Decreased Ethylene Biosynthesis, and Induction of Aerenchyma, by Nitrogen- or Phosphate-Starvation in Adventitious Roots of Zea mays L.[1]

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ABSTRACT

Plants of Zea mays L. cv TX5855 were grown in a complete, well oxygenated nutrient solution then subjected to nutrient starvation by omitting either nitrate and ammonium or phosphate from the solution. These treatments induced the formation of aerenchyma close to the apex of the adventitious roots that subsequently emerged from the base of the shoot, a response similar to that shown earlier to be induced by hypoxia. Compared with control plants supplied with all nutrients throughout, N- or P-starvation consistently depressed the rates of ethylene release by excised, 25 mm apical segments of adventitious roots. Some enzymes and substrates of the ethylene biosynthetic pathway were examined. The content of 1-amino cyclopropane-1-carboxylic acid (ACC) paralleled the differences in ethylene production rates, being depressed by N or P deficiency, while malonyl-ACC showed a similar trend. Activity of ACC synthase and of ethylene forming enzyme (g^{-1} fresh weight) was also greater in control roots than in nutrient starved ones. These results indicate that much of the ethylene biosynthetic pathway is slowed under conditions of N- or P-starvation. Thus, by contrast to the effects of hypoxia, the induction of aerenchyma in roots of Zea mays by nutrient starvation is not related to an enhanced biosynthesis and/or accumulation of ethylene in the root tips.

Many dryland species are exposed to temporary periods of oxygen deficiency during their growth following heavy rain, irrigation, or flooding, when the soil becomes water saturated (10, 17). Some species respond to this situation by forming roots with continuous, gas-filled channels in the roots (19). These channels improve the internal supply of oxygen, the oxygen originating from the atmosphere or photosynthesis and passing from leaves to roots down a concentration gradient (1). The flooding resistance of a wide range of monocot and dicotyledonous species, both herbaceous and woody, is often associated, in part, with the capacity to develop such aerenchymatous roots (8, 9, 16, 17, 19, 23). Additionally, metabolic acclimation to low oxygen improves the tolerance of roots to anoxia (29).

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The gaseous plant hormone, ethylene, is associated with a wide variety of responses in higher plant cells (3). In earlier reports we showed that aerenchyma formation in adventitious (nodal) roots of maize by cell lysis is induced by a partial oxygen deficiency (hypoxia) and is closely related to enhanced endogenous concentrations of ethylene (11). Hypoxia clearly stimulates the biosynthesis of ethylene in growing maize roots (2, 11, 12, 20) as in other responsive plant tissues (4, 5, 7). Low exogenous concentrations of ethylene under aerobic conditions (1–5 μL L^{-1} in air) induce aerenchyma (18) that is structurally indistinguishable from that induced by hypoxia (11, 12); furthermore, hypoxically induced aerenchyma formation in the apices of growing roots is blocked by inhibitors of ethylene biosynthesis or ethylene action (12, 18, 20).

However, Konings and Verschuren (21) showed that formation of aerenchyma in the seminal roots of maize was stimulated under fully aerobic conditions by omission of either a nitrogen source (nitrate or ammonium) or phosphate. Similar results were obtained with the adventitious roots of another maize variety (13), while omission of potassium under similar conditions did not induce aerenchyma.

In this paper we compare the mechanism of cell lysis in maize root tips, induced by nitrate- or phosphate-deficiency, to that induced by oxygen shortage. We show that unlike hypoxia, N or P deficiency inhibits the ethylene biosynthetic pathway.

MATERIALS AND METHODS

Plant Growth Conditions

Caryopses of maize (Zea mays L. cv TX 5855) were surface sterilized for 2 min in 10% chlorox, washed in tap water 4 h, and allowed to imbibe and germinate in shallow dishes lined with paper and moistened with distilled water at 25°C in the dark in an incubator. At 4 d from the start of imbibition, germinated plants were transferred from the incubator to stainless steel mesh and suspended in the dark at 25°C over an aerated (1×) nutrient solution comprising (mM): KNO_3, 0.1; $Ca(NO_3)_2$, 0.4; $NH_4H_2PO_4$, 0.1; $MgSO_4$, 0.05; Fe as Fe-EDTA, 0.1; and micronutrients. After 1.5 d, plants were exposed to light (650 μmol photons m^{-2} s^{-1} PAR, 14 h light period) in a controlled environment room with day/night conditions of 25°C/20°C, RH = 75/65. After a further 12 h, plants were transferred in groups of 4 to 2-L volumes of the 1× nutrient solution. After an additional 24 h, the solution

