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Enhanced ethylene production by primary roots of *Zea mays* L. in response to sub-ambient partial pressures of oxygen


ABSTRACT

Ethylene production by primary roots of 72-h-old intact seedlings of *Zea mays* L. cv. LG11 was studied under ambient and sub-ambient oxygen partial pressures (pO₂) using a gas flow-through system linked to a photoacoustic laser detector. Despite precautions to minimize physical perturbation to seedlings while setting-up, ethylene production in air was faster during the first 6h than later, in association with a small temporary swelling of the roots. When roots were switched from air (20-8kPa O₂) to 3 or 5kPa O₂ after 6h, ethylene production increased within 2-3h. When the roots were returned to air 16 h later, ethylene production decreased within 2-3h. The presence of 10kPa CO₂ did not interfere with the effect of 3kPa O₂. Transferring roots from air to 12.5kPa did not change ethylene production, while a reduction to 1 kPa O₂ induced a small increase. The extra ethylene formed in 3 and 5kPa O₂ was associated with plagiotropism, swelling, root hair production, and after 72 h, increased amounts of intercellular space (aerenchyma) in the root cortex. Root extension was also slowed down, but the pattern of response to oxygen shortage did not always match that of ethylene production. On return to air, subsequent growth patterns became normal within a few hours. In the complete absence of oxygen, no ethylene production was detected, even when anaerobic roots were returned to air after 16h.

Key-words: *Zea mays* L.; roots; flooding; environmental stress; ethylene; oxygen; carbon dioxide; aerenchyma; photoacoustic laser detector.

INTRODUCTION

Soil waterlogging significantly reduces the yield of most arable crops and pastures worldwide. Water itself is not toxic, but in excess, it asphyxiates roots by displacing soil oxygen and by impeding gaseous exchange between plant roots, the rhizosphere and the aerial environment. In well-structured, freely drained soils, the interstitial pores are gas-filled and interconnected with the atmosphere above the soil surface (Gambrell, Delaune & Patrick 1991). Consequently, plant roots are surrounded by a stable gaseous atmosphere virtually identical to that above ground (pO₂: 20-8kPa) (Greenwood 1970). As the soil becomes increasingly wet, pore spaces become water-filled and oxygen is depleted by biological and chemical oxidations. Large fluxes of gas cannot take place in the liquid phase; the diffusion of oxygen and carbon dioxide in water being 1/10000 the rate of diffusion into the gaseous soil atmosphere (D_{water}/D_{air}: 1-13 \times 10^{-4}) (Greenwood 1961; Grable 1966). Hence, further diffusion of gases into and out of the soil is restricted by the presence of free water in the pores and oxygen availability declines, whilst carbon dioxide and the products of microbial and root system respiration accumulate (Ponnamperuma 1984). Plants require metabolic and/or morphological adaptations to survive such conditions. Many of the latter are mediated via plant hormones, most notably ethylene (Jackson 1985, 1990; Voesenek et al. 1992).

In maize roots, there is evidence of causal relationships between oxygen supply, rate of ethylene biosynthesis and the extent of aerenchyma development. When nodal roots of maize were exposed to partial oxygen shortage, aerenchyma developed in the cortex, in association with faster ethylene production (Jackson 1982; Jackson et al. 1985). A similar increase in ethylene production was observed in roots of barley (Jackson et al. 1984) and in the stems of deep water rice (Metraux & Kende 1983). However, not all plant tissues respond to oxygen deficiency in this manner. In mungbean hypocotyls (Imaseki, Watanabe & Odawara 1977), rice coleoptiles (Raskin & Kende 1983) and banana fruit (Banks 1985), ethylene evolution declines rather than increases with decreasing partial pressures of oxygen. Furthermore, ethylene biosynthesis requires molecular oxygen for the conversion of methylthioribose phosphate to...
Single seedlings with roots 20–30 mm long were sealed into glass cuvettes (see Fig. 1 and Moss, Hall & Jackson 1988). Each cuvette consisted of two chambers. The upper contained the caryopsis and damp vermiculite into which secondary roots and coleoptile extended, and was open to the ambient atmosphere. The primary root was sealed in the lower chamber through which chosen gas mixtures were passed. The lower chamber had an internal volume of $14 \times 10^{-6}$ m$^3$ and contained $1–2 \times 10^{-6}$ m$^3$ of distilled water to ensure high internal humidity. Seedlings were sealed into the cuvettes with a slurry of plaster of Paris around the base of the radicle (Fig. 1b). Once the plaster of Paris had set, a layer of damp vermiculite was placed over the caryopsis and the root compartment darkened by wrapping it with aluminium foil.

