Plant Physiol. (1980) 65, 322-326
0032-0889/80/65/0322/05/$00.50/0

Xylem Transport of 1-Aminocyclopropane-1-carboxylic Acid, an Ethylene Precursor, in Waterlogged Tomato Plants[1]

Received for publication June 8, 1979 and in revised form September 25, 1979

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ABSTRACT

Waterlogging is known to cause an increase in ethylene synthesis in the shoot which results in petiole epinasty. Evidence has suggested that a signal is synthesized in the anaerobic roots and transported to the shoot where it stimulates ethylene synthesis. Experimental data are presented showing that 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, serves as the signal. Xylem sap was collected from detopped tomato plants (*Lycopersicon esculentum* Mill. cv. VFN8). ACC in the sap was quantitated by a sensitive and specific assay, and its tentative chemical identity verified by paper chromatography. ACC levels in both roots and xylem sap increased markedly in response to waterlogging or root anaerobiosis. The appearance of ACC in the xylem sap of flooded plants preceded both the increase in ethylene production and epinastic growth, which were closely correlated. Plants flooded and then drained showed a rapid, simultaneous drop in ACC flux and ethylene synthesis rate. ACC supplied through the cut stem of tomato shoots at concentrations comparable to those found in xylem sap caused epinasty and increased ethylene production. These data indicate that ACC is synthesized in the anaerobic root and transported to the shoot where it is readily converted to ethylene.

A characteristic response of tomato to waterlogging is epinasty of the petioles (10). Recent studies have demonstrated that epinastic growth of the petioles is a response to accelerated rates of ethylene synthesis (2, 8). Anaerobic conditions in the root zone are sufficient to cause elevated ethylene synthesis and epinasty in the shoot regardless of whether the anaerobiosis is imposed by waterlogging (8, 11), or by flushing with N_2 (2, 8, 9, 14). Indirect evidence suggested that a signal from the anaerobic roots is transported to the shoot where it stimulates ethylene synthesis (2, 8, 9). Using plants with a divided root system, Jackson and Campbell (8) demonstrated that the transport of such a signal was probably through the xylem.

A burst of ethylene is often observed when plant tissues are transferred from anaerobic to aerobic conditions (4). Such a surge of ethylene evolution following an anaerobic incubation has been observed in excised tomato roots (9). Recently, Adams and Yang (1) have identified ACC[2] as an immediate precursor of ethylene in apple tissue. ACC accumulates under anaerobic conditions and is rapidly converted to ethylene in aerobic tissues.

The present work was undertaken to test whether ACC is the signal which is synthesized in anaerobic roots and transported in the xylem to the shoot, where it would be rapidly converted to ethylene.

MATERIALS AND METHODS

Plant Material. Tomato plants (*Lycopersicon esculentum* Mill., cv. VFN8) were grown for 5 weeks in soil in a growth chamber. The environmental conditions were: light, 500 μE m^{-2} s^{-1} PAR; temperature, 25 C; photoperiod, 16 h; RH, 39%. Plants were at the five- to seven-leaf stage when used for experiments. Other plants were grown under similar conditions in aerated nutrient solution. Plants for ACC feeding experiments were grown in soil in a greenhouse under natural lighting (25 C minimum).

Waterlogging Treatments. Plants grown in 10-cm pots were placed inside 13-cm pots and the larger pots were filled with distilled H_2O. The plants were sumberged to the cotyledonary node. O_2 was not measured, but it is well established that O_2 levels fall rapidly in waterlogged soils (8). With solution culture plants, anaerobic stress was imposed by switching the aerating gas from air to N_2. Measurements with an O_2 electrode indicated that anaerobic equilibrium was established within 20 min.

Ethylene Measurements. Ethylene production by excised petioles was estimated by the procedure of Jackson and Campbell (7). Proximal sections (5 cm) of the second, third, and fourth oldest petioles were enclosed in a 4.1-ml test tube, flushed with ethylene-free air, and capped with a serum stopper. After 30 min, a 0.5-ml gas sample was taken with a gas-tight syringe. The ethylene content was determined by gas chromatography with a flame ionization detector. All data are corrected for any ethylene present in blank tubes and are expressed on a fresh weight basis. In some cases the tubes were flushed and resampled at hourly intervals after the initial 30-min period.

