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Induction of Enzymes Associated with Lysigenous Aerenchyma Formation in Roots of *Zea mays* during Hypoxia or Nitrogen Starvation[1]

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Either hypoxia, which stimulates ethylene biosynthesis, or temporary N starvation, which depresses ethylene production, leads to formation of aerenchyma in maize (*Zea mays* L.) adventitious roots by extensive lysis of cortical cells. We studied the activity of enzymes closely involved in either ethylene formation (1-aminocyclopropane-1-carboxylic acid synthase [ACC synthase]) or cell-wall dissolution (cellulase). Activity of ACC synthase was stimulated in the apical zone of intact roots by hypoxia, but not by anoxia or N starvation. However, N starvation, as well as hypoxia, did enhance cellulase activity in the apical zone, but not in the older zones of the same roots. Cellulase activity did not increase during hypoxia or N starvation in the presence of aminoethoxyvinylglycine, an inhibitor of ACC synthase, but this inhibition of cellulase induction was reversed during simultaneous exposure to exogenous ethylene. Together these results indicate both the role of ethylene in signaling cell lysis in response to two distinct environmental factors and the significance of hypoxia rather than anoxia in stimulation of ethylene biosynthesis in maize roots.

When seminal or newly emerging adventitious roots of maize extend under hypoxic conditions, i.e. during exposure to an environment that is partially deficient in dissolved O_2, aerenchyma form by a process involving extensive lysis of cells of the cortex, beginning about 10 mm behind the root tip (McPherson, 1939; Drew et al., 1979; Konings, 1982). Temporary exposure of adventitious roots to nutrient solutions deficient in N (nitrate and ammonium) or P also causes aerenchyma formation in the absence of any O_2 shortage (Konings and Verschuren, 1980; Drew et al., 1989). However, under both O_2 shortage and N or P deficiency, aerenchyma formation appears to be triggered by ethylene. With hypoxia, ethylene biosynthesis is accelerated, endogenous concentrations increase, and application of low concentrations of ethylene (1 μL L^{-1} ethylene in air) stimulates aerenchyma formation in well-aerated, complete nutrient solution (Drew et al., 1979; Jackson et al., 1985; Atwell et al., 1988). Inhibitors of ethylene action (Ag^+) or biosynthesis (AVG) effectively block aerenchyma formation in hypoxic roots (Drew et al., 1981; Konings, 1982; Jackson et al., 1985). However, whereas hypoxia stimulates ethylene production, N or P deficiency depresses it (Drew et al., 1989); instead, they strongly enhanced the sensitivity of cortical cells to ethylene, causing a more rapid lysis of cells in the presence of very low concentrations of the gas (He et al., 1992). As with hypoxia, inhibitors of ethylene-mediated responses (Ag^+ or AVG) block aerenchyma formation in roots growing in N- or P-deficient solution, suggesting that ethylene is involved in aerenchyma formation under these conditions as well (He et al., 1992).

In the present study, we examined in maize adventitious roots: (a) the activity of ACC synthase, an essential and rate-limiting enzyme in ethylene biosynthesis, during hypoxia and anoxia and compared the responses with those to N deficiency; and (b) whether cellulase, an enzyme involved in cell-wall lysis, is stimulated by hypoxia or N deficiency via ethylene-dependent signaling.

MATERIALS AND METHODS

Plant Growth Conditions and Experimental Treatments

Caryopses of maize (*Zea mays* L. cv TX 5855) were germinated and grown in a complete nutrient solution in a controlled-environment room at 25°C as described earlier (Drew et al., 1989). For N deficiency, plants were transferred 10 to 11 d from the start of imbibition from this nutrient solution to one of similar composition but lacking a N source (NH_4^+ and NO_3^-). Control plants were always maintained with the complete nutrient solution. In the majority of experiments, plants were grown in groups of four in 2-L volumes of nutrient solution (Drew et al., 1989).

However, to minimize internal transport of O_2 from leaves to roots, some experiments were done in a "closed system," in which shoots and roots were in the same gaseous atmosphere. In these experiments, groups of 24 plants were supported by a 2-cm-thick expanded polystyrene block that floated on the nutrient solution (10 L) contained in a 40-L glass cylinder. Aluminum foil covered the lower portion of the outside of the glass jars to exclude light from roots and solution. The glass cylinders were each fitted with a plate glass lid with inlet and outlet tubes. Silicon rubber was used to make the lids air tight at the junction of the cylinder walls. Gases were passed into the nutrient solution by the inlet tube that extended into the solution.

