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Lactic Acid Efflux as a Mechanism of Hypoxic Acclimation of Maize Root Tips to Anoxia

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ABSTRACT

Hypoxic pretreatment (3 kPa oxygen) of maize (*Zea mays* L.) root tips improved their survival time in a subsequent anoxic incubation from 10 h to more than 3 d, provided that glucose was added to the medium to sustain metabolism. The glycolytic flux (lactate + ethanol) was the same in both pretreated and untreated root tips during the 1st h after transfer to anoxia. It was only after 2 h that it declined sharply in untreated tips, but was sustained in pretreated ones. Right after the transition from normoxia to anoxia of untreated root tips, the only fermentative product detected was lactic acid, which accumulated in a 7:1 proportion after 30 min in tissue and medium, respectively. It took 10 min before ethanol could be detected and 20 min for it to be produced at its maximum rate at the expense of lactate production, which slowed down. In contrast, in hypoxically pretreated root tips, ethanol was produced at a maximum rate right after the transfer to anoxia. Concurrently, low amounts of lactic acid were produced that accumulated in a 1:1 proportion after 30 min in tissue and medium, respectively. This large efflux of lactic acid could account for the higher cytoplasmic pH values always found in pretreated tissues. The presence of cycloheximide during pretreatment abolished this difference, suggesting that the greater efficiency of lactate efflux was linked to protein synthesis. The role of lactate in cytosolic pH regulation and in sensitivity to anoxia is discussed.

Most mesophytes, including maize, are intolerant to anoxic conditions. However, pretreatment at low oxygen pressure induces mechanisms that improve their survival capacity under subsequent anoxic conditions. These mechanisms lead to two kinds of survival strategies. One consists of the formation of lysigenous cortical aerenchymas, helping to maintain a high respiration rate in the tissues by improving internal oxygen transport (5). This is a slow (days) and nonreversible mechanism induced by ethylene (4). The second strategy consists of the metabolic acclimation that apparently improves the energy relationships of anoxic tissues and contributes to a more prolonged survival in the absence of oxygen (9, 25). This response is fast (hours) and involves the induction of a limited number of "anaerobic polypeptides" (23). These polypeptides are different from the "heat shock proteins" (14), and those that have been identified are enzymes involved in the glycolytic and fermentative pathways (18, 19, 24). However, the significance of these proteins in tolerance to anoxia has rarely been examined and remains unclear. The results obtained by Hanson and colleagues (6, 7) on hypoxically inducible LDH[1] in barley roots and aleurone tissues imply some unrecognized function for this enzyme. Furthermore, there is evidence that ADH levels, which are known to increase dramatically during hypoxic acclimation, are not correlated with the better survival capacity of acclimated maize root tips in anoxia (22).

Metabolic studies of survival of plant tissues in the absence of oxygen have focused mainly on the possible toxicity of the end-products of fermentation. In the 1970s, Crawford (1) proposed that sensitivity to anoxia was proportional to the rate of ethanol production. This idea is now ruled out (8) because of the high diffusion rate of ethanol in the external medium and its low toxicity for plant tissues in anoxia, as illustrated by rice, which is extremely resistant to anoxia despite a very high rate of ethanol production (19). However, ethanol can be indirectly toxic to some tissues by its metabolism to acetaldehyde when they are returned to aerobic conditions, as illustrated with carrot cells (15). More recently, Roberts et al. (21), studying the cytoplasmic acidosis of maize root tips by ^{31}P NMR during the transition from normoxia to anoxia, concluded that the initial acidosis observed is attributable to a transient accumulation of lactic acid, which provides the signal triggering ethanol production (2). The death of the root tips in the absence of oxygen is attributed to a poor regulation of cytoplasmic pH, which is controlled by the balance between lactic and ethanol fermentation (20). However, recent studies also using ^{31}P NMR show that the fast initial cytoplasmic acidification and lactate accumulation do not have similar time courses (13, 28); therefore, lactic acid alone does not account for the initial cytoplasmic acidification in anoxic maize root tips.