Compressed air or oxygen in a balance of nitrogen (Hoekloos, Schiedam, The Netherlands) was scrubbed of trace hydrocarbon contaminants with a platinized catalyst at 400°C and with ‘Ethysorb’ (Stayfresh Ltd, London, UK) to remove ethylene. Mixtures containing carbon dioxide were passed through the platinized catalyst alone, since Ethysorb also removes carbon dioxide. Gases, flowing at $1 \times 10^{-3}$ m$^3$ h$^{-1}$, were humidified before entering the cuvettes by bubbling through distilled water in a sealed serum vial. Out-flowing gases passed from the cuvettes into an eight-way switching valve, where the gas streams to be measured were directed in sequence into the PA-detector. Flows not under measurement were vented into the atmosphere. Before entering the PA-detector, gases passed over potassium hydroxide pellets to remove carbon dioxide, and over CaCl$_2$ desiccant. Removal of carbon dioxide and water is important since both interfere with ethylene detection, increasing the background signal and lowering the sensitivity of the apparatus. The gas stream was also passed through a cold trap at $-70^\circ$C to remove ethanol and other large molecular weight volatiles, which may also interfere with PA detection of ethylene. The apparatus was checked to ensure that low partial pressures of ethylene were not frozen-out along with ethanol in the cold trap.

The laser-driven intracavity photoacoustic detector

Ethylene concentration in out-flowing gas streams from the cuvettes was measured by a laser-driven PA-detector (Harren et al. 1990). Briefly, the detector operates as follows. A mechanically-chopped carbon dioxide waveguide laser beam is directed into a photoacoustic cell (PA-cell) containing a gas sample. Ethylene in the sample absorbs at the emission frequencies of the laser (9–11 µm). Ethylene molecules are excited by the laser beam from the ground state into a higher vibrational state. De-excitation processes redistribute the energy via collisions with other molecules in the gas sample, causing an increase in the kinetic energy of the molecules, and hence, a concurrent increase in temperature. In a closed, resonant PA-cell, the increase in temperature raises the pressure which, when modulated at an audio frequency, can be detected by a microphone. To maximize the microphone signal, a mechanical beam-chopper was tuned to the resonance frequency of the PA-cell, creating an acoustic ‘standing wave’ inside the resonator (length 100 mm; diameter...
Figure 1. (a) The primary root of a single, intact seedling of *Zea mays* sealed into a glass cuvette. The root was isolated in the lower chamber through which a humidified gas stream of known partial pressure of oxygen or carbon dioxide was passed. (b) Detail showing seedling sealed into the cuvette with plaster of Paris around the base of the caryopsis.

6 mm) of the PA-cell. To further increase the sensitivity, the PA-cell was enclosed within the laser cavity. The resultant high intracavity laser power (>100 W) enables detection of ethylene down to 0.041 pmol m\(^{-3}\) (Harren et al. 1990). The PA-detector was calibrated against known amounts of ethylene (0.003–0.0005 mm\(^3\)) in air and the response was checked for linearity.

**Ethylene measurement**

Ethylene from two separate, single roots was measured in each treatment. A sealed cuvette without a root was included to provide a reference for changes in background. This background value also accounted for any trace contamination of inflowing gas mixtures with ethylene and was assumed to represent the zero baseline from which subsequent root production values were calculated. Experiments lasted approximately 28h and involved measurements of ethylene from a single root and respective reference cuvette in each of two gas mixtures. In most cases, the first and last 6h of the experiment were performed in air with the treatment mixtures supplied for the intervening 16h. All times are approximate. Gas streams from root and empty (reference) cuvettes were measured consecutively over a period of 1h, and the sampling order of the cuvettes was randomized.