Other plants were treated in an "open system," in which

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Abbreviation: AVG, aminoethoxyvinylglycine.

no lid was in place on the 40-L cylinder, so that only roots received the gassing treatment, while the shoots were exposed to air. To make roots hypoxic, air (20.6% [v/v] O_2) was mixed with N_2 gas from pressurized cylinders to provide 4% (v/v) O_2, which was passed into the nutrient solution. Gas-flow rates were regulated with electronic mass-flow controllers and sparged at 1 L min^{-1} for the large containers and 200 mL min^{-1} for the smaller ones. For anoxic treatments, O_2-free pre-purified N_2 (99.97%) was supplied to plants at the same flow rates as cited above. In all experiments, adventitious (nodal) roots were excised from the first or second whorl above the coleoptile node for assay of enzyme activity.

Measurement of ACC Synthase and Cellulase Activities

ACC synthase activity was determined by the in vivo method of Cohen and Kende (1987), in which excised root segments were incubated in a N_2 atmosphere for 6 h, and the production of ACC was measured to give an estimate of enzyme activity. Cellulase activity was measured by a modification of the method used by Kawase (1979). Root tips from treated maize plants (1 g fresh weight, 25 mm or 10 mm from apex as indicated in figure legends) were excised in a cold room (4°C) and macerated with a pinch of river sand in 5 mL of 0.03 M K-phosphate buffer at pH 6.1 containing 1 M NaCl (final concentration). The extract was centrifuged at 3000*g* for 10 min, and 4 mL of the supernatant solution was added to 25 g of 3% sodium carboxymethyl cellulose of medium viscosity (Sigma). Cellulase activity in the supernatant solution was assayed by measuring the change in the viscosity of carboxymethyl cellulose during 2 h of incubation at 25°C using a viscometer. Cellulase activity was expressed as units g^{-1} fresh weight by comparison with a standard cellulase preparation from *Aspergillus niger* (Sigma C7377, Sigma).

RESULTS

ACC Synthase Activity Was Stimulated by Hypoxia but Inhibited by Long-Term Anoxia

With hypoxia (4% O_2 in gas mixture) in an open system (only roots received the gassing treatment; shoots were in air), ACC synthase activity in the root tips of maize showed a steady increase beginning after about a 4-h delay (Fig. 1A). Treatment with N_2 gas to give a very low O_2 concentration in the solution (extreme hypoxia) produced only a transient increase in ACC synthase activity at 12 h, followed by a depression of enzyme activity to levels below controls (Fig. 1B). Hypoxia (4% O_2) for 24 h, followed by N_2 gas, gave high levels of ACC synthase activity at the beginning and then showed a steady decrease with time (Fig. 1B). N deficiency caused little change in ACC synthase activity (Fig. 1A).

In a closed system (shoot and roots in the same controlled atmosphere), ACC synthase activity in root tips increased rapidly with hypoxia (4% O_2) and levels remained considerably higher than in the controls (Fig. 2). When hypoxia for 24 h was followed by N_2 gas, ACC synthase activity was high at the beginning like that in the open-system treatment. However, ACC synthase activity did not show any transient increase when initially treated with N_2 gas, but instead showed a steady decrease to very low levels at the conclusion.

Cellulase Activity Was Stimulated by Hypoxia, by Exogenous Ethylene, or by N Deficiency

All treatments (hypoxia, exogenous ethylene, and N deficiency) stimulated cellulase activity in the root tips of maize. Cellulase reached the highest levels (about 70 × 10^{-3} units g^{-1} fresh weight) in the 10-mm apical zone with hypoxia or exogenous ethylene for 8 d and N deficiency for 12 d (Fig. 3). There was no change in cellulase activity in controls. Along the whole root length, the highest cellulase activity was located in the 0- to 10-mm apical zone with hypoxia, exogenous ethylene, and N deficiency (Fig. 4). Cellulase activity showed a similar pattern in all the treatments with continuous decrease from tip to base, whereas the controls showed the opposite, with minimum activity in root tips and a gradual increase toward the base (Fig. 4).

