedlings, might reflect a more limited diffusion from petioles than from leaves as well as a reduced access to O_2 under immersed conditions. However, because of the ability of stems and petioles to accumulate large amounts of HRGP in infected plants, the suitability of this assay to measure both the synthesis of HRGP and of ethylene was investigated.

HRGP Synthesis in the Petiole Assay. The incorporation of [^{14}C]hydroxyproline into cell wall proteins was first followed in infected petioles together with the incorporation of [^{14}C]proline, since peptidyl hydroxyproline originates from peptidyl proline. It was found that, over an incubation time of at least 8 h (Fig. 3),

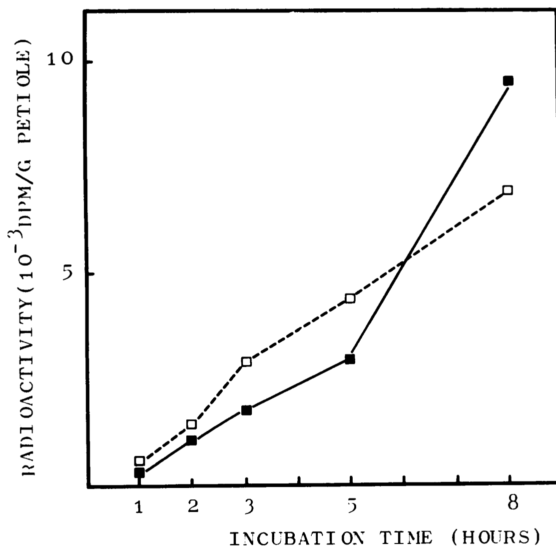

FIG. 3. Deposition of [^{14}C]proline (□) and [^{14}C]hydroxyproline (■) in the cell wall of infected petioles as a function of time of incubation in the presence of [^{14}C]proline. The two amino acids were separated by paper chromatography of a cell wall hydrolysate as described.

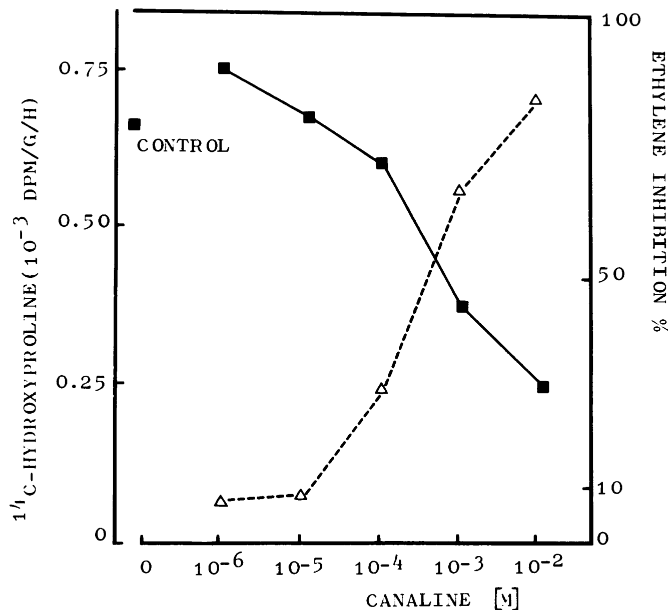

FIG. 4. Percent inhibition of ethylene production (△) and [^{14}C]hydroxyproline deposition (■) in the cell wall of infected petioles incubated in the presence of increasing amounts of L-Can. [^{14}C]Hydroxyproline was measured as indicated in Figure 3.

both amino acids were incorporated about linearly into the cell wall, thus indicating that the synthesis of cell wall HRGP was accurately measured under the conditions of the assay. These conditions were applied to the simultaneous determination of ethylene and of HRGP synthesis, in petioles, with time after inoculation (Fig. 2). It was shown that the increased amount of ethylene produced was paralleled by an increased HRGP deposition into the cell wall. The rate of hydroxyproline accumulation, as measured by radiolabeled techniques, was in good agreement with results already obtained by using colorimetric techniques (19). In the presence of 0.1 mM cycloheximide, the amounts of ethylene and HRGP were lowered by 50 and 90%, respectively. These levels were naturally very low in control, healthy petioles. From these analyses, it was concluded that the conditions of the petiole assay were satisfactory to investigate the effect of the *in vivo* produced ethylene on the synthesis of cell wall HRGP. This assay was employed routinely in all other experiments.

Cell Wall HRGP Synthesis as Related to Ethylene Synthesis. Three inhibitors of ethylene biosynthesis (L-Can, BITC, and the rhizobitoxine analog AVG), and a precursor of ethylene in higher plants (ACC), were used in an attempt to assess the regulatory role of endogenous ethylene in the synthesis of HRGP. Each inhibitor was used at concentrations ranging within values found in the literature. The results of typical experiments with L-Can and BITC are given in Figures 4 and 5. The synthesis of ethylene in infected petioles was severely affected by both inhibitors, which is in agreement with data reported by several authors (20, 21). The deposition of [^{14}C]hydroxyproline and [^{14}C]proline was simultaneously and similarly depressed. Thus, at 10^{-3} and 10^{-2} M L-Can, ethylene was inhibited by 66 and 84%, hydroxyproline by 40 and 61%, and proline by 31 and 54%. The effects of BITC were still more drastic since the levels of ethylene, hydroxyproline, and proline were depressed by 93, 94, and 93% respectively in the presence of 0.153×10^{-3} M BITC in the medium.