In this article, we examine the effects of a hypoxic pretreatment (acclimation) of maize (*Zea mays* L.) root tips on their metabolism and their survival in the absence of oxygen, in comparison with nonacclimated tips. We show that an external supply of sugar is necessary for survival of acclimated root tips. We also show a clear difference in the kinetics of lactic acid and ethanol accumulation between the kinds of root tips. This difference is enhanced by a more efficient efflux into the external medium of lactic acid produced in anoxia by acclimated tissues. This efflux seems linked to protein synthesis during hypoxic pretreatment. The role of

[1] Abbreviations: LDH, lactate dehydrogenase; ADH, alcohol dehydrogenase; NHPT, not hypoxically pretreated; HPT, hypoxically pretreated; AEC, adenylate energy charge; DMO, 5,5-dimethyl oxalolidine-2–4-dione; ΔpH, change in pH.

lactic acid in the death of nonacclimated tissues in the absence of oxygen is discussed according to these results and the recent literature.

MATERIALS AND METHODS

Plant Material and Pretreatments

Maize (*Zea mays* L. DEA, Pioneer) seeds were germinated in the dark at 25°C on wet filter paper. Three days after imbibition, germinated seedlings were transferred to 0.7 L of mineral solution (19) in 1-L gas-tight jars fitted with inlet and outlet tubing for gas flow. Seedlings were inserted individually through holes of an expanded polystyrene "raft" floating on the surface of the solution, which was bubbled (at about 10 $L \cdot h^{-1}$) with compressed gas mixtures of either 50% (v/v) O_2 in N_2 (NHPT root tips) or 3% (v/v) O_2 in N2 (HPT root tips), equivalent to O_2 partial pressure of 3 kPa, which is approximately one-seventh of that in ambient air (20.6 kPa). According to reference 26, the 50% O_2 treatment had no deleterious effect on the rate of root tip elongation as compared with air treatment. This treatment using an O_2 concentration above the critical O_2 pressure (30 kPa) was deliberately chosen to ensure that tissues would never experience hypoxia. During pretreatments, the same gaseous atmosphere was maintained around the shoots and the roots to avoid internal O_2 movement from one to the others. In some experiments, cycloheximide (15 $\mu g \cdot mL^{-1}$) was added to the medium to block protein synthesis during pretreatment. Unless otherwise stated, 3-mm excised primary root tips (2.2 mg fresh weight·tip-1) were used in most experiments.

Anaerobic Treatments

Root apices were placed in 5- or 10-mL disposable syringes containing 4 or 8 mL of 5 mM Mops buffer (pH 6.2), which was prepared in the above nutrient medium containing 10 $mL \cdot L^{-1}$ of an antibiotic-antimycotic mixture (Sigma A-7292), and were either supplemented or not with 100 mM glucose. The syringes with rubber puncture caps were fitted with 12 × 0.45 mm needles on rubber vacuum tubes flushed with N_2 containing less than 4 $\mu L \cdot L^{-1}$ O_2.

Survival Assessment and Adenine Nucleotide Determinations

After various incubation times in anoxia, batches of 10 root tips were transferred for 30 min to air to allow respiration to resume and the ATP to increase in living cells. After 15 min, the roots were dipped in boiling water for 3 min and the volume of the extract was adjusted to 5 mL for 10 tips. ATP was assayed by a bioluminescent luciferin-luciferase reaction. The root tips were considered dead when their residual ATP content represented 10% or less of the initial value found before the anoxic treatment, and according to the concurrent loss of tissue turgidity.

In some experiments, root tips were excised after various hypoxic pretreatments, and their AEC was determined after 1 h of incubation in anoxic conditions according to ref. 28.