Induction of Cellulase Activity Was Blocked by AVG

Addition of AVG, an inhibitor of ethylene biosynthesis, blocked any induction of cellulase activity in response to hypoxia or N deficiency (Fig. 5). However, the effect of AVG could be overcome by simultaneous exposure to exogenous ethylene 1 μL L^{-1} in air or in the 4% O_2 mixture.

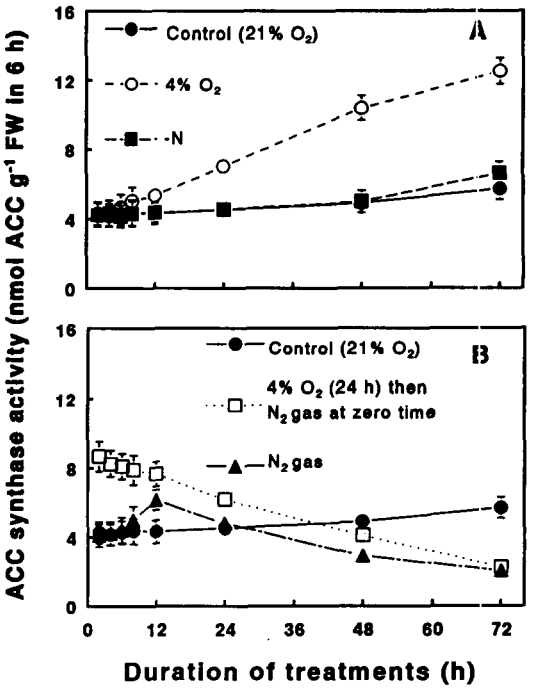

Figure 1. ACC synthase activity in intact maize root tips from plants given different treatments in a 40-L volume open system (only roots received gas treatments; shoots were in air). Segments were 0 to 25 mm from apex. A and B indicate different gassing treatments in the same experiment. Vertical bars indicate SE (n = 6).

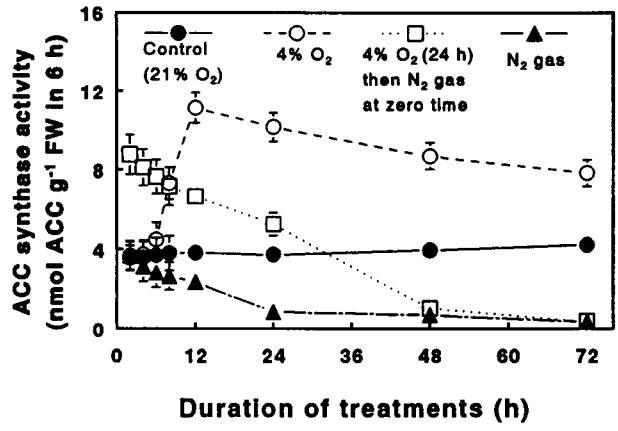

Figure 2. ACC synthase activity in intact maize root tips from plants given different treatments in a 40-L volume closed system (roots and shoots were in the same controlled atmosphere). Segments were 0 to 25 mm from apex. Vertical bars indicate SE (n = 6).

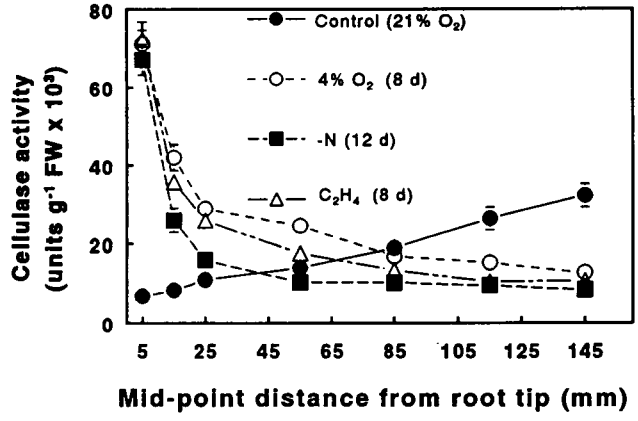

Figure 4. Cellulase activity of segments (10-mm tips) of intact roots with N starvation, hypoxia (4% O_2), and exogenous ethylene in air (1 μL L^{-1}) at different locations along the root axis. Roots were in 2-L volumes of nutrient solution. Vertical bars indicate SE (n = 6).