These data were interpreted taking into account the effects of the same inhibitors on total protein synthesis (Table I). At concentrations ranging from 10^{-6} to 10^{-3} M, where L-Can efficiently inhibited the incorporation of [^{14}C]hydroxyproline into the cell wall, no noticeable effect on protein synthesis was observed. However, this did not hold true at higher concentrations (10^{-2} M) or in the case of BITC which displayed strong inhibitory effects, whatever the concentrations used.

In search of a milder way of modulating the ethylene pathway, experiments were designed in which the incubating medium was supplemented either in AVG, the rhizobitoxine ethylene inhibitor analog (17) or in ACC, an *in vivo* precursor of ethylene (4). The levels of ethylene, proline, and hydroxyproline were increasingly depressed in the presence of increasing concentrations of AVG in the medium: 10^{-8} to 10^{-5} M. Results of typical experiments indicated that ethylene was inhibited by 63% when AVG was used at the concentration of 10^{-5} M (Fig. 6); hydroxyproline and proline deposition in the cell wall were simultaneously inhibited by 21 and 22%, respectively. It has been demonstrated by Mattoo *et al.* (18) that protein synthesis is not affected when AVG is used at concentrations ranging up to 10^{-4} M. Although sharing common structural features with canaline, AVG was more efficient at low concentrations since both ethylene and hydroxyproline were only slightly affected upon incubation with 10^{-5} M L-Can.

The use of ACC provides another convenient means of modifying the internal level of ethylene. From Table II, it can be seen that this compound stimulated both the synthesis of ethylene and of cell wall HRGP in control, healthy petioles. Again the two parameters were closely related even though ACC did not affect their levels to the same extent, a fact already found with externally supplied ethylene (9). It is thought that the 0.5-fold increase in hydroxyproline which occurred within a 24 h in these conditions is significant when compared to the 10-fold accumulation of hydroxyproline which is found in infected seedlings 7 d after inoculation. Altogether these data are in favor of a regulatory role for ethylene in the biosynthesis of cell wall HRGP in plants.

DISCUSSION

It has been known for a long time that plants may release large amounts of ethylene on wounding (1), artificial (7) or natural aging like ripening (12), senescence (26), treatment with noxious chemicals (2), and infection with microorganisms (22). In this respect, the increased production of this hormone by melon seedlings infected with *Colletotrichum lagenarium* gives a further example of stress-induced ethylene production. In a number of these situations, the synthesis and/or activity of some proteins and enzymes such as phenylalanine ammonia lyase (14), peroxidases

Table I. *Effect of L-Canaline and of BITC on the Incorporation of [^{14}C]Leucine into the Total Protein Fraction*

Radioactivity	L-Can (M)						BITC (M × 10^{-6})				
	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	0	19.1	38.3	76.5	153
% Radioactivity uptake[a]	65	65	68	69	64	60	82	71	67	66	67
% Radioactivity incorporated[b]	6.5	7.7	6.4	7.8	6.8	3.5	9.7	4.9	4.4	2.0	0.4

[a] Radioactivity expressed in dpm/g incubated petioles as a percentage of the total radioactivity (4.5 × 10^6 dpm) added to the incubation medium.

[b] Radioactivity expressed in dpm/g incubated petioles as a percentage of the radioactivity uptake. In the same conditions, in the presence of canaline, for instance, incorporation of proline in the cell wall ranged from 0.34 to 0.39, 0.35, 0.31, 0.26, 0.18%; and incorporation of hydroxyproline ranged from 0.14 to 0.15, 0.13, 0.13, 0.09, 0.06% at the various concentrations of the inhibitor.

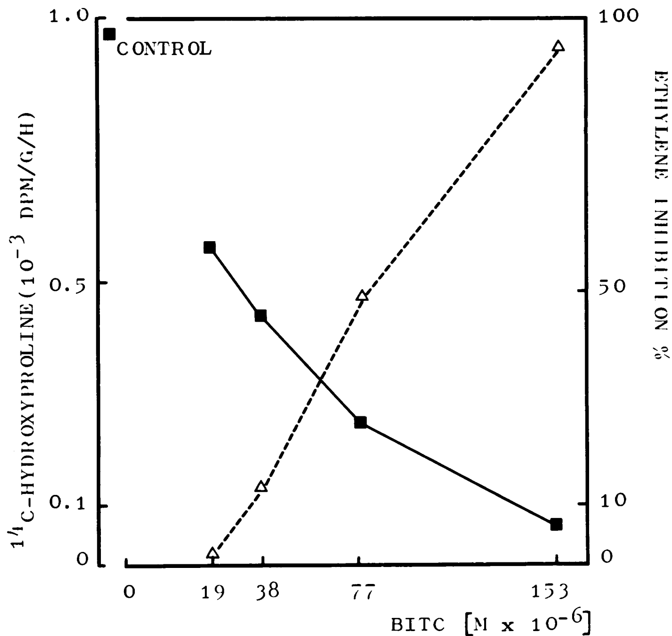

FIG. 5. Percent inhibition of ethylene production (△) and [^{14}C]hydroxyproline deposition (■) in the cell wall of infected petioles in the presence of increasing amounts of BITC. [^{14}C]Hydroxyproline was measured as indicated in Figure 3.

