How Maize Seeds and Seedlings Cope with Oxygen Deficit

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When flooding occurs, the O_2 concentration in the soil declines gradually (Blackwell, 1983) and roots undergo a period of hypoxia before O_2 is fully depleted from the soil and anoxia is reached. (We use the terms hypoxia and anoxia as described by Pradet and Bomsel (1978). Hypoxia is a condition in which the concentration of O_2 limits ATP production in the mitochondria. Anoxia is defined as a condition in which the concentration of O_2 is so low that virtually no ATP is produced by the mitochondria.) When these conditions are simulated in the laboratory, by pretreating roots with low O_2 before subjecting them to anoxia, root tips survive longer than those made anoxic without a hypoxic pretreatment. This response was first described by Saglio et al. (1988) and has been termed "hypoxic acclimation."

There is a pronounced difference in the responses of acclimated and unacclimated seminal maize (*Zea mays* L.) roots to anoxia. At the onset of anoxia, energy production declines, there is acidosis of the cytoplasm, and cell death occurs within 15 to 20 h (Johnson et al., 1989; Roberts et al., 1984a, 1984b; Saglio et al., 1988). Thus, after 24 h of anoxia, there is little remaining cellular organization of unacclimated root tip cells. The only recognizable organelle is the nucleus (Fig. 1). In contrast, acclimated root tips survive 96 h of anoxia (Johnson et al., 1989). Acclimated root tips subjected to anoxia appear to maintain normal cellular organization through 48 h (Fig. 1) and possess higher ATP concentrations and higher rates of anaerobic metabolism than unacclimated root tips (Hole et al., 1992; Johnson et al., 1989; Saglio et al., 1988).

Clearly, hypoxia induces physiological responses that give rise to improved resistance to anoxia, and the identification of those responses has been the focus of research for several years. The present report discusses the role of hypoxia in the induction of anaerobic metabolism. This is not intended to be a general review of the response of plants to O_2 deficit (for such a review, see Kennedy et al., 1992; Ricard et al., 1994). We first examine how different methodologies to induce O_2 deficit can give rise to contrasting conclusions. Second, we present data suggesting that seeds and young seedlings are more resistant to anoxia than older seedlings. We conclude with a review of our data and those of others concerning the role of hypoxia in extending survival of anoxia in maize seminal root tips.

METHODS OF INDUCING O_2 STRESS

While the anaerobic response of maize has been the subject of extensive study (reviewed in Bailey-Serres et al., 1988), a wide variety of methods have been used to induce O_2 deficit. In general, germinated seedlings of varying ages are completely immersed in solutions that are either left open to the air, sealed without sparging, or sparged with gases (for examples, see Hwang and VanToai, 1991; Peschke and Sachs, 1994; Rowland and Strommer, 1986; Russell and Sachs, 1992; Sachs et al., 1980). Interestingly, the method that is used to induce O_2 stress can result in measurable differences in the extent and localization of the response.

There is evidence that cells within the root apical meristem, where respiration rates are high, can be subjected to a mild hypoxia when grown at ambient O_2 concentrations at room temperature (Andrews et

al., 1993). Our method is to pretreat seedlings by sparging with 40% O_2 for 18 h to ensure that they are fully aerobic before the onset of the experimental hypoxic treatment, anoxic treatment, or both. Seedlings are not immersed, but are placed on floats in containers so that the roots, but not the shoots, are immersed in solution. The containers are then sealed and continuously sparged with gas of known composition. Thus, even though the shoots are not submerged, they are exposed to the same gaseous atmosphere as the roots. For the hypoxic pretreatment (HPT), seedlings are sparged with 4% O_2 for 18 h before the anoxic treatment. Seedlings that are not hypoxically pretreated (NHPT) are transferred directly from 40% to 0% O_2 (100% N_2).