Increase in ACC Synthase Activity with Hypoxia Preceded the Increase in Cellulase Activity

With hypoxia, the rise in ACC synthase activity preceded that of cellulase in the root tips (Fig. 6). The ACC synthase activity increased perceptibly at 12 h of treatment, whereas cellulase activity began to increase at about 48 h. ACC synthase activity was almost unchanged by N deficiency, whereas cellulase activity in these roots was higher than in controls from d 3 onward.

DISCUSSION

The present results support the hypothesis that ethylene signals the formation of aerenchyma in response to either hypoxia or N deficiency, and induces a rise in activity of cell-degrading enzymes such as cellulase. Because of the complete disappearance of cell walls and protoplasts of the cortical cells that are affected in aerenchyma formation, leaving prominent gas-filled voids, it seems reasonable to suppose that a wide array of cell-degrading enzymes are involved. Indeed, cell-wall disappearance takes place relatively late in the process of cell lysis (Campbell and Drew, 1983) so that cellulase is unlikely to have a primary role.

Increased activity of ACC synthase occurs with hypoxia rather than anoxia (Figs. 1 and 2). Even in the open system, in which internal diffusion from leaves to roots could take place in intercellular spaces and thereby increase O_2 concentrations in roots (Armstrong and Beckett, 1987; Erdmann and Wiedenroth, 1988), ACC synthase activity in 25-mm root tips showed only a transient rise when roots were sparged with N_2 gas. With strictly anaerobic conditions in the closed system, ACC synthase activity was strongly depressed, even in roots of plants previously acclimated by hypoxia and with a high initial enzyme activity (Fig. 2). When roots were sparged with 4% O_2, the increase in ACC synthase activity was more gradual in the open system than in the closed

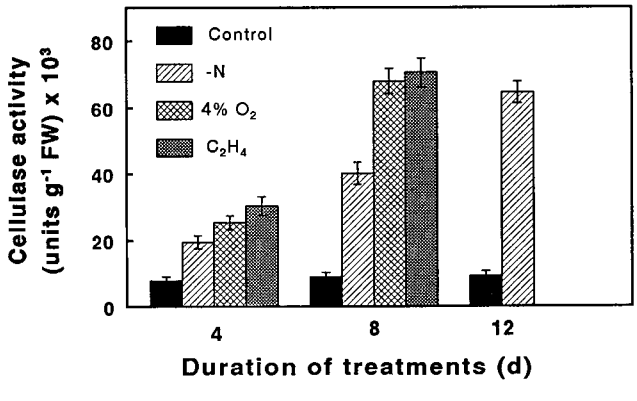

Figure 3. Cellulase activity of segments (10-mm tips) of intact roots with N starvation, hypoxia (4% O_2), and exogenous ethylene in air (1 μL L^{-1}). Roots were in 2-L volumes of nutrient solution. Vertical bars indicate SE (n = 6).

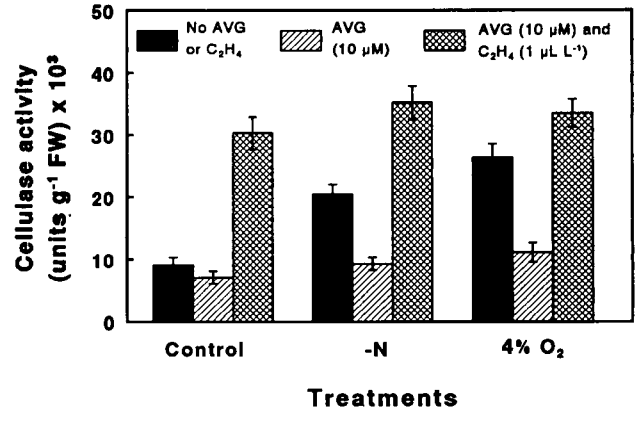

Figure 5. Inhibition of ethylene-dependent cellulase activity by AVG in root segments (10-mm tips) of intact maize roots. Treatments were for 4 d. Roots were in 2-L volumes of nutrient solution. Vertical bars indicate SE (n = 6).