Epinasty Measurement. Epinasty was defined as the change in the angle between the petiole and the stem as measured with a transparent protractor. In flooding experiments, epinasty refers to the difference between the petiole angles of the third oldest leaf of treated and control plants. In ACC feeding experiments, epinasty is the change in the angle of a given petiole from zero time.

Collection of Xylem Sap. Plants were detopped just below the cotyledonary node and latex tubing was attached to the stump. The tubing was connected through a rubber stopper to a 30-ml test tube. A second tube through the stopper was connected to a vacuum line. The vacuum (50 mm Hg) was applied continuously for the 3-h collection period. The vacuum technique was employed as exudation due to root pressure often ceases in flooded plants (Bradford, unpublished observations). For uniformity, sap from both treated and control plants was collected under vacuum. The collection tubes contained 1 ml of 0.3 N HCl to stabilize the ACC and were stored at −15 C. Since the exudation rate varied with the treatment, the ACC data are expressed as nmol h^{-1}, which is calculated as the concentration in the exudate times the exudation rate. The values should therefore represent the rate of ACC

[1] This work was supported in part by National Science Foundation Grant PCM 78-09278.

[2] Abbreviation: ACC: 1-aminocyclopropane-1-carboxylic acid.

synthesis in the root and export through the stem.

ACC Assay. ACC was assayed by the method of Lizada and Yang (16), which is based on the liberation of ethylene from ACC with NaOCl in the presence of $HgCl_2$. Ethylene released was determined by gas chromatography. The acidified xylem sap was assayed directly without purification. For the assay of ACC in the root, the extract was first fractionated by ion exchange resin as described below. The efficiency of the conversion of ACC to ethylene was determined by adding 1 nmol of authentic ACC as an internal standard to another sample solution, which was then degraded to yield ethylene with the NaOCl reagent (16). The yield ranged from 25 to 65% in various samples. The amount of ACC in the sample was calculated from the quotient of ethylene released/efficiency of the conversion of ACC to ethylene. This assay method has been shown to be highly specific for ACC with virtually no interference by other compounds existing in xylem sap (Fig. 1) or other tissue extracts (16).

Extraction and Chromatography. Roots grown in solution culture were homogenized in 5% 5-sulfosalicylic acid (3 ml/g tissue) and centrifuged at 30,000*g* for 10 min. An aliquot of the supernatant was passed through an ion exchange resin (Dowex 50-X8, H^+ form) column, washed with water, and eluted with 2 N NH_4OH (16). The eluate was evaporated to dryness and dissolved in 2 ml of H_2O for ACC assay and paper chromatography.

Samples of root extract and xylem sap were spotted on Whatman 3MM chromatography paper and developed in a descending manner with 1-butanol-acetic acid-water (4:1:1, v/v). The chromatograms were cut into sections which were eluted three times each with 0.5 ml H_2O. The eluates were assayed for ACC by the standard method. Authentic ACC was located by ninhydrin spray or by elution from the chromatogram followed by the assay as performed for the unknown samples.

ACC Feeding. Shoots were excised just below the cotyledons and immediately recut under water above the cotyledonary node. The lower leaves were removed, leaving three young leaves per cutting. Each cutting was inserted through a Parafilm cover into a 125-ml Erlenmeyer flask containing the appropriate concentration of ACC. Petiole angles of the second oldest remaining leaves and total weight per flask were determined initially and after the 6-h uptake period. Experiments were performed on a laboratory bench with diffuse fluorescent light at 25 C. ACC was purchased from Calbiochem.

Statistical Analysis. The data were analyzed by the analysis of variance procedure. When applicable, single degree of freedom comparisons and linear regressions were employed. Since the ACC levels in xylem sap from control plants were generally below detection, error bars indicating ± 1 SE are included with ACC curves for flooded plants to give an estimate of the experimental error.

RESULTS

Inasmuch as many organic compounds, including amino acids, are known to be present in xylem sap (18), it is important to establish whether there are any other compounds which may release ethylene upon reaction with NaOCl reagent (16). Paper chromatography of xylem sap from flooded plants indicates that only the region corresponding to ACC released significant ethylene in the assay method (Fig. 1). As expected for ACC, the ethylene-releasing compound in the xylem sap was adsorbed on cation ion exchange resin, and following elution and paper chromatography it had an R_f identical to authentic ACC (Fig. 1). These results, coupled with the observation that the release of ethylene with NaOCl reagent is highly specific for ACC (16), indicate that the ethylene-releasing compound is indeed ACC. Only trace amounts of ACC were detected in control sap. Roots treated anaerobically for 12 h in solution culture showed an 11-fold increase in ACC content. ACC content after 12 h treatment under air or N_2 was 0.09 ± 0.03 and 1.02 ± 0.32 nmol/g fresh weight, respectively. The ethylene-releasing compound in the root extract also had an R_f identical to that of authentic ACC (Fig. 1). These results indicate that ACC is present at elevated levels in both anaerobic roots and xylem sap, and that other compounds present in the extracts do not interfere with the assay.