Alcohol dehydrogenase has long been used as a marker for fermentation and we have compared the induction of its message (*adh1*) using our methods with those used by other researchers. Both submerged and NHPT-treated plants show a similar response in root tips and root axes (Fig. 2). There is little *adh1* transcript in root tips in submerged or NHPT plants at 24 h of treatment (Fig. 2), but there is induction in the root axes. The root tips of HPT seedlings contain a higher concentration of *adh1* transcript after 24 h than root axes or shoots. This result suggests that, when seedlings are submerged in a buffer solution, the dissolved O_2 is depleted at a higher rate than necessary for hypoxic acclimation to occur. However, note that the seedlings for the immersion treatment had been pretreated with 40% O_2 . As already noted, root tips of maize seedlings that are maintained at 25°C in 21% O_2 are already under hypoxia (Andrews et al., 1993) so that seedlings that had not been pretreated with 40% O_2 before immersion probably more closely mimic the HPT treatment.

Although the root tips die, the root axes of NHPT plants survived more than 24 h of anoxia (Fig. 2) and have survived as long as 70 h (Sachs et al., 1980). We have found that the zone of maximal ADH activity in the seminal root is not uniform and changes during 24 h of anoxia (Fig. 3). Primary root axes, 8 cm long, were divided into 2-cm segments after the 0.5-mm root tips sections had been removed, and ADH activity then was determined in each segment during 24 h of anoxia (Fig. 3). At time 0, corresponding to the 18-h 40% O_2 pretreatment, there was a slightly higher level of ADH enzyme activity in the root axes than in the root tips. During anoxia, there was a transient rise in ADH activity in the root tip through 6 h; then activity declined to near 0 at 18 h. The 2-cm section adjacent to the root tips exhibited a decline throughout the treatment; however, ADH activity increased in the more distal segments of the root through 24 h.

RESPONSE OF SEEDS AND YOUNG SEEDLINGS TO ANOXIA

In most of our work, we have used root tips from roots that are 8 to 10 cm long (≈ 5 days after germination). Recently, we compared the response of these root tips (L) with that of root tips from shorter roots (S) (≈ 3 days after germination). Interestingly, S roots did not need a hypoxic pretreatment to survive 12 h of anoxia, whereas L root tips required 18 h of hypoxia to survive (Andrews et al., 1994b). This finding has lead us to examine the response of unimbibed seeds and germinating seeds to anoxia. In this experiment, unimbibed maize seeds or seeds that had germinated for 1, 2, or 3 days were placed in anoxia for 2, 4, or 6 days, then returned to ambient air. Survival was gauged as the ability of the roots to resume growth. While they did not germinate during the anoxic treatment, seeds that were imbibed during anoxia germinated when returned to air (Table 1) and retained this ability even after 10 days of anoxia (data not shown). Leblova (1978) suggested that imbibed, germinated seeds in general are anaerobic

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Fig. 1. Electron micrographs of maize root cells ($\times 7200$). (A) Hypoxic pretreatment (HPT) after 24 h of anoxia; (B) HPT after 48 h anoxia; (C) after the 18 h HPT; (D) Nonhypoxically pretreated (NHPT) maize root tip cells after 24 h of anoxia. All are sections from the central core of the meristematic zone.

until radicle emergence. While this observation is not accurate for all seeds, maize seeds are known to have a relative high rate of fermentation in air and can germinate at low O_2 concentrations (Al-Ani et al., 1985; Raymond et al., 1985). Since they did not germinate during anoxia, perhaps maize caryopses extend the anaerobic period of germination until O_2 is present and germination can proceed.

Caryopses that were germinated in air for 1 to 3 days survived at least 2 days of anoxia (Table 1). Seeds that were germinated for 3 days before anoxia survived anoxia for only 2 days. This decline in survival coincides with root emergence, which occurs between 2 to 3 days at 25C. Therefore, while very young roots are more resistant to anoxia than are roots from older seedlings, they are not nearly as resistant as seeds.