was replaced by a 5× nutrient solution, and after another day the full strength (10×) nutrient solution was applied. Iron and micronutrients remained at the 1× concentration. Two to 3 d later (at 10–11 d from the start of imbibition), when the first whorl of adventitious (nodal) roots had extended about 50 mm and four leaves were visible, the following treatments were imposed: (a) some plants were transferred to solution lacking a source of nitrogen. The N-deficient solution comprised a 10× solution in which NaH_2PO_4 replaced $NH_4H_2PO_4$; $CaSO_4$ replaced $Ca(NO_3)_2$; and KCl replaced KNO_3, all at the same molarity; (b) some plants were transferred to phosphate-free solution, prepared by substituting in the 10× solution NH_4NO_3 for $NH_4H_2PO_4$; (c) controls were maintained in fresh 10× nutrient solution.

Measurement of Ethylene Production by Excised Roots

At different times from the start of the nutrient deficiency treatments, the apical 25 mm of the nodal roots originating at the first, second or third whorl were excised. These sections were transferred (0.5 g FW[3]) to 10-mL volume glass vials with 100 μL of distilled water to humidify the atmosphere and sealed with a rubber serum stopper. After 60 min of incubation, a 1-mL volume of gas was withdrawn with a hypodermic needle and syringe and immediately replaced with 1 mL of clean air. The sample was injected into a gas-chromatograph, with N_2 as carrier gas and flame ionization detector (27). After an additional 60 min of incubation, a second sample was taken. The amount of ethylene produced was quantified by reference to analysis of standard concentrations of ethylene gas in air as described (27).

Estimation of ACC and MACC

ACC content was estimated by a modified Lizada and Yang method (24, 28). Root segments (the apical 25 mm [1.0 g FW]) were excised and ground with pestle and mortar in excess liquid nitrogen. The residue was homogenized in 8 mL of 80% (v/v) methanol in water using a Brinkmann Polytron homogenizer and centrifuged at 10,000g for 15 min. The supernatant was stored while the pellet was homogenized (in 2 mL of 80% methanol) and centrifuged again as described above. Half of the combined supernatants (5 mL) were passed through a SEP-PAC C_{18} cartridge that had been preconditioned with 100% methanol followed by distilled water. Samples were transferred to a 50-mL Erlenmeyer flask, to which was added 3.0 mL of $HgCl_2$ solution (3.3 μmol mL^{-1}) before sealing with a serum stopper. At 0.3-mL volume of a 2:1 (v/v) solution of 5% NaOCl in saturated NaOH solution was injected into each flask. A 1-mL volume of gas from the head space was removed at 2 h with a needle and syringe and analyzed for ethylene content by gas chromatography. The efficiency of conversion of ACC to ethylene, tested by measuring the release of ethylene from a known amount of ACC (4.0 nmol) was high (78–80%), and values given in this paper are uncorrected. For estimation of MACC (15) we used the

[3] Abbreviations: FW, fresh weight; ACC, 1-amino cyclopropane-1-carboxylic acid; EFE, ethylene forming enzyme; MACC, malonyl-amino cyclopropane-1-carboxylic acid; SAM, S-adenosylmethionine.

same 80% (v/v) methanol extract as for ACC determination. A 5-mL volume was heated at 100°C in the presence of hydrochloric acid (final concentration, 2 M) for 3 h. The solution was neutralized with NaOH, and the remainder of the procedure was exactly as described above for ACC, beginning with addition of $HgCl_2$ and then NaOCl.

Estimation of ACC Synthase and EFE

ACC synthase was estimated by an *in vivo* method (7). Two sets of apical 25 mm root segments (each 1.0 g FW) were placed upright in separate 50 mL-volume glass vials and sealed with rubber septum caps. One vial was continuously purged with humidified air, while the other received humidified O_2-free N_2-gas (1 L min^{-1}). After 6 h incubation at 25°C, tissue was ground in liquid N_2 as described above for estimation of ACC. The difference in ACC content between the two sets of roots gives an estimate of ACC synthase activity. EFE activity was estimated from the ability of root segments to convert exogenous ACC to ethylene. Apical 25 mm root segments (0.5 g FW) were placed in a 10-mL vol tube with 1 mL of ACC solution containing 1 μmol and sealed with a rubber septum. The rate of ethylene production was measured by withdrawing a 1.0-mL volume with a syringe and found constant up to at least 2 h.