**Data manipulation and calculation of ethylene production rates**

Root length, appearance and flow-rates were assessed at each change of treatment gas. Flow rates were adjusted as necessary to restore a rate of 1 \times 10^{-3} m^3 h^{-1}. Root fresh weight was measured at the end of each experiment. The mean initial fresh weight of roots was obtained from 10 roots of similar length to those in the cuvettes at the beginning of the experiment. Ethylene concentration in out-flowing gas streams was determined on the basis of the difference in laser signal (converted into equivalent cubic millimetres of ethylene) between root and reference cuvettes. Ethylene concentrations were converted into production rates (mm\(^3\) g\(^{-1}\) fresh weight h\(^{-1}\)) which incorporated estimates of increases in root biomass based on changes in length. The relationship between length and
weight was calculated from each phase of the experiments (i.e. first 6 h of air, 16 h treatment period and second 6 h of air). Each experiment was repeated two to five times with similar results. Representative data are presented.

Assessment of cortical aerenchyma

In a separate experiment, caryopses were germinated as described previously and seedlings with primary roots 20–30 mm long transferred to opaque, acrylic boxes (6.6×10⁻³ m³). These allowed the primary roots of up to 10 intact seedlings to be treated with a flow of 3 kPa O₂ in a dark, moist atmosphere for 72 h prior to analysis of cortical gas space. Precautions were taken to ensure that the portion of root analysed had completed its longitudinal growth phase before being exposed to the treatment and had not already responded to endogenous ethylene up to that time. This was accomplished by growing the seedling roots in an air atmosphere enriched with the volatile ethylene antagonist 2,5-norbornadiene (2×10⁻⁴ m⁻³; Aldrich Chemical Co. Ltd, Dorset, UK) for 48 h. The roots were marked 10 mm behind the tip with a slurry of charcoal powder after 24 h, and again after 48 h. The zone of root between the two marks was examined for aerenchyma after a further 72 h in either air or 3 kPa O₂, flowing at 3×10⁻³ m⁻³ h⁻¹. A 10-mm section from the middle of the marked zone was sectioned transversely with a razor blade and examined with a low-power microscope connected to a video camera and Optomax Model 5 image analyser (Analytical Measuring Systems, Cambridge, UK). The percentage area of the cortex that comprised intercellular space formed by cell collapse was scored.

RESULTS

Ethylene production

There were clear trends in ethylene production by primary roots of maize in response to different partial pressures of oxygen. Ethylene production in air (20.8 kPa O₂) was high at the start of most experiments, but declined during the first 6 h. When air was passed over the roots throughout the experiment, production continued to decline at a rate of approximately 0.00015 mm³ h⁻¹ (Fig. 2a). Switching to 12.5 kPa O₂ after 6 h in air did not affect this pattern of decline during the next 16 h and the resultant trends in air and 12.5 kPa O₂ were more or less identical (Fig. 2b). However, treatment with 5 kPa, 3 kPa and probably 1 kPa O₂ caused ethylene production to increase (Fig. 2c,d,e). The most pronounced stimulation occurred in 3 kPa O₂, where levels increased within the first hour of treatment and remained at a high level 0.008–0.01 mm³ g⁻¹ h⁻¹ for the entire 16-h treatment. The effect of 5 kPa O₂ was similar, but production gradually declined with time of exposure. A small stimulation of production was also apparent when roots were switched into 1 kPa O₂. Ethylene synthesis was arrested completely by the absence of oxygen (Fig. 2f). Ethylene production rates soon returned to normal when, after 16 h in 5 or 3 kPa O₂, roots were returned to air. Production of ethylene after 16 h of anoxia did not increase when the roots were returned to air, and remained at or close to zero for the remaining 6 h of the experiment.

The possibility that carbon dioxide might interfere with the ability of the roots to produce ethylene in response to 3 kPa O₂ was tested by combining 3 kPa O₂ with either 0.1 or 10 kPa CO₂. These mixtures were supplied for 16 h before the roots were returned to air. The marked decrease in ethylene production observed upon switching back to air (Fig. 3a) indicates that the difference in ethylene production in air or 3 kPa O₂ is retained in the presence of carbon dioxide. These partial pressures of carbon dioxide also failed to change rates of ethylene production in air (Fig. 3b).