Extraction and Assay of LDH and ADH

Batches of 10 tips were ground in an ice-cold homogenizer in 200 μL of 0.1 M Tris-HCl buffer, pH 8.5, containing 10 mM Na borate, 10 mM DTT, 5 $mg \cdot mL^{-1}$ BSA, and 15% (v/v) glycerol according to ref. 6. LDH was assayed in the pyruvate to lactate direction as well as in the reverse one, and ADH activity was assayed in the ethanol to acetaldehyde direction according to ref. 7.

Determination of Lactic Acid and Ethanol

Root apices (20–30) were placed in agitated sealed penicillin vials containing 2 mL of Mops buffer (pH 6.2) in nutrient medium, supplemented with 100 mM glucose. Anoxia was obtained by flushing for 5 min with N_2 before starting the experiment in a confined atmosphere. After various incubation times in anoxia, the medium was collected and kept frozen until assayed. For lactic acid determinations, the root tips (20–30) were rapidly rinsed and blotted before grinding in 250 μL of 1.2 M perchloric acid at 4°C. After three rinses with 250 μL of perchloric acid, the brei was neutralized by adding about 300 μL of 5 M KOH in the presence of 10 μL of 0.05% methylorange, and then centrifuged for 15 min at 8000*g* at 4°C in an Eppendorf 5414 centrifuge. The supernatant was frozen until further analysis.

Lactic acid and ethanol determinations were performed according to ref. 27. Samples were read on a double-beam spectrophotometer against a reference containing the extract without enzymes to balance the color shift of the extract, which was not stable with time.

Assessment of Cytoplasmic pH and Membrane Permeability

The cytoplasmic pH was estimated by the distribution of DMO between the tissues and the external medium according to references 3 and 11. The pH could be calculated according to the following simplified formula, adapted to the 3 mm maize root tips, in which the vacuolar volume could be considered as low:

$$pH_i = pK_a + \log[C_i/C_e \times (1 + 10\ pK_e - pK_a) - 1]$$

where pK_a is the pK of the probe (pK DMO = 6.4 at 25°C), C_i and C_e are the internal and external concentrations of the probe at equilibrium, and pK_e is the pK of the external medium.Thirty to fifty root tips were placed in 5-mL disposable syringes in 2 mL of 10 mM Mes/BTP (pH 5.42) containing 0.5 mM $CaSO_4$ and 2.5 mM K_2SO_4 and were continuously bubbled by a gas mixture of the desired composition (50:50 O_2:N_2 or N_2). Labeled [^{14}C]DMO (Commissariat à l'Energie Atomique, 27 μCi/mmol, 6 μM final concentration) was injected into the medium at time zero. After various times, the root tips were rinsed three times for 1 min with 20 mL of the same ice-cold buffer, which contained 6 μM unlabeled DMO, and then were immediately placed in a vial containing 2 mL of scintillation liquid (ACS II, Amersham Corp.). Radioactivity was determined 2 h later by scintillation counting, because 99% of the radioactivity was extracted in the scintillation liquid by that time, as checked by counting the vials after

removing the tissues. The data obtained in normoxic conditions were used for assessment of the cytosolic volume, assuming a value of 7.5 for the cytosolic pH.

To check for the possible induction of a modification of membrane permeability induced during hypoxic pretreament, influx of labeled DMO in HPT and NHPT root tips and its subsequent efflux were studied in oxygenated conditions as described above. After loading with [^{14}C]DMO, the root tips were rinsed once with 20 mL of the same medium devoid of DMO and the radioactivity was measured at various times in the tissues and the medium separately.

RESULTS

Sugar Supply and Survival in Anoxia

According to the stability of their ATP content and the turgidity of the tissues (not shown), the results in Figure 1 show that the survival of NHPT root tips in anoxia was very short. It was as short as 10 h in the absence of added glucose in the external medium, and did not last more than 13 h in the presence of 100 mM glucose. HPT root tips did not survive very much better in the absence of exogenous glucose, but unlike NHPT root tips, they were able to survive up to 3 d in the presence of glucose.