Measurement of Aerenchyma Formation

Nodal roots from the first or second whorl were marked with carbon (charcoal slurry) 10 mm behind the tip so that the subsequent extension could be followed during the experiment. At different times after the imposition of nutrient deficiency, or other treatment, these marked roots were excised, and transverse sections cut by hand at zones that were 1, 2, 3, or more days old (11). Camera lucida drawings were made of the transverse sections, identifying the areas that comprised intact cells, cells in the process of lysing, and clearly delineated gas-filled spaces (lacunae). These areas were quantified for each section.

RESULTS

Aerenchyma Formation in Roots during Nutrient Deficiency

After only 4 d of nutrient deprivation, the newly emerging adventitious roots showed distinctive changes in structure (Fig. 1). The fractional area of the root occupied by gas spaces (or lacunae) was greatly increased by N-deficiency when a nitrogen-source (nitrate and ammonium) was omitted from the nutrient solution. The 'collapsed cells' refer to areas in the cross-section where cells were disintegrating but had not yet coalesced to form clearly-defined, gas-filled lacunae. A relatively small area of collapsed cells was invariably found in roots growing in solution sparged with air, and these areas were increased only a little by N-deficiency, presumably because of rapid transition from cell collapse to formation of gas spaces. The total area of lysed cells approached 35%, which is close to the maximum degradation of the cortex we observe in maize roots (cf. ref. 11). The extent of cell break-

down was greatest in the midcortex, with no signs of lysis in the hypodermis and epidermis or in the endodermis or stele. Thus, at the light microscope level, the appearance of aerenchymatous roots induced by nutrient deprivation was identical to that induced by hypoxia.

Aerenchyma formation induced by P-deficiency initially was always less than that induced by N-deficiency (Fig. 1), but with increasing age the extent of breakdown with P-deficiency became similar. Thus in root segments that were 5 to 7 d old, the aerenchymatous structure appeared very similar for the two nutrient treatments.

Ethylene Production by Excised Roots

At 1 d from the start of nutrient deprivation, cumulative ethylene production during a 6 h period was already slower for nodal root tips excised from plants deprived of N or P (Fig. 2). As the plants developed, successive whorls of nodal roots emerged. For each, a similar pattern of ethylene production was observed, with the lowest rate always found in N-deprived roots. Following excision, the root tips released ethylene rapidly (Fig. 3). Ethylene production peaked at 2 h and then subsided to a more steady state. However, treatment differences generally were consistent despite extended periods of nutrient starvation, with ethylene production by controls always in excess of that by −N and −P plants. Thus, the treatment differences established at 3 d of nutrient deprivation with the first whorl of nodal roots were also found with long periods of nutrient deprivation in the second and third whorl of nodal roots.

The initial peak in ethylene release was probably due to wounding. If we examine the rate during the period 4 to 5 h after excision (Fig. 4), when wound ethylene release is essentially complete, we see a consistent picture of inhibition of ethylene production by the root tips of maize deprived of N or P.

Root Content of ACC and MACC

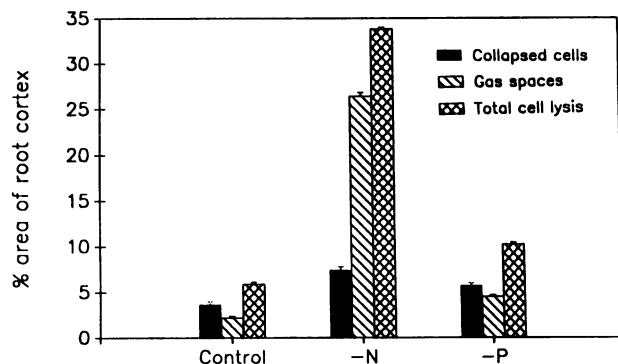The amount of ACC per g FW (Fig. 5) was always greater in controls than in the roots starved of N or P. Only a low
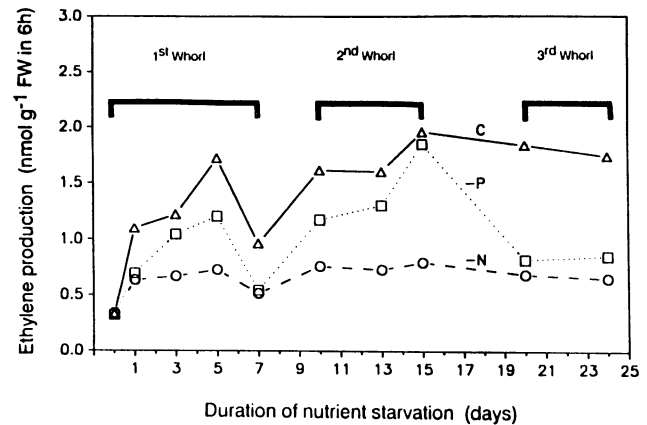