Root elongation

Root extension was inhibited at all oxygen concentrations below 20.8 kPa, compared to aerobic controls (Fig. 4). At 12.5 kPa O₂, extension rates were slowed from 1.50 mm h⁻¹ to 1.18 mm h⁻¹ (a reduction of 21%), while more severe inhibitions were obtained with smaller oxygen partial pressures. The 3 or 5 kPa O₂ treatments gave extension rates of 0.46 and 0.41 mm h⁻¹, respectively, whilst 1 kPa reduced root growth to 0.29 mm h⁻¹, only 19% of the elongation rate in air. Almost no growth was observed in the complete absence of oxygen. In most cases, post-treatment root extension rates were similar to those observed during treatment (Fig. 4). However, after 16 h of anoxia, no further growth was observed while recovery from 16 h of 5 kPa O₂ was sufficient to raise rates of extension 2.5-fold to rates approaching those of roots given air throughout. Thus, treatment with partial pressures of oxygen less than 5 kPa limited the competence of the roots to recover strongly during the first 6 h after hypoxia.

Root morphology

Whilst roots exposed to anoxia, 1 kPa and 12.5 kPa O₂ resembled those grown in air in all respects except root length, oxygen partial pressures of 3 and 5 kPa resulted in other changes in root morphology. These included swelling of tissue behind the root tip, proliferation of root hairs and plagiotropic deflection of the root tip (Fig. 5). When roots given 3 kPa O₂ for 72 h were sectioned in a zone that lay 1 cm behind the root tip at the time treatments began, the extent of cortical collapse to form aerenchyma was considerably greater than that in roots grown in air (Table 1).

The morphology of tissue produced after returning to air was similar to air-grown roots, in all cases, except
Ethylene production by roots of *Zea mays* exposed to various partial pressures of oxygen. All experiments commenced with an acclimatization period of approximately 6h in air, followed by 16h of treatment and lastly a second 6h period in air to assess the response to a return to air. Representative data from the primary root of a single, intact seedling are displayed for each separate treatment. Each experiment was repeated two to five times with similar results. All data have been compensated against a reference gas-sample passed through an empty cuvette, to account for any changes in background or contamination of the gas-stream at source. Arrows indicate start and finish of the 16-h treatment period.
Figure 3. Effect on ethylene production by roots of *Zea mays* of (a) switching to air after 16-h exposure to 3 kPa O\textsubscript{2} in the presence of 0-1 or 10 kPa CO\textsubscript{2}, or (b) switching to air after 16-h exposure to 0-1 or 10 kPa CO\textsubscript{2} in air. Arrows indicate the time at which roots were returned to air: dashed arrows refer to 10 kPa CO\textsubscript{2} treatments, and solid arrows to 0-1 kPa CO\textsubscript{2} treatments.

Figure 4. Root extension (mm h\textsuperscript{-1}) of *Zea mays* seedlings during 16 h of treatment at the indicated partial pressure of oxygen, and during 6 h when roots were returned to air.
Ethylene production by hypoxic maize roots

Air 12.5 kPa 5 kPa 3 kPa 1 kPa

Figure 5. Roots of Zea mays after 16h of treatment at various sub-ambient partial pressures of oxygen. Roots grown in 3 and 5kPa O₂ are thickened, plagetropic and have many root hairs compared to those grown in 12.5 and 1kPa O₂. Roots in 12.5kPa O₂ were slightly shorter but otherwise similar in appearance to roots grown in air throughout. Root lengths are not to scale.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aerenchyma area (percentage of total cortical area)</th>
<th>±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-8kPa O₂</td>
<td>0.796</td>
<td>0.280</td>
</tr>
<tr>
<td>3.0kPa O₂</td>
<td>11.863</td>
<td>2.006</td>
</tr>
</tbody>
</table>

* Means of several sections from each of 10 roots. Sections analysed were 24h old when first treated with 3kPa O₂ or with air, and 96h old when sectioned.

after anoxia. When anoxic roots were returned to air, a browning of the tissues at the apex was observed, which gradually progressed acropetally in association with tissue contraction. It is not clear whether anoxic roots died during anoxia per se or whether post-anoxic oxidative injury caused the tips to die.