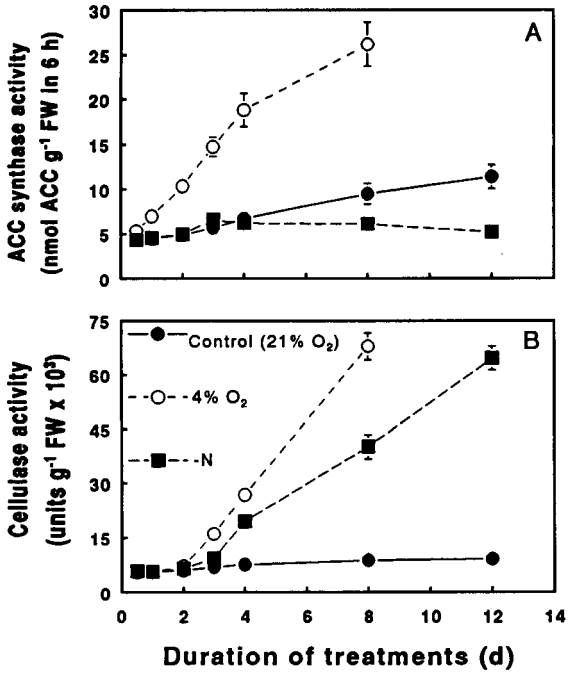

Figure 6. Enzyme activities in intact maize roots (25-mm tips) in a 40-L volume open system (only roots received gassing treatments; shoots were in air). A, ACC synthase; B, cellulase. Vertical bars indicate SE (n = 6).

system. This can be accounted for by the internal transfer of O_2 from leaves and stem to the roots in the open system (Raymond et al., 1978; Armstrong, 1979; Erdmann and Wiedenroth, 1988) so that root tips were under a smaller O_2 deficit. That deficit would have become greater with time because of an increased path length for O_2 diffusion in the extending roots (Armstrong, 1979).

The present results are consistent with earlier reports that low O_2 concentrations in the root environment of tomato, brought about by sparging with N_2 gas (Wang and Arteca, 1992) or by waterlogging of soil (Bradford and Yang, 1980), lead to either an increase in ACC synthase activity in roots (Wang and Arteca, 1992) or to large increases in ACC in xylem sap (Bradford and Yang, 1980), presumably reflecting the greater ACC synthase activity. Research with tomato has been concentrated on the conversion of ACC to ethylene and its role in epinasty (Jackson, 1985). However, in maize roots, elevated concentrations of ACC have been shown to be closely related to enhanced rates of ethylene production during induction of aerenchyma (Atwell et al., 1988). Additionally, maize roots respond to mechanical impedance by greater ACC synthase activity and enhanced ethylene production (Sarquis et al., 1991, 1992). ACC synthase activity was also enhanced by submergence or low O_2 concentrations in internodes of deep-water rice (Cohen and Kende, 1987), where the additional production of ethylene stimulated internode growth. In all the above circumstances where enhancement of ACC synthase activity was found under low-O_2 conditions, it seems likely that the plant tissue was under hypoxia rather than anoxia. In maize roots it is clear that strictly anaerobic conditions do not promote the activity of this enzyme.

Hypoxia stimulates formation of aerenchyma in roots of maize in nutrient solution by enhancing ethylene biosynthesis and increasing the endogenous ethylene concentration (Drew et al., 1979; Atwell et al., 1988). By contrast, temporary deprivation of N or P does not stimulate the ethylene biosynthetic pathway (Figs. 5 and 6; see also Drew et al., 1989) but greatly enhances the sensitivity of ethylene-responsive cells of the root cortex, leading to cell lysis and aerenchyma formation (He et al., 1992). The present results implicate ethylene in the induction of cellulase activity, even under conditions where ethylene biosynthesis is slowed. Addition of AVG to inhibit ACC synthase activity effectively cuts down on induction of cellulase activity by hypoxia or N deficiency (Fig. 5). However, this effect can be overcome by simultaneous exposure to ethylene, showing that AVG is not exerting a generalized inhibitory effect on metabolism.