Figure 2. Ethylene production by excised, adventitious root tips of maize with different durations of nutrient starvation. Production was measured over a 6 h interval. Measurements were made on first, second, and third whorls of roots, depending on plant age, using the apical 25 mm of each root. C, control; −P, phosphate starved; −N, NO_3^-- and NH_4^+-starved.

amount of ACC was in the −N roots. The content of ACC increased with time in controls in −P plants, but it should be recognized that three different whorls of roots were excised during the experiment (see Fig. 2 legend). The content of MACC was also greater in control roots (Fig. 5) except for the initial period (first whorl of roots).

Activity of ACC Synthase and EFE

Control roots showed higher activity of ACC synthase than roots from either of the nutrient-starvation treatments (Fig. 6). For most analyses, −P plants gave the lowest activity. Activity of EFE (Fig. 6) showed a distinctly higher value in controls at all times. The lowest activity was in −N roots, reflecting the ethylene production rates which were also lowest with this treatment.

DISCUSSION

Because of the marked stimulation of aerenchyma formation by transient starvation of the roots of a source of nitrogen or phosphate (Fig. 1), we expected to find a comparable enhancement of ethylene production. However, omission of N or P from the nutrient solution for only 3 d resulted in a depression of ethylene production (Figs. 2–4) which was maintained for each successive whorl of adventitious roots that emerged from the stem base for the duration of the starvation treatment (up to 24 d). Our results may seem to be at variance with those of others (6, 14) who found that it was the presence of phosphate that inhibited ethylene biosynthesis in a wide range of higher plant tissues, including carrot root and etiolated stem segments of pea (*Pisum sativum*) and tomato (*Lycopersicon esculentum*). Inhibition of ethylene production by phosphate also takes place in fruit tissues (6, 14) where it acts on the conversion of ACC to ethylene (14). However, the concentrations of phosphate that were used were high (50–100 mM), greatly in excess of those that plant roots would encounter under physiological conditions; commonly, hydro-
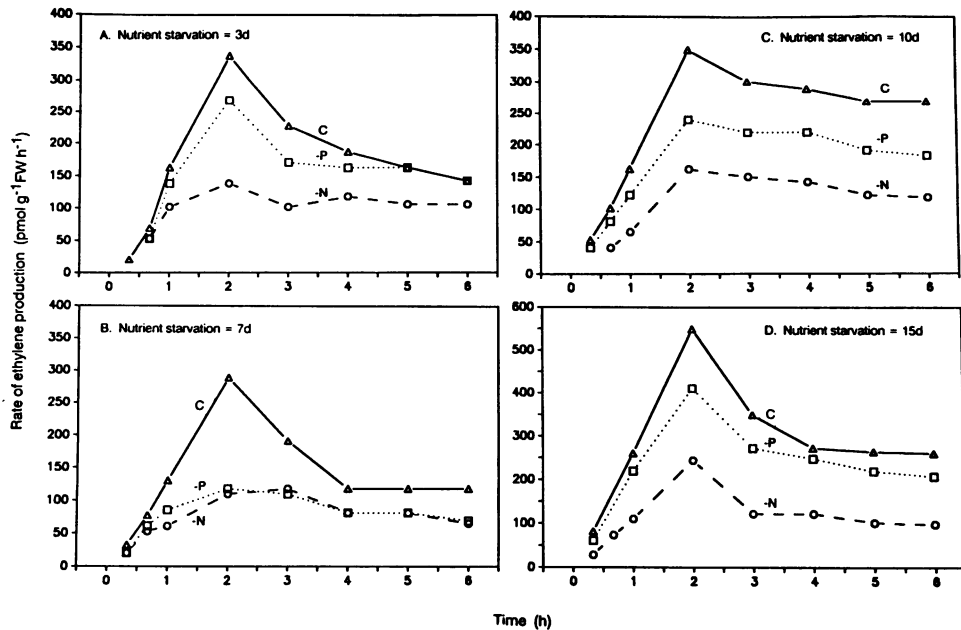