DISCUSSION

The time-courses of ethylene emanation from single root axes of intact seedlings (the first of their kind) were made possible by the very high sensitivity of the laser detector to ethylene. The enclosure of roots in through-flow cuvettes ensured that the roots suffered a minimum of physical perturbation. Problems arising from the excision of segments that characterize the conventional headspace methodology were, thus, avoided. Nevertheless, ethylene production rates declined during the first 5h after transferring the seedlings to the flow-through cuvettes. We suppose that this early high production rate was a response to the, albeit gentle, procedures involved in transferring seedlings to the cuvettes. The enhanced rates may well have been physiologically significant since they were associated with the production of a short region of swollen tissue and prominent root hair production. Such features are typical ethylene effects on roots. In air (20-8kPa O₂), the roots soon grew away normally, leaving behind a swollen segment marking the position of the root tip at the time of transfer. This subsequent normal growth was associated with a low but slowly declining rate of ethylene production. After 6h in air, roots transferred to 12.5kPa O₂ for 16h also continued to form ethylene at slow rates. Therefore, the slightly slower rate of root extension by these roots cannot be attributable to any extra ethylene production. Instead the inhibition was probably a consequence of oxygen shortage inhibiting the action of oxidases (e.g. indole acetic acid oxidase) with low affinities for oxygen. The absence of swelling, root hair production and abnormal gravitropism supports the view that roots in 12.5kPa O₂ produce insufficient ethylene for marked physiological activity. This finding differs slightly from that of previous experiments (Jackson et al. 1985) with excised segments of maize root tip,
that showed a small increase in ethylene when oxygen supply was reduced from 20-8 kPa to 12.5 kPa O₂.

Evidence that oxygen partial pressures below 12.5 kPa can stimulate ethylene production is seen in (1) the marked upward shift in ethylene emanation when the oxygen supply was lowered to 3 or 5 kPa O₂, and (2) an equally prompt downward shift in ethylene formation when the roots were returned to air 16 h later. Earlier work with excised root segments also showed increased production in response to 3 or 5 kPa O₂ (Jackson 1982; Jackson et al. 1985; Atwell, Drew & Jackson 1988). Although the increases in ethylene in the present study were smaller than previously reported, they appear to be sufficient for physiological activity since the roots quickly became swollen and plagiotropic while forming numerous root hairs. All these phenomena can be reproduced in maize roots by applying small amounts of ethylene in air (Bucher & Pilet 1982; Moss et al. 1988).

Furthermore, when roots grown in air or 3 kPa O₂ for 72 h were sectioned through 4-d-old tissue, the latter contained increased amounts of aerenchyma in the cortex. Previous studies with inhibitors of ethylene production have attributed aerenchyma formation in oxygen-deficient roots to the action of ethylene (Jackson et al. 1985).

The extent to which the extra ethylene formed in 3 or 5 kPa oxygen was responsible for inhibiting root elongation is not clear. Previous studies with roots of barley (Hordeum vulgare) indicated that a lack of oxygen rather than ethylene was the more important (Jackson et al. 1984).

There is a notable inconsistency between the present results and those of Jackson et al. (1985) with respect to the effects of 1 kPa O₂. In the present work, decreasing oxygen partial pressures from those of air to 1 kPa did not change ethylene production substantially. A slight increase was seen, but there was no discernible decrease on returning to air. Previous work, with excised root segments showed a large promoting effect (Jackson et al. 1985). However, in accord with earlier findings, no evidence of physiological responses to ethylene were seen under these small oxygen partial pressures, i.e. no swelling or plagiotropism. The very slow rate of root extension and the lack of ethylene responsiveness of roots in 1 kPa O₂ were probably consequences of metabolic lesions, especially to energy metabolism and unsaturated fatty acid synthesis, that could be expected to interfere with growth processes (Vartapetian, Maziak & Lance 1978). However, measurements of root extension show clearly that such lesions were not sufficiently severe to stop growth completely. As little as 0.1 kPa O₂ was found to be sufficient to sustain slight extension over 16 h (result not shown) and others have also shown that some root growth is possible provided only a very small amount of oxygen is present (Laan, Clement & Blom 1991; Waters et al. 1991). In our experiments, it was unlikely that the roots received any supplementary oxygen by internal transport from the shoots via aerenchyma since 16 h treatment was insufficient to enhance intracellular gas space formation. In their absence, the small, unconnected intercellular pores will be highly limiting to oxygen transport (Armstrong & Beckett 1985). Thus, we conclude that the stimulation of ethylene production by 1-0 kPa O₂ previously reported was an artifact of the method employed.