Fig. 2. *Adh1* mRNA levels (as cpm · μg⁻¹ total RNA) in root tips, root axes, and shoots of maize seedlings using two methods to induce O₂ stress. For the submerged treatment, seedlings were first pretreated with 40% O₂, then completely submerged in a beaker filled with a buffered solution. The solution was neither sparged nor the beaker sealed for 24 h. Nonhypoxic pretreatment and hypoxic pretreatment were as described in the text.

Fig. 3. Distribution of ADH activity during 24 h of anoxia of nonhypoxically pretreated maize seminal roots. Distances from root tip.

ROLE OF HYPOXIA IN INDUCING FERMENTATION

Oxidative phosphorylation is minimal when O₂ is limited and roots must maintain ATP production through glycolysis-fermentation (Johnson et al., 1989; Roberts et al., 1989; Saglio et al., 1980). ATP production through these pathways is too limited to sustain root growth (net yield is 2 mol ATP/mol glucose for fermentation vs. ≈36 by oxidative phosphorylation), and extended periods of O₂ stress result in cell injury and, subsequently, death (Drew, 1988; Jackson and Drew, 1984; Saglio et al., 1984). In plants, the major product of fermentation is ethanol (Smith and Ap Rees, 1979), although conversion of pyruvate to lactate under anoxia does occur and may play a critical role in hypoxia-induced anoxia tolerance. The pathways for these conversions are summarized in Fig. 4. Briefly, pyruvate can be converted into lactate by lactate dehydrogenase (LDH). Alternatively, pyruvate is converted by pyruvate decarboxylase (PDC) to acetaldehyde and then by alcohol dehydrogenase (ADH1 and ADH2) to ethanol. ADH1 specific activity is 10- to 20-fold higher than that of ADH2 and is the predominant enzyme that catalyzes this reaction (Freeling and Bennett, 1985; Sachs et al., 1985).

Induction of ethanolic fermentation

A key feature that occurs during hypoxia is the induction of the ethanolic fermentative pathway (Hole et al., 1992; Xia and Saglio, 1992). After 18 h of HPT, ≈75% of the CO₂ produced in an atmosphere

Table 1. Survival of seeds after exposure to anoxia for 2, 4, or 6 days. Seeds were germinated in petri dishes in distilled water at 25°C for either 1, 2, or 3 days before transfer into anoxia. For 0 time, unimbibed seeds were placed in an anaerobic workbench before distilled water (sparged with nitrogen) was added. After the anoxic treatment, seeds were returned to ambient air and survival was based on the ability of the seeds to germinate or on regrowth of roots. Roots emerged between 1 and 2 days of germination and root length at 3 days was ≈1 cm. In comparison, average root length of seedlings following germination at 28°C is ≈5 cm after 3 days.

Days of germination before anoxia	Days in anoxia		
	2	4	6
	Germination (%)		
0	98.1 ± 3.2	90.2 ± 4.6	91.3 ± 7.6
1	81.5 ± 5.3	88.3 ± 8.2	52.6 ± 7.9
2	76.4 ± 6.1	66.5 ± 5.9	13.2 ± 8.2
3	88.3 ± 2.9	6.2 ± 3.2	8.2 ± 4.2

Fig. 4. Overview of ethanolic and lactate fermentative pathways. Thick arrows mark the normal flow of carbon through oxidative phosphorylation. During hypoxia there is an induction of a sustained ethanolic fermentation (thin arrows). Whereas ethanol can freely diffuse from cells, lactate cannot and hypoxia appears to induce a transport mechanism to remove lactate from the cells (Xia and Roberts, 1994).

of 4% O₂ can be accounted for through fermentation by excised maize root tips (Table 2). Both HPT and NHPT root tips show similar initial increases in the rate of glycolysis and ethanol production during the first hour of anoxia (Hole et al., 1992). However, HPT root tips can sustain a higher rate of ethanol production than NHPT root tips (Hole et al., 1992; Xia and Saglio, 1992). Additionally, HPT root tips from intact maize plants have higher levels of ATP and total adenylates than do NHPT root tips (Johnson et al., 1989).