Figure 1. Aerenchyma formation in adventitious root tips of maize with deprivation of NO_3^- and NH_4^+ (−N) or phosphate (−P). Plants were starved of nutrients for 4 d. Transverse sections were made of the first whorl of adventitious roots in 4 d old zones located 110 mm (−N), 90 mm (−P), and 90 mm (control) behind the root apex. Bar indicates SE of mean (n = 10). Total cell lysis equals collapsed cells plus gas spaces.

Figure 3. Rate of ethylene production by excised adventitious root tips of maize with time. Plants were starved of N or P for different periods (A-D). Roots used were 1st whorl (A), 2nd whorl (B and C), 3rd whorl (D).
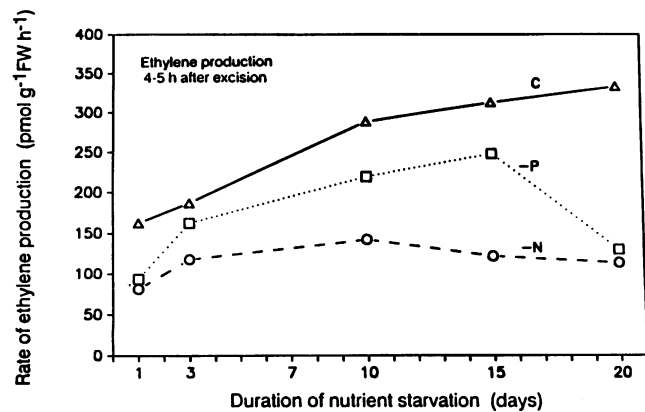

Figure 4. Rate of ethylene production by excised adventitious root tips of maize, between 4 and 5 h after excision, with different durations of nutrient starvation.

Figure 5. ACC and MACC concentrations in maize root tips with different durations of nutrient starvation. Analyses were of the 25 mm apical zone, excised from first, second, or third whorls of roots, as defined in Figure 2.

ponic solutions contain 1 mM phosphate but soil solution phosphate concentration is much lower, between 10^{-4} and 10^{-2} mM. Thus, the present experiments deal with stress resulting from suddenly depriving normally growing plants of P, whereas the previous reports (6, 14) involve the quite different situation of treating plants with unusually high levels of P.