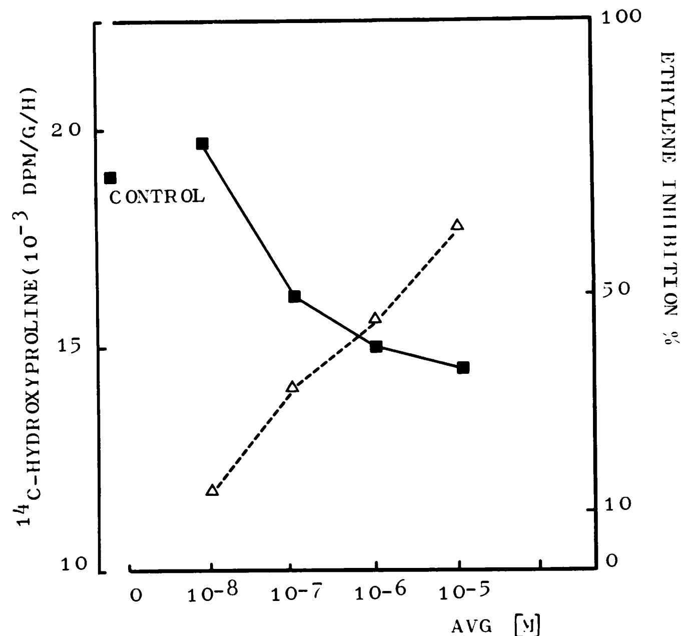

FIG. 6. Percent inhibition of ethylene production (△) and [^{14}C]hydroxyproline deposition (■) in the cell wall of infected petioles incubated in the presence of increasing amounts of AVG. [^{14}C]Hydroxyproline was measured after ion exchange chromatography as described under "Materials and Methods."

(6), β-1,3-glucanase and chitinase (3), and ribonuclease (25) are enhanced. Indirect evidence suggests that ethylene plays a major role in regulating the levels of these proteins since exogenously added ethylene stimulates their synthesis and/or activity. This also happens in the case of HRGP which accumulates in the cell wall during infection (11, 19) and upon treatment of seedlings with ethylene (9, 24). In none of these instances, however, has the role of *in vivo*-produced ethylene been investigated.

Recent progress in the elucidation of the pathway of ethylene biosynthesis, together with the availability of inhibitors of this pathway (17) provide a means of investigating the regulatory role of endogenous ethylene. As indicated below, ethylene is generally synthesized from methionine, which is the most common precursor of this hormone in plants (16), through a series of steps involving *S*-adenosylmethionine (SAM) and ACC (4): methionine → SAM → ACC → ethylene (4, 15). Several inhibitors acting at different points in this pathway have been described, notably rhizobitoxine and its analogs (17), L-canaline (20), and benzylisothiocyanate (21). Because of this knowledge, experiments were designed with the purpose of elucidating the role of endogenous ethylene in the accumulation of HRGP, into the cell wall, in diseased plants. The results indicated that, even at low concentrations, L-canaline and the ethoxy analog of rhizobitoxine, AVG, efficiently inhibit the production of ethylene and the synthesis of cell wall HRGP.

Table II. *Effect of ACC on the production of Ethylene and on the Deposition of HRGP in the Cell Wall of Control, Healthy Petioles*

[^{14}C]Hydroxyproline was measured as in Figure 6.

ACC	Ethylene	[^{14}C]Hydroxyproline
M	*nl/g·24 h*	*dpm/g·24 h*
0	0.95	109,084
10^{-6}	1.18	137,266
10^{-5}	12.20	161,604

However, in doing such experiments, one should be very careful to avoid possible effects of the inhibitors on total protein synthesis which would result in an effect on the synthesis of the ethylene-protein forming system itself as well as on other proteins. Thus, protein synthesis was depressed by high amounts of L-Can and by BITC.

In our experiments, the effect of ethylene on the biosynthesis of HRGP was fully confirmed by feeding plants with ACC, the natural precursor of the hormone. In these conditions, both ethylene and [^{14}C]hydroxyproline incorporation into the cell wall were stimulated. Because experiments lasted for a relatively short time (4 or 5 h), it is believed that ethylene is not a remote signal, but a signal which directly affects some step controlling the

biosynthesis of HRGP.

Besides giving the first evidence of the *in vivo* effect of ethylene on the hydroxyproline glycoprotein synthesis, this paper provides another model system for studying the biochemistry of ethylene-mediated responses in diseased plants.

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