All of these data indicate that fermentation is induced during hypoxia, and it is not surprising that genes involved in either carbohydrate metabolism, glycolysis, or fermentation have been identified as being among those induced by anaerobic stress. These genes include those for glucose phosphate isomerase (Kelley and Freeling, 1984a), pyruvate decarboxylase (*pdc*) (Kelley, 1989; Laszlo and Lawrence, 1983), glyceraldehyde-3-phosphate dehydrogenase (*gpc 3 & 4*) (Russell and Sachs, 1992), aldolase (*ald*) (Kelley and Freeling, 1984b), alcohol dehydrogenase (*adh1* and *adh2*) (Dennis et al., 1985; Ferl et al., 1980; Gerlach et al., 1982; Rowland and Strommer, 1986; Sachs et al., 1980), Suc synthase (Springer et al., 1986), and lactate dehydrogenase (Good and Paetkau, 1992); their induction under O₂-limited conditions in maize seedlings has been well characterized at the molecular level. There is also evidence for both transcriptional and translational regu-

Table 2. Carbon dioxide evolution, ethanol production, and O₂ uptake by maize root tips in 4% O₂ after 18 h hypoxic pretreatment. (Determinations were made as described in Hole et al., 1992.)

Criterion	Quantity ($\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$)
CO ₂ evolved	16.9 $\pm$ 1.03
Ethanol production	13.9 $\pm$ 0.87
O ₂ uptake	3.7 $\pm$ 1.17

^aFresh weight basis.

lation (Bailey-Serres and Freeling, 1990; Bailey-Serres et al., 1988; McElfresh and Chourey, 1988; Sachs et al., 1980). Genes from the glycolytic pathway and the fermentation pathway share a consensus sequence in the promoter that may be required for anaerobic expression (Walker et al., 1987).

We have examined the induction of the glycolytic genes for aldolase (*ald1*) and enolase (*eno1*) as well as for *adh1*, *adh2*, and *pdh* from the ethanolic fermentative pathway in root tips of intact maize seedlings (Andrews et al., 1993, 1994b). In general, mRNAs from genes of the fermentative pathway were induced during hypoxia and showed a broadly similar pattern of expression. When expressed on the basis of relative induction, *adh2* and *pdh* were induced to a higher degree than *adh1*, *eno1*, and *ald1* (Fig. 5). Both *adh2* and *pdh* reach a peak of induction at 12 h (16- and 13-fold, respectively). *Adh2* declined to near the initial aerobic level by 18 h. After the 18-h treatment, *pdh* mRNA had declined below that of the initial aerobic level (data not shown). *Adh1* transcripts reached a maximum induction of $\approx$ 3-fold at 6 h and had declined slightly by 18 h (Fig. 5). There was only slight induction of *eno1* and *ald1* (Fig. 5). These transcripts are abundant under aerobic conditions, and further induction may not be as critical if the need for pyruvate under hypoxia does not exceed that needed under aerobic conditions. It is also important to note that mRNAs for all three genes specific for the ethanolic fermentative pathway were present, albeit in very low amounts, after the 40% O₂ pretreatment (time point 0, Fig. 5).

During anoxia, HPT root tips had a greater induction of transcripts than did NHPT root tips (Fig. 6). *Adh1* showed a slight increase of $\approx$ 3-fold through 18 h, then declined in HPT root tips, but declined throughout the treatment in NHPT root tips (Fig. 6). *Adh2* transcripts in HPT root tips reached a maximum induction at 6 h, $\approx$ 13 fold over the initial aerobic level, and had declined to near 0 at 48 h. In NHPT root tips, *adh2* reached a maximum induction at 24 h. *Ald1* was induced during anoxia in both HPT and NHPT root tips, although induction was greater and was maintained for a longer period of time in HPT root tips (Fig. 6). Neither *pdh* nor *eno1* was induced during anoxia in either HPT or NHPT root tips, and both declined through the duration of anoxia.