Within 12 h after flooding, ACC flux in the xylem had already increased dramatically, while epinasty was still not detectable (Fig. 2). At subsequent times, changes in ACC flux preceded similar changes in epinasty by about 12 h. ACC in xylem sap of control plants was barely detectable if present at all. The relationship between ACC flux and epinasty is shown clearly in Figure 3, where epinasty at a given time is plotted as a function of the ACC flux measured 12 h previously. The degree of epinastic growth was highly correlated with the ACC flux.

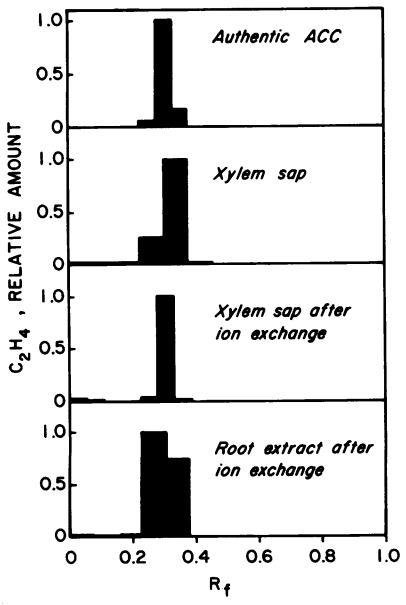

FIG. 1. Histograms of ethylene released from fractions obtained by paper chromatography of xylem sap and root extract. Ordinate represents relative amount of ethylene released from each fraction following ACC assay.

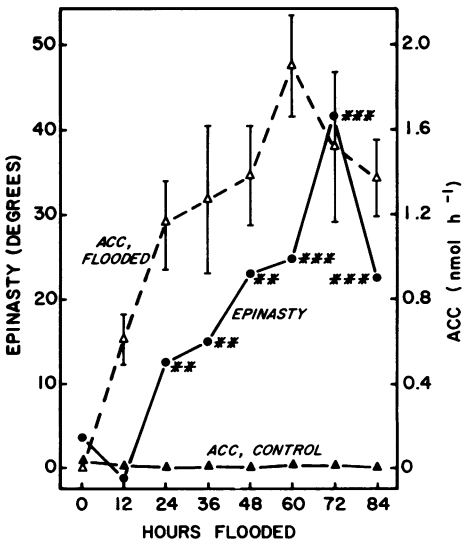

FIG. 2. Time course of appearance of ACC in xylem sap and development of epinasty following flooding. Epinasty is expressed as the difference between petiole angles of treated and control plants. Statistical significance of difference between treatment and control: **, $P < 0.01$; ***, $P < 0.001$.

The immediate cause of epinasty is increased ethylene levels in the petiole. ACC flux in relation to ethylene production rate was therefore examined. An increase in ACC export from the root preceded the increase in ethylene synthesis caused by waterlogging (Fig. 4). The decline in ACC flux after about 60 h was observed in several experiments (*e.g.* Fig. 2). Development of epinasty followed a time course virtually identical to that of ethylene synthesis (data not shown). The close correlation between the rate of ethylene synthesis and epinasty has been well documented (9).

To determine more precisely the time course of ACC accumulation during anaerobiosis, detached root systems in solution culture were flushed with either air or N_2. Plants (two per treatment) were detopped at zero time and fractions of xylem sap were collected for 2-h intervals up to 16 h. There was a linear increase in the ACC flux between 2 and 12 h which leveled off after about 14 h (Fig. 5). Root resistance of anaerobic roots increased rapidly for the first 4 h, then slowly decreased until it was less than that for control roots (Fig. 5). A similar pattern of a rise and fall in root resistance was observed in plants flooded in soil, but with a much slower time course. Large increases in exudation rates (decreases in root resistance) were consistently observed after 36–48 h of flooding. Similar effects of anaerobiosis on root resistance have been reported previously (17).