Anoxic Energy Metabolism, ADH and LDH Activities

We followed the in vitro activities of ADH and LDH during hypoxic pretreatment. As shown in Figure 2, both activities increased up to 48 h in hypoxia. The maximum increase in LDH activity was low (2-fold) in contrast to ADH activity, which increased up to 7-fold.

In anoxia, HPT root tips have a higher AEC, as already reported (25), but it is interesting to note the time course of this modification. Figure 2 shows that hypoxic pretreatment induced an increase in the AEC value in anoxia. The increase was maximal after 3 h of pretreatment and remained stable for longer pretreatment periods. Survival experiments (not reported here) show that after a hypoxic treatment as short as 3 h, maize root tips are acclimated to anoxia and can survive more than 24 h in the absence of O_2. At the same time (3 h of hypoxic pretreatment), in vitro LDH and ADH activities are not very different from those found in the NHPT root tips.

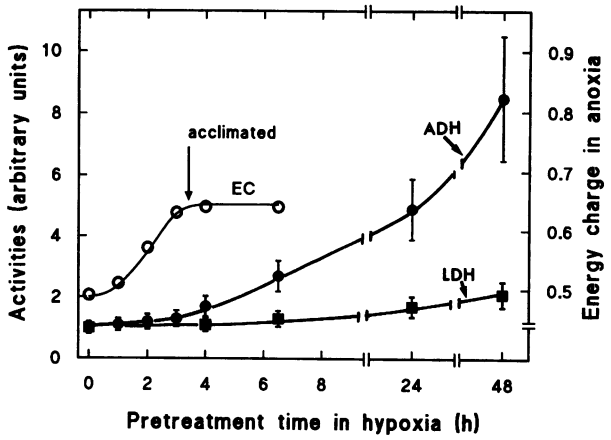

Figure 2. Variation of different metabolic markers as a function of the hypoxic (3% O_2) pretreatment time. O, AEC values were obtained after transfer of the root tips to anoxia for 1 h. One arbitrary unit for ADH and for LDH corresponded to 0.24 and 0.05 μmol min^{-1} mg^{-1} protein, respectively. Data are means of two replicates. Bars represent the measurement interval. For AEC, the interval was less than the width of symbol.

Kinetics of Lactic Acid and Ethanol Accumulation

The above results prompted us to study the kinetics of ethanol and lactic acid production in anoxia, which gives a good assessment of the glycolytic flux in maize root tips (27). After the transfer of NHPT root tips to nitrogen, the first end product synthesized at a measurable rate was lactic acid (Fig. 3A). Ethanol was produced at a significant rate only after a

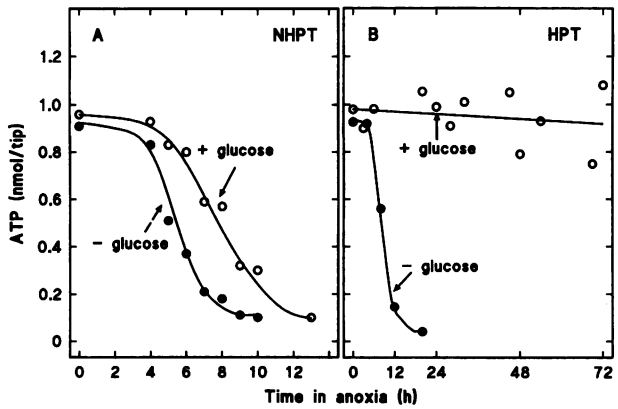

Figure 1. Survival in anoxia of freshly excised root tips. A, Not acclimated in hypoxia (NHPT root tips); B, acclimated for 18 h in 3 kPa O_2 (HPT root tips). O, In the presence of 100 mM added glucose; ●, in the absence of glucose.