We have also examined how hypoxia and anoxia change the activity of the enzyme ADH (Andrews et al., 1993; Johnson et al., 1989, 1994) and have recently extended this to PDC. During hypoxia, ADH and PDC activity increased throughout 18 h in root tips of intact seedlings. During anoxia, these same root tips showed a higher level of ADH and PDC activity through 96 h than did NHPT root tips (Johnson et al., 1989). In NHPT root tips, ADH1 activity peaked at 6 h of anoxia and declined thereafter (Andrews et al., 1993), whereas PDC activity was low at the onset of anoxia and did not increase.

Gauging the relative importance of *adh1* and *pdh* in regulating ethanolic fermentation is of interest. Roberts et al. (1989), using a series of isogenic maize lines that differed in only ADH activity, showed that only a low level of ADH was necessary for survival so that the significance of the large increase in ADH activity remains unclear. Unacclimated *adh1-null* root tips will only survive 6 to 12 h of anoxia (Johnson et al., 1994; Roberts et al., 1984a, 1984b). However, *adh1-null* root tips survived anoxia longer after hypoxic acclimation (Johnson et al., 1994). PDC increased linearly with time for the duration of hypoxia in intact root tips of wild type, and *adh1-null* and the concentration of PDC more closely paralleled ethanol production than did the concentration of ADH. This result suggests that regulation of *pdh* may play a key role in inducing fermentation.

Fig. 5. Relative levels of *adh1*, *adh2*, *pdh*, *ald1*, and *eno1* mRNA in root tips during hypoxia. Message levels are expressed relative to the level after the 40% O₂ pretreatment.

Fig. 6. Relative levels of *adh1*, *adh2*, *pdh*, *ald1*, and *eno1* mRNA in root tips during anoxia of HPT and NHPT. Message levels are expressed relative to the level after the 40% O₂ pretreatment.

Evidence for induction of a lactate transport mechanism during hypoxia

A determinant of cell death under anoxia is cytoplasmic acidosis (Roberts et al., 1984a, 1984b). Davies (1980) first proposed that the initial production of lactate under anoxia results in a lowered cytosolic pH, activates pyruvate decarboxylase and ethanolic fermentation, and inhibits lactate fermentation. Roberts et al. (1984a, 1984b) showed that, in unacclimated maize root tips, the cytosolic pH declined during the first 20 min of anoxia and then stabilized coincident with a burst of lactate production. Periods of anoxia longer than 10 h resulted in a further acidification of the cytoplasm and cell death.

The improved regulation of cellular pH following hypoxic acclimation may be attributed in part to the removal of lactate from the cells. Xia and Saglio (1992) and Xia and Roberts (1994) have shown that lactate is transported out of HPT root tips, whereas lactate accumulated in NHPT root tips. Xia and Roberts (1994) suggest that hypoxia may induce synthesis of a lactate transporter, thus minimizing cellular accumulation of lactate and cytoplasmic acidosis.

In contrast, Saint-Ges et al. (1991) have shown that cytosolic pH

changes before lactate levels rise and the change in pH coincides with a decrease in ATP. Production of alanine and regulation of malate levels also are involved in regulation of cytoplasmic pH under conditions of low O₂ (Roberts et al., 1992).

CONCLUSIONS

The procedures used to induce O₂ deficit must be taken into consideration when comparing results from different laboratories. In many cases, the exact O₂ concentration is not controlled and it is probable that some degree of acclimation has occurred in many of the studies of the anaerobic response in maize.

Maize seeds and young seedling roots are more resistant to anoxia than older seedlings. Older maize seedling roots require a hypoxic pretreatment to survive anoxia. This acclimation is associated with the induction of genes that are involved in ethanolic fermentation, thus allowing for ATP production during anoxia. Acclimation also appears to induce the transport of lactate from root cells, thus improving the regulation of the cytoplasmic pH during anoxia.

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