Is the timing of changes in ACC synthase activity, ACC accumulation, ethylene production, and aerenchyma formation consistent with our hypothesis? In the closed system (Fig. 2), where hypoxia of the root apex was closely controlled, a rise in ACC synthase activity was detectable 6 to 9 h from the start of treatment. The precise timing of the rise in ACC synthase activity is uncertain because an in vivo method was used that required incubation of root segments for 6 h before assay. Given that uncertainty, increased levels of ACC between 1.75 and 3 h following the onset of hypoxia, and increased ethylene production at 2.25 h (Atwell et al., 1988), seem to be in reasonable agreement. The first signs of aerenchyma formation, at 10 mm behind the root tip (Campbell and Drew, 1983), correspond to about 12 h of hypoxia.

Working with sunflower stems, Kawase (1979, 1981) found that exogenous ethylene, or exclusion of O_2 from part of the stem or stem and roots, led to lysis of cells of the stem cortex and increased activity of cellulase. Our results confirm that in maize roots, treatments that give rise to aerenchyma formation (4% O_2, exogenous ethylene, N deficiency) all stimulate cellulase activity in the apical zone (Figs. 3–6). The greatest cellulase activity following the above treatments is in the tips, with a progressive decline toward the base. This suggests that cellulase activity was lost soon after cell-wall degradation was complete, usually within 50 mm of the tip. By contrast, the gradual rise in cellulase activity in controls, from tip to base, accords with the lysis of some cortical cells, which we observe in older root zones even under well-oxygenated conditions.

During hypoxia, the rise in ACC synthase activity precedes that of cellulase activity in root tips (Fig. 6), which is consistent with induction of the latter enzyme by ethylene. Yet it is noticeable that in this open system both enzymes gradually increased in activity with time, probably as a consequence of the gradual increase in O_2 deficit in the root-tip zone, as discussed above. Also as mentioned above, there can be no doubt that many degradative enzymes are induced under these conditions, and cellulase may increase relatively late in the process of cell lysis. It is interesting to note the parallel between aerenchyma formation and the signaling by ethylene of cell lysis in the leaf abscission zone, which also involves

induction of cellulase activity (Horton and Osborne, 1967; del Campillo et al., 1990; del Campillo and Lewis, 1992).

Our studies with aerenchyma formation in maize roots point to at least two signal-transduction pathways that should be considered. The first involves steps between hypoxia and stimulation of ACC synthase activity. Little is known about this pathway, although it appears that anoxia itself is unlikely to be the signal; acclimated primary root tips of maize remain viable for up to 96 h under anoxia (Johnson et al., 1989), but ACC synthase activity is reduced under anoxia (Fig. 2). It is not known whether both transcription and translation are affected. The second pathway is that between ethylene and the cell-degrading enzymes that it induces. It seems reasonable to propose that an early step in this pathway involves binding of ethylene to receptor molecules in target cells in the root cortex. Presumably, part of this pathway behaves differently in N- or P-starved roots that become much more sensitive to low concentrations of ethylene. We have attempted to characterize the ethylene-binding properties of N-starved roots relative to those of unstarved controls, based on published procedures (Sanders et al., 1990, 1991), but we found no evidence to support the hypothesis that N-starved roots bind ethylene differently than controls (C.-J. He, P.W. Morgan, W.R. Jordan, and M.C. Drew, unpublished data).

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LITERATURE CITED

Armstrong W (1979) Aeration in higher plants *In* HW Woodhouse, ed, Advances in Botanical Research. Academic Press, New York, pp 225–331

Armstrong W, Beckett PW (1987) Internal aeration and the development of stelar anoxia in submerged roots: a multi-shelled mathematical model combining axial diffusion of oxygen in the cortex with radial losses to the stele, the wall layers and the rhizosphere. New Phytol **105:** 221–245

Atwell BJ, Drew MC, Jackson MB (1988) The influence of oxygen deficiency on ethylene synthesis, 1-aminocyclopropane-1-carboxylic acid levels, and aerenchyma formation in roots of *Zea mays*. Plant Physiol **72:** 15–22

Bradford KJ, Yang SF (1980) Xylem transport of 1-aminocyclopropane-1-carboxylic acid, an ethylene precursor, in water logged tomato plants. Plant Physiol **65:** 322–326

Campbell R, Drew MC (1983) Electron microscopy of gas space (aerenchyma) formation in adventitious roots of *Zea mays* L. subjected to oxygen shortage. Planta **157:** 350–357