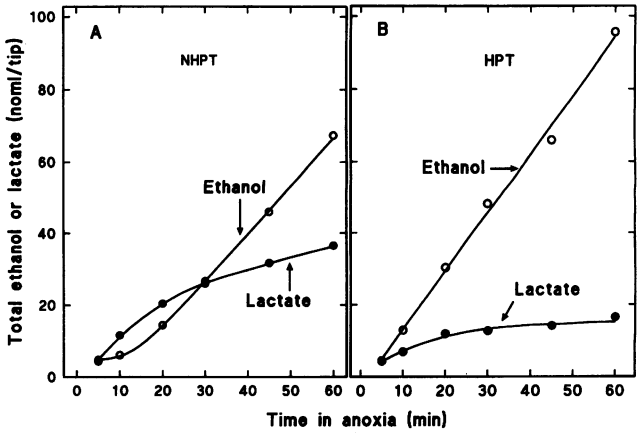

Figure 3. Time course of total (root tips + medium) ethanol and lactate production by acclimated (HPT) and nonacclimated (NHPT) maize root tips after transfer to anoxia. Each data point was obtained from 20 to 30 root tips and is the mean of triplicate measurements. SE was of the same order of magnitude as the symbols.

10- to 15-min lag period. In contrast, with HPT root tips (Fig. 3B), ethanol was produced at its maximum rate almost immediately after the transfer to nitrogen. Total lactic acid production by HPT root tips was low in comparison with NHPT root tips, but in both treatments the production rate of lactate decreased rapidly. Despite very different kinetics for lactic acid and ethanol synthesis by NHPT and HPT root tips, the sum of the two metabolites gives almost identical values (Fig. 3) during the 1st h of incubation in nitrogen (104 and 111 nmol·tip^{-1}·h^{-1} for HPT and NHPT root tips, respectively), thereby showing that the fermentative flux was not modified by hypoxic pretreatment. For longer anaerobic treatments, however, the rate of ethanol production declined in both kinds of roots, but remained about 4-fold higher in HPT (34.5 nmol·tip^{-1}·h^{-1}) as compared to NHPT (8.75 nmol·tip^{-1}·h^{-1}) root tips (Fig. 4). Results concerning the distribution of lactic acid between the tissues and the surrounding medium are shown in Figure 5. The HPT root tips (18 h at 3 kPa O_2) accumulated only a small amount of lactate, but despite its lower concentration, the initial rate of lactate efflux (12.5 and 4.4 nmol·tip^{-1}·h^{-1} for HPT and NHPT root tips, respectively) was 3-fold higher than in NHPT root tips, and 65% of the lactate produced after 80 min of anoxia was in the medium. The amount of total lactate produced in anoxia depended on the time of hypoxic pretreatment. With a short acclimation treatment (4 h in hypoxia), lactate production was almost the same as with NHPT root tips (Fig. 6). But here again, 70% of the lactic acid was found in the medium after 80 min of anoxia. With NHPT roots, this proportion was inverted.

A similar experiment was done with root tips incubated in the presence of cycloheximide during acclimation pretreatment to stop the synthesis of proteins induced in hypoxia. As shown in Figure 6, the total lactate production was reduced to 41 and 57% of the control in NHPT and HPT root tips, respectively, indicating an overall effect of cycloheximide on the metabolism. However, the large efflux of lactate from HPT root tips was abolished and the percentage found inside and outside of the tissues was close to the control, suggesting that lactate efflux in HPT root tips might be linked to protein synthesis.

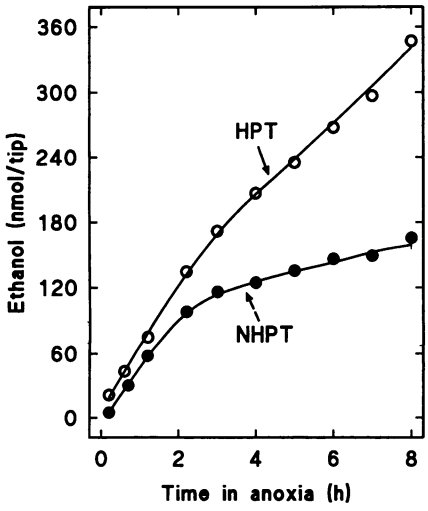

Figure 4. Time course of total ethanol production by acclimated (HPT) and nonacclimated (NHPT) maize root tips during long-term incubation time in anoxia. Each data point was obtained from 20 to 30 root tips.