Results with all of the components associated with the ethylene biosynthetic pathway that we examined, both substrates like ACC and MACC (Fig. 5) and enzymes such as ACC synthase and EFE (Fig. 6), suggest that there was an overall depression of the pathway in nutrient-starved roots. Levels of ACC and rates of ethylene production in our control roots were similar to previously published values for maize root tips (2, 11, 18). The response to nutrient deprivation therefore contrasts sharply with that to oxygen deficiency (hypoxia). In maize (2), ethylene production rates as well as ACC concentration were raised many-fold in the roots when
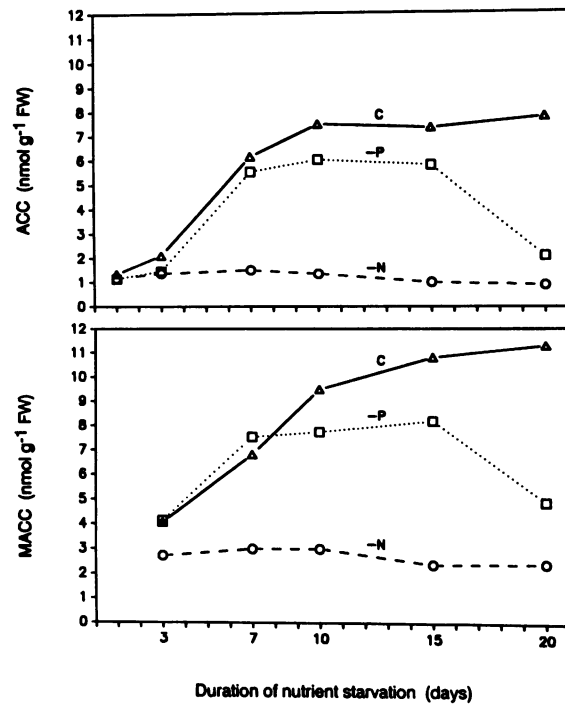
in nutrient solution sparged with 5% (v/v) oxygen in nitrogen (about one-quarter the oxygen concentration in air). Flooding of the root system in tomato strongly stimulated production of ACC by roots and its transport in the transpiration stream to shoots (4, 5). Likewise, in the hypoxic internodes of submerged, deep water rice, promotion of ethylene synthesis was accompanied by enhancement of ACC synthase activity (7,
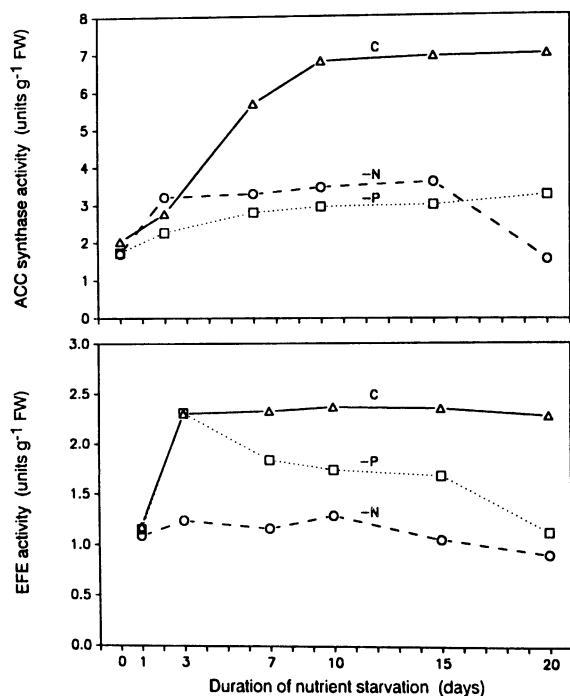
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Figure 6. Activity of ACC synthase and EFE in maize root tips with different durations of nutrient starvation. Analyses were of the 25 mm apical zone, excised from first, second, or third whorls of roots, as defined in Figure 2. A unit of enzyme activity is equivalent to a conversion rate of 1 μmol h^{-1}.

25). One hypothesis suggested by the unlikely combination of nutrient deprivation causing increased aerenchyma formation and decreased ethylene synthesis, is that sensitivity to ethylene increases in response to treatment. This possibility is under investigation.

Although not all the components of the ethylene biosynthetic pathway have been examined in maize roots, there is sufficient information from characterization of ACC, ACC synthase, and EFE (this paper and ref. 2), as well as the action of the inhibitor of ACC synthase (AVG) (18, 21) to assume that essentially the same pathway from methionine to ethylene is followed in maize roots as in many other plant tissues especially fruits that have been more fully described (14, 15, 26, 31). The present results can therefore be examined in relation to what is known concerning the regulation of this pathway in other plant tissues. In excised wheat leaves during water deficit, ACC and ethylene production rapidly increased then declined (15). This decrease in ACC could be accounted for by malonylation of new and previously synthesized ACC to yield MACC, which accumulated to concentrations greatly in excess of the ACC. However, the decline in content of ACC in maize roots with P or N deficiency (Fig. 5) is not associated with a reciprocal rise in MACC content, but rather the two metabolites decrease approximately in parallel.

The rate of production of ethylene (Fig. 4) after its initial wound ethylene response (22) seems to reflect closely the concentration of ACC when the different nutrient treatments are compared (Fig. 5). This suggests that the conversion of SAM to ACC is inhibited or modified somehow by nutrient

deprivation. SAM is recognized to be a precursor for both the ethylene biosynthetic pathway, and for formation of spermidine and other polyamines (26, 30). Inhibition of the conversion of SAM to ACC in orange peel discs shunted SAM into spermidine (14). It would be interesting to know whether nutrient deprivation of maize roots stimulates diversion of SAM to polyamines rather than ethylene biosynthesis.

ACKNOWLEDGMENTS

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