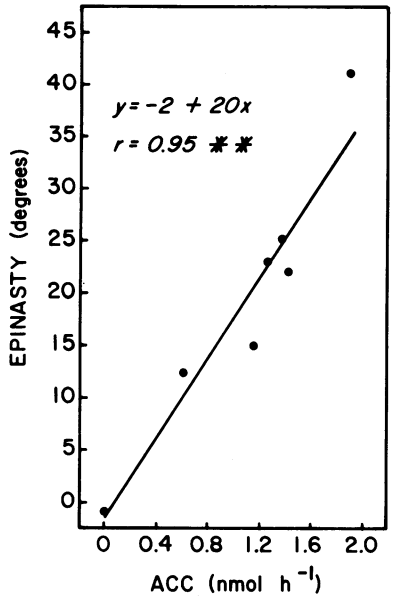

FIG. 3. Correlation between ACC flux and epinasty. Epinasty is plotted as a function of ACC flux measured 12 h prior to epinasty measurement. Each point represents the mean of four plants each for epinasty and ACC flux. Data from Figure 2.

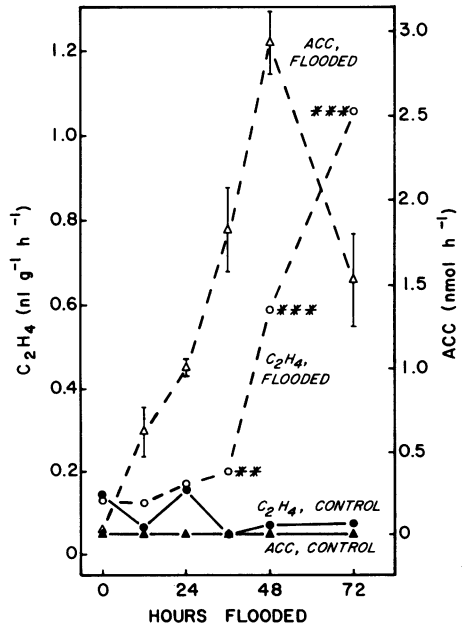

FIG. 4. Time course of appearance of ACC in xylem sap and changes in petiolar ethylene production following flooding. Statistical symbols as in Figure 2.

Since the conversion of ACC to ethylene requires O_2 (1), draining the flooded pots should readmit O_2 to the root zone and allow metabolism of ACC in the root. If accelerated shoot ethylene synthesis is due to ACC from the anaerobic root, both ACC flux and ethylene synthesis should decline rapidly after draining the pots. When flooded plants were drained after 30 h of flooding, the ACC flux decreased more than 7-fold during the next 6 h, and fell to zero on subsequent days (Fig. 6). Ethylene synthesis rates also declined rapidly to the control level, in marked contrast to the continuously flooded situation (Fig. 4).

The results presented strongly support the hypothesis that ACC is the signal from anaerobic roots which causes increased ethylene synthesis in the shoot. If ACC is the signal, supplying ACC alone to the tomato shoot should reproduce the flooding symptoms. Concentrations of ACC exceeding 3 μM were found in flooded xylem exudate. When ACC at comparable concentrations was fed to tomato cuttings through the transpiration stream, both epinasty and increased ethylene production were observed (Table I). The measurements were taken at a time when epinastic growth was rapidly occurring. Epinasty may be a more sensitive indicator of internal ethylene levels than is the method used for ethylene determination, as epinasty was detected at a lower ACC concentration than was the stimulation in ethylene production. Only a small increase in ethylene production rate is sufficient to give a maximal epinastic response.

High ethylene production rates by petioles require a continuous supply of ACC. When petioles were excised from shoots supplied with ACC through the transpiration stream, the ethylene production rates fell rapidly to the control level (Fig. 7). A similar rapid decline was observed when flooded plants were drained (Fig. 6).