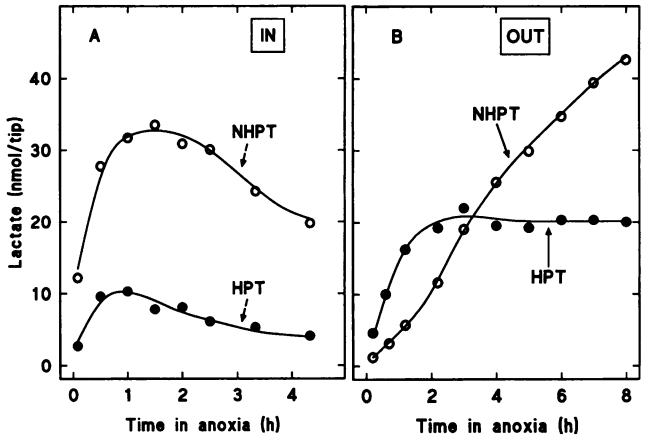

Figure 5. Time course of lactic acid accumulation inside (A) or outside (B) of acclimated (HPT) and nonacclimated (NHPT) maize root tips in anoxia. Each data point was obtained from 20 to 30 root tips.

Cytoplasmic pH

Root tips (3 mm) are mainly meristematic tissues with very small vacuoles, and vacuolar pH is very acidic in comparison to the cytoplasmic pH. Therefore, most of the internal DMO should be located in the cytosol (3, 11). The main problem concerns the determination of the cytosolic volume for cal-

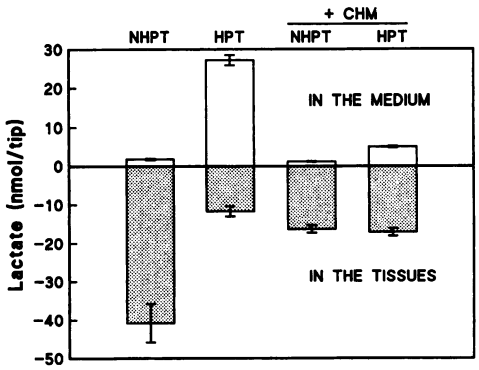

Figure 6. Effect of cycloheximide (CHM) during hypoxic pretreatment on lactic acid excretion by anoxic maize root tips. Maize seedlings growing for 14 h in nutrient medium bubbled with a 50% O_2 gas mixture were pretreated as follows: NHPT roots were grown for an additional 6 h in the same conditions. HPT roots were grown for 2 h in the same conditions, followed by 4 h of bubbling with a 3% O_2 gas mixture. NHPT + CHM, pretreated in the presence of cycloheximide (15 μg mL^{-1}). HPT + CHM, pretreated in the presence of cycloheximide. After pretreatment, the tips were excised and rinsed before anoxic treatment. Lactic acid was assayed in the tissues and in the medium after 80 min of incubation as described in "Materials and Methods." Data are means ± SE for triplicate samples of 30 root tips.

culation of the DMO internal concentration. However, the cytoplasmic pH of many tissues in normoxia is very well regulated and stable around values close to 7.5 (10, 20). This value has recently been confirmed with normoxic maize root tips of the same genotype used in this experiment, even after a preincubation time of 1 h in anoxic conditions (28). The internal concentration of labeled DMO in root tips reached an equilibrium value after 2 h in anoxia, regardless of whether DMO had been loaded in the root tips prior to anoxia or added in anoxia (data not shown). Assuming that the equilibrium value in normoxia corresponded to a cytoplasmic pH of 7.5, the pH after 2 h of anoxia was calculated with NHPT and HPT 3-mm root tips (Table I). All determinations gave higher cytoplasmic pH values (0.1–0.15 pH units) for HPT than for NHTP tissues in anoxia.