Accumulation of carbon dioxide commonly occurs in flooded soils (Grable 1966) and within the roots of flooded plants, where it may influence ethylene production, metabolism and action (Smith et al. 1985; Sisler & Wood 1988; Hall 1991; Mattoo & White 1991), although the mechanisms are uncertain and probably various. However, little is known about the influence of elevated CO₂ concentrations on root-ethylene production at low partial pressures of oxygen. Thus, it was important to check whether carbon dioxide could interfere with the response of maize roots to oxygen. Our results showed that an increase in carbon dioxide concentration to 0.1 or 10 kPa did not affect ethylene production rates in air or 3 kPa O₂. Hence, the build-up of carbon dioxide in flooded soils is unlikely to interfere with increased ethylene production caused by partial oxygen shortage.

Since ethylene production is extinguished by anoxia there is, obviously, an absolute requirement for some molecular oxygen. Thus, the stimulation of ethylene production by partial oxygen shortage is something of a paradox. However, our results show unequivocally that such a stimulation occurs, and under our experimental conditions, is concomitant with changes in root morphology which may be associated causally with the presence of increased amounts of ethylene. The dependence on molecular oxygen, observed in our experiments, and the evidence of Jackson et al. (1985), that inhibitors of ACC-synthase, such as 1-aminoethoxyvinylglycine (AVG), suppress ethylene production during hypoxia, strongly suggest that biosynthesis in hypoxic maize roots occurs via ACC and the ethylene-forming enzyme complex (EFE). Furthermore, a positive correlation, during hypoxia, between endogenous ACC concentrations, ACC-synthase activity and ethylene production has been observed by several authors (Cohen & Kende 1987; Atwell et al. 1988; Wang & Arteca 1992), which suggests that synthesis in oxygen-deficient cells may be regulated by ACC-synthase. This could be brought about by enhanced transcription of one or more genes coding for ACC-synthase, by post-translational modification of existing mRNA, or by modification of an inactive form of the enzyme. The possibility of down-regulated polyamine biosynthesis releasing additional S-adenosyl methionine for conversion to ACC has been discounted by Jackson & Hall (1993). The ACC oxidizing EFE is thought to be a dioxygenase with considerable sequence homology to flavanone 3-hydroxylase (Hamilton, Lycett & Grierson 1990; Ververidis & John 1991), and its action to be absolutely dependent on molecular oxygen (McKeon & Yang 1987). According
to McGarvey & Christoffersen (1992), the $K_m$ of EFE from avocado fruit for oxygen is $4.6 \pm 0.8 \text{kPa}$. Therefore, assuming that EFE from maize roots has a similar $K_m$, its activity at $3 \text{kPa} \text{O}_2$ would be less than half of that in air. However, EFE appears to be present in quantities in excess of those required to convert the additional ACC to ethylene, since the capacity of roots exposed to $3 \text{kPa} \text{O}_2$ to convert exogenous ACC is similar to that in air (Atwell et al. 1988).

The complete picture may need to take into account spatial separation of the sites of ACC synthesis and its oxidation to ethylene. For example, ACC may accumulate in the anoxic root tip or stele (Jackson 1989) as a result of increased ACC synthase production (Wang & Arteca 1992) or activity. The extra ACC produced in this way may then diffuse into better aerated cortical tissues where it is converted to ethylene in the presence of small amounts of oxygen. Further work is needed to clarify some of these possibilities.

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