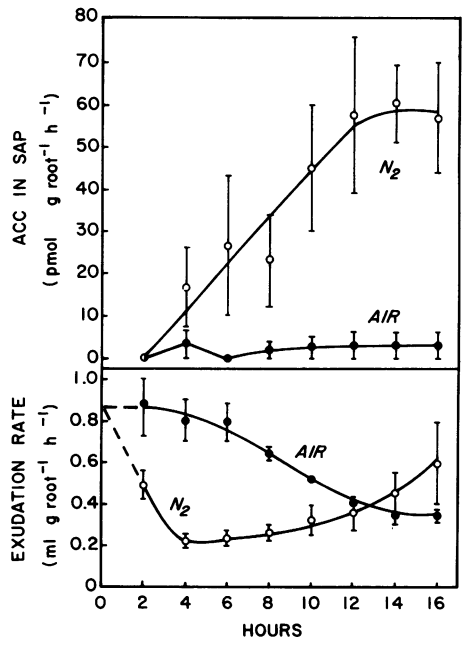

FIG. 5. Export of ACC (top) and flux of xylem sap (bottom) from roots in solution culture bubbled with air or N_2. Linear regression coefficient for N_2 treatment is 0.96 ($P < 0.001$). Average root weight: air, 13.9 g; N_2, 10.8 g. Error bars indicate ± 1 SE.

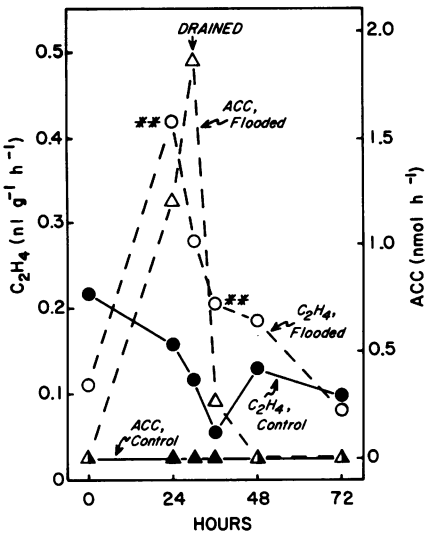

FIG. 6. Effect of flooding and draining on ACC flux and ethylene production. Water was drained 30 h after flooding as indicated. Error bars are not shown on ACC curve for clarity, but standard errors are similar to those in Figure 4. Statistical symbols as in Figure 2.

Table I. *Effects of ACC on Ethylene Production and Epinasty of Tomato Shoots*

Shoot cuttings took up ACC solutions through the transpiration stream for 6 h. ACC flux was calculated from the product of transpiration rate and ACC concentration. Epinasty indicates the increase in petiole angle from zero time. Ethylene production is the rate during the first 30 min following excision of the petioles.

ACC	ACC Flux	Epinasty	C_2H_4 Production
μM	*nmol* h^{-1}	*degrees*	*nl g fresh weight*$^{-1}$ h^{-1}
0	0	8	0.37
1	2.0	12*	0.33
10	19	22***	0.99***
100	190	22***	11.9***

* Difference from control significant at $P < 0.05$; *** difference from control significant at $P < 0.001$.

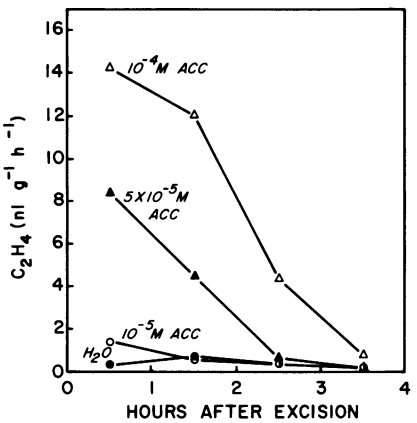

FIG. 7. Ethylene production from petioles excised from shoots which had been supplied with ACC for 6 h through transpiration stream. Linear trend for increase in ethylene production with ACC level for 0.5 h data significant at $P < 0.001$.

This is in contrast to the effect of IAA, where ethylene production from excised petioles remained fairly constant over the 3.5-h measurement period (3). The pattern shown in Figure 7 is indicative of the substrate role of ACC as compared to the presumed enzyme-inducing effect of IAA (19, 20).