Cohen E, Kende H (1987) In vivo 1-aminocyclopropane-1-carboxylate synthase activity in internodes of deep water rice. Enhancement by submergence and low oxygen levels. Plant Physiol **84:** 282–286

del Campillo E, Lewis LN (1992) Identification and kinetics of accumulation of proteins induced by ethylene in bean abscission zones. Plant Physiol **99:** 955–961

del Campillo E, Reid PD, Sexton R, Lewis LN (1990) Occurrence and localization of 9.5 cellulase in abscising and nonabscising tissues. Plant Cell **2:** 245–254

Drew MC, He CJ, Morgan PW (1989) Decreased ethylene biosynthesis, and induction of aerenchyma, by nitrogen- or phosphate-starvation in adventitious roots of *Zea mays* L. Plant Physiol **91:** 266–271

Drew MC, Jackson MB, Giffard S (1979) Ethylene-promoted adventitious rooting and development of cortical air spaces (aerenchyma) in roots may be adaptive responses to flooding in *Zea mays* L. Planta **147:** 83–88

Drew MC, Jackson MB, Giffard SC, Campbell R (1981) Inhibition by silver ions of gas space (aerenchyma) formation in adventitious roots of *Zea mays* L. subjected to exogenous ethylene onto oxygen deficiency. Planta **153:** 217–224

Erdmann B, Wiedenroth EM (1988) Changes in the root system of wheat seedlings following root anaerobiosis. 3. Oxygen concentration in the roots. Ann Bot **62:** 277–286

He C-J, Morgan PW, Drew MC (1992) Enhanced sensitivity to ethylene in nitrogen- or phosphate-starved roots of *Zea mays* L. during aerenchyma formation. Plant Physiol **98:** 137–142

Horton RF, Osborne DJ (1967) Senescence, abscission and cellulase activity in *Phaseolus vulgaris*. Nature **214:** 1086–1088

Jackson MB (1985) Ethylene and responses of plants to soil waterlogging and submergence. Annu Rev Plant Physiol **36:** 145–174

Jackson MB, Fenning TM, Drew MC, Saker LR (1985) Stimulation of ethylene production and gas-space (aerenchyma) formation in adventitious roots of *Zea mays* L. by small partial pressures of oxygen. Planta **165:** 486–492

Johnson J, Cobb BG, Drew MC (1989) Hypoxic induction of anoxia tolerance in roots of *Zea mays*. Plant Physiol **91:** 837–841

Kawase M (1979) Role of cellulase in aerenchyma development in sunflower. Am J Bot **66:** 183–190

Kawase M (1981) Effect of ethylene on aerenchyma development. Am J Bot **68:** 651–658

Konings H (1982) Ethylene-promoted formation of aerenchyma in seedling roots of *Zea mays* L. under aerated and non-aerated conditions. Physiol Plant **54:** 119–124

Konings H, Verschuren G (1980) Formation of aerenchyma in roots of *Zea mays* in aerated solutions, and its relation to nutrient supply. Physiol Plant **49:** 265–270

McPherson DC (1939) Cortical air spaces in the roots of *Zea mays* L. New Phytol **38:** 190–202

Raymond PH, Bruzau F, Pradet A (1978) Etude du transport d'oxygène des parties aeriennes aux racines à l'aide d'un paramètre du metabolisme: la charge enérgétique. CR Acad Sci Paris **286:** 1061–1063

Sanders IO, Harpham NVJ, Raskin I, Smith AR, Hall MA (1991) Ethylene binding in wild type and mutant *Arabidopsis thaliana* (L.) Heynh. Ann Bot **68:** 97–103

Sanders IO, Ishizawa K, Smith AR, Hall MA (1990) Ethylene binding and action in rice seedlings. Plant Cell Physiol **31:** 1091–1099

Sarquis JI, Jordan WR, Morgan PW (1991) Ethylene evolution from maize (*Zea mays* L.) seedling roots and shoots in response to mechanical impedance. Plant Physiol **96:** 1171–1177

Sarquis JI, Morgan PW, Jordan WR (1992) Metabolism of 1-aminocyclopropane-1-carboxylic acid in etiolated maize seedlings grown under mechanical impedance. Plant Physiol **98:** 1342–1348

Wang TW, Arteca RN (1992) Effects of low O_2 root stress on ethylene biosynthesis in tomato plants (*Lycopersicon esculentum* Mill cv Heinz 1350). Plant Physiol **98:** 97–100