Membrane Permeability

Figure 7 shows that the time courses of DMO influx or efflux were identical in both kinds of root tips, indicating that membrane permeability to weak acids had not been modified by the hypoxic pretreatment.

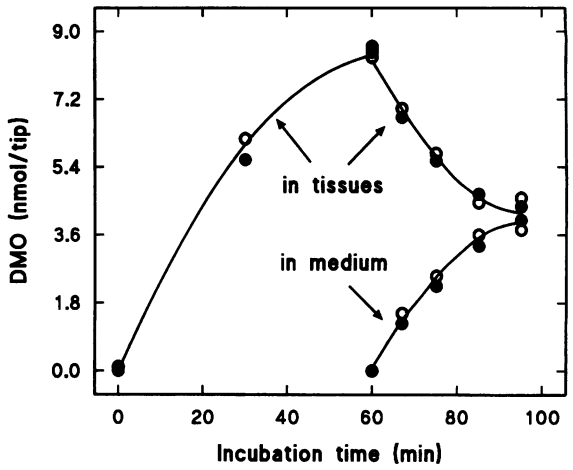

Figure 7. Time courses of DMO accumulation and efflux with maize root tips. The labeled DMO was added at time zero to the medium and root tips were incubated in oxygenated conditions. At the times indicated by the arrow, root tips were rinsed and transferred in the same medium devoid of DMO for efflux measurements. The radioactivity was determined in root tips and in medium separately. O, NHPT root tips; ●, HPT root tips. Data were obtained from 30 root tips.

DISCUSSION

These results show that two conditions are required for the expression of tolerance to anoxia in maize root tips. They need exposure for a few hours to low oxygen partial pressure, and they need an external carbohydrate supply, presumably to sustain the glycolytic rate needed for maintenance of cellular metabolism during long-term incubation in anoxia. However, the sugar supply of the tissues is not immediately involved in the mechanism of acclimation to anoxia. The kinetic parameters of sugar membrane transport are not modified by the hypoxic pretreatment, as reported in ref. 30, and so it is not possible to suggest that there is a better sugar uptake by HPT root tips.

The AEC values found in anoxia are correlated with the glycolytic rate with untreated maize root tips (27). However, because the rate of ethanol + lactate production was identical for HPT and NHPT tissues during the 1st h of anoxia, the higher AEC values found with HPT root tips cannot be explained by an increase in the glycolytic rate. They are, rather, the result of a new steady-state, reached after the transfer to anoxia, between the energy-consuming and the energy-regenerating pathways (16). This new steady-state was different in the two kinds of root tips because, as a result of differential pretreatments, the tissues were not metabolically identical before transfer to anoxia.

In addition, these results demonstrate that the increase in LDH and especially in ADH activities did not induce any modification of the glycolytic flux. Therefore, these enzymes do not seem to be directly involved in the mechanisms of resistance to anoxia, as already reported (22).

The fact that the glycolytic rate decreased sharply in NHPT root tips after 2 h of anoxia suggests the occurrence of some harmful event earlier in these tissues. This event could very well be an over-acidification of the cytosol as a consequence of the accumulation of lactic acid. There was a clear difference between the kinds of roots as far as kinetics of lactate and ethanol production are concerned. NHPT root tips presented the classical sequence described in vitro by Davies et al. (2) and demonstrated in maize root tips by Roberts and his colleagues (20–22). Fermentation started with lactic acid production, and it was only after a 10- to 15-min lag period that

Table I. *Cytosolic pH of Maize Root Tips Estimated from the DMO Distribution*

Cytosolic pH was determined as described in "Materials and Methods" after 2 h of incubation in the presence of labeled DMO with or without 100 mM glucose. Cytosolic volume was calculated from normoxic controls, assuming a value of 7.5 for cytosolic pH. Data are the means ± SE. In parentheses, *n* is the number of independent repetitions.