DISCUSSION

It is now well established that waterlogging causes a rise in internal ethylene levels which in turn stimulates epinastic growth of the petioles (2, 8, 11). Various sites have been suggested as the source of the excess ethylene. Following the discovery of ethylene in anaerobic soils, Jackson and Campbell (6) demonstrated that ethylene could enter the plant from the root zone and cause epinasty in the shoot. Kawase (12, 13), on the other hand, proposed that water prevents the escape of ethylene from the root. The resulting accumulation of ethylene would cause the gas to diffuse internally from the root to the shoot. However, subsequent investigations showed that neither ethylene in the root zone nor a water barrier was required for the elevated ethylene production and epinasty in the shoot (2). Rather, accelerated ethylene production in the shoot is a result of O_2 deprivation of the root (2, 8, 9). An ethylene-promoting signal moving from the anaerobic root through the xylem to the shoot was proposed by Jackson and Campbell (8). Transferring excised tomato roots from an anaerobic to an aerobic environment caused a burst of ethylene production, suggesting that a precursor was accumulating during anaerobiosis (9).

Adams and Yang (1) have recently elucidated the metabolic role of ACC in ethylene biosynthesis based on the observation that ACC accumulated under anaerobiosis. They have demonstrated the following pathway for ethylene biosynthesis in apple tissue: methionine → *S*-adenosylmethionine → ACC → ethylene. O_2 is required for the conversion of ACC to ethylene. ACC caused a marked increase in ethylene synthesis in a wide variety of plant tissues and organs (5). These observations implicate ACC as a likely candidate to be the ethylene-promoting signal from anaerobic roots.

Data presented in this paper have shown that: (*a*) ACC levels in the roots increase during anaerobiosis; (*b*) ACC is exported from anaerobic roots in the xylem sap (Figs. 1, 2, 4–6); (*c*) ACC appears in the xylem sap prior to increases in ethylene production and epinasty (Figs. 2–4); (*d*) to support an ethylene production rate of 1 nl g^{-1} h^{-1}, which causes a maximal epinastic response (Table I), requires an ACC flux of 0.6 nmol h^{-1} for a 15-g shoot based on a mole-to-mole conversion; thus, observed ACC fluxes of up to 3 nmol h^{-1} should be sufficient to sustain the elevated rates of ethylene synthesis; (*e*) draining flooded plants results in a rapid, simultaneous decrease in ACC flux and ethylene production (Fig. 6); (*f*) ACC fed through the transpiration stream causes epinasty and increased ethylene synthesis (Table I); and (*g*) petioles are dependent upon an ACC flux for continued high rates of ethylene synthesis (Fig. 7). These results strongly support the view that ACC from the anaerobic roots is responsible for elevated ethylene levels in the shoot.

There are two possibilities to account for the accumulation of ACC in anaerobic roots. First, lack of O_2 prevents conversion of ACC to ethylene, so that the ethylene normally synthesized in the root would instead accumulate as ACC. In addition, anaerobiosis may actually stimulate the ethylene biosynthetic pathway, again resulting in ACC accumulation when O_2 is limiting. The normal rates of root ethylene synthesis are insufficient to account for the increase in shoot ethylene evolution (2). However, as reported here, ACC export from anaerobic roots is adequate to sustain the ethylene synthesis rate of the shoot. Thus, anaerobic stress not only blocks conversion of ACC to ethylene, but also accelerates ACC synthesis. This is suggested by Figures 2 and 4, where ACC export from the root increased for up to 60 h before declining. Furthermore, the observations of Kawase (14) are consistent with the notion of an anaerobic stimulation of ACC synthesis in sunflower stems. The mechanism of regulation of ACC synthesis in anaerobic tissues remains to be elucidated.

The classical definition of a hormone is an endogenous compound which is synthesized at one site and is transported to another site where it exerts a physiological effect. Due to its

gaseous nature, ethylene exerts a physiological effect at a site where it is synthesized. Thus, this classical definition does not apply to ethylene. The present work has shown that the ethylene precursor, ACC, is synthesized in the anaerobic root, transported, and exerts its effect through conversion to ethylene in the aerobic shoot.

Auxin, gibberellins, cytokinins, and ABA have all been found in xylem sap (15). However, King (15) has stated that "although there is much suggestive evidence it is not yet possible to conclude that vascular transport of a known plant growth substance is indisputably involved in the control of plant growth." The results reported herein clearly show that ACC is a root-synthesized compound and is transported in the xylem to the shoot, where it regulates growth responses through conversion to ethylene. All five classes of plant hormones can now be said to be transported in the xylem.

Acknowledgments—We thank D. O. Adams, A. C. Cameron, N. E. Hoffman, Y.-B. Yu, C. Lizada, and J. Rudich for many stimulating discussions. K.J.B. gratefully acknowledges support from a National Science Foundation Graduate Fellowship.

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