Treatment	NHPT	HPT	pH
With glucose			
Normoxia	7.5	7.5	
Anoxia	6.82 ± 0.014 (n = 6)	6.91 ± 0.007 (n = 8)	0.09
Without glucose			
Normoxia	7.5	7.5	
Anoxia	6.89 ± 0.011 (n = 8)	7.03 ± 0.024 (n = 6)	0.14

ethanol production started with a concomitant inhibition of lactic acid production. With 18-h HPT root tips, this sequence no longer existed; ethanol production started at its maximum rate immediately after transfer to anoxia, whereas lactic acid production remained comparatively low and almost stopped after 60 to 80 min of anoxia. The maximum concentration of lactic acid always remained much lower in HPT (8 mM) than in NHPT (30 mM) root tips, assuming a cytosolic volume of 1 μL per root tip.

However, there is now evidence that lactic acid alone cannot account for all the fast cytosolic acidification induced by the transfer to anoxia. Recent kinetic studies of the variation of cytoplasmic pH, nucleotides (^{31}P NMR), and lactate during normoxic and anoxic transition in maize root tips (28) show that lactate levels and cytoplasmic pH do not follow the same time courses. The drop in cytoplasmic pH during the course of an aerobic/anaerobic transition (10 min) can be explained by factors other than lactic acid, such as proton build-up or phosphate release (for review and speculation see refs. 10, 17, and 28). The effect of lactic acid on the cytosolic pH should then consist of supplementary acidification occurring during the first hours of anoxic incubation after the main drop in pH. This over-acidification may produce irreversible damage, as illustrated by the decline in metabolic activity in NHPT root tips and their subsequent death. The lower concentration of lactic acid in HPT root tips will contribute to the maintenance of a higher cytoplasmic pH in these tissues. As already reported (17) with anoxic maize root tips, the observed ΔpH is smaller than that expected from lactic acid accumulation in the tissues. Part of the lactic acid may be sequestered in the vacuole, which represents about 70% of the volume in 4-mm maize root tips, according to reference 17. That would lower its actual concentration in the cytoplasm. Another explanation could be the consumption of excess protons by metabolic processes (12) other than alanine accumulation, which was too slow in our material (data not shown) to contribute significantly to the pH regulation. However, even if this ΔpH is not that large (0.1–0.15 pH units), it may be essential at this critical pH threshold for maintenance of an active metabolism and good tissue survival.

This lower accumulation of lactic acid in HPT root tips appears to be the result of two associated mechanisms: a lower production of lactic acid, especially with long treatment (18 h) of HPT root tips, and an increase in lactic acid efflux in the external medium, even with short treatment (4 h) of HPT root tips. It is interesting to note that both strategies are encountered in some cereal organs reported to be very resistant to anoxic conditions. In rice embryos that produce only trace amounts of lactic acid, ethanol fermentation occurs without a lag phase (18), exactly as in HPT maize root tips. On the contrary, in barley aleurone layers (7), the large amounts of lactic acid produced in anoxia do not accumulate in the tissues and are efficiently secreted in the medium. According to the results obtained with DMO, showing that membrane permeability to weak acids was not modified by hypoxic pretreatment, this enhanced lactic acid efflux in our material cannot be attributed to an increase in free diffusion through the plasma membrane. The higher cytosolic pH (0.1–0.15 pH unit) of HPT root tips should lead, on the contrary, to a higher retention of lactic acid in the cytosol. The effect of cycloheximide suggests, rather, that protein synthesis might be involved in this excretion, which should then be a kind of detoxification system induced during the acclimation hypoxic treatment. It is interesting to notice that in mammals (29), hypoxic treatments induce proteins involved in the detoxification of cells and the resistance to a number of drugs. Do similar mechanisms exist in plant cells, and would this be precisely the case with the lactic acid excretion system hypoxically induced in maize root tips?

These results on lactate and cytoplasmic pH are consistent with the hypothesis (12, 13, 21) that cytoplasmic acidosis and intracellular lactate accumulation are determinants of anoxic intolerance of plant tissues. Further experiments are needed to prove the molecular reality of the excretion system.

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