Anaerobic Metabolism in Germinating Seeds of *Echinochloa crus-galli* (Barnyard Grass)\(^1\)

**METABOLITE AND ENZYME STUDIES**

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**ABSTRACT**

*Echinochloa crus-galli*, a problem weed in rice fields, has the rare ability to germinate and to grow in a totally oxygen-free environment. After 7 days growth in the light or dark under \(N_2\), *E. crus-galli* var. *oryzicola* produces a 2- to 3-centimeter nonpigmented shoot.

Ethanol, malate, and lactate were measured in seeds germinated under \(N_2\) and air, and compared with changes in the activities of alcohol dehydrogenase, malate dehydrogenase, NADP-malic enzyme, and phosphoenolpyruvate carboxylase. During a 7-day time course, ethanol levels increased 661 micromoles per gram dry weight under \(N_2\), with no increase under air. Alcohol dehydrogenase activity increased 5.5 micromoles per gram dry weight per minute in \(N_2\) compared to 1.0 in air. Corresponding increases for lactate were 7.9 micromoles per gram dry weight under \(N_2\) and 2.7 under air, and for malate, 5.3 micromoles per gram dry weight under \(N_2\) and 0.4 under air.

Although 85% of the ethanol produced by the seedlings was found in the external solution under \(N_2\), the seeds still contained 90 times more ethanol under anaerobic conditions than under air. No phosphoenolpyruvate carboxylase activity was detected and phosphoenolpyruvate carboxylase activity was 10 times less under \(N_2\) than air. Malic enzyme activity increased 5-fold under anaerobic conditions, comparable to the change under aerobic conditions. Proposed adaptive mechanisms of flood-tolerant species to anaerobiosis are discussed.

Higher plants have an absolute requirement for \(O_2\) for growth and metabolism. Although many tissues can tolerate anaerobiosis for a limited time, growth is inhibited. In germinating seeds it has been suggested (1) that anoxia occurs after imbibition and before rupture of the seed coat, but prolongation of this natural anaerobic period by continued soaking results in death. The well known exception to this is *Oryza sativa* (rice) which has the rare ability to germinate and grow in an anaerobic environment. The rice coleoptile is the only plant organ reported which can grow in anoxia (22). Recently, however, we (18) reported that two varieties of the *Echinochloa crus-galli* complex, common weeds of rice fields, can also germinate and grow for long periods of time in a completely \(O_2\)-free environment.

\(E. crus-galli\) var. *oryzicola* (hereafter oryzicola) seeds germinate as well and produce as large a seedling under \(N_2\) as rice, despite their much smaller seed size (18). Like rice (19, 26), under anaerobiosis, the primary leaves do not emerge from the coleoptile and no root growth occurs. In oryzicola (25), however, the mesocotyl elongates and, if the seed coat is removed, the radicle emerges under anaerobic conditions. Neither occurs with rice. These adaptations of oryzicola to low \(O_2\) may account for its success in flooded rice fields where it can reduce yields by up to 40\% (16, 24). Little is known about the adaptive mechanisms and regulation of anaerobic energy production in germinating seeds, and much of what is known about anaerobic metabolism has come from studies on the influence of flooding on plant roots (7, 15).

In a widely accepted theory proposed by Crawford and colleagues (7–9, 21), flood tolerance in plants is thought to result from decreased ethanol production, while still allowing substrate level ATP formation to continue as an energy source. In roots, this adaptation is dependent on low ADH\(^3\) activity (4, 11, 20) and the ability to reroute glycolytic intermediates to alternate end products such as lactate, malate, or other organic acids. In addition, malate may be linked to other substrate level phosphorylations or oxidized with an alternate electron acceptor (12). Crawford (21) has also proposed that flood-tolerant species lack malic enzyme which would convert malate to pyruvate and, in the absence of \(O_2\), to ethanol.

Contrary to Crawford’s (21) metabolic theory of flooding tolerance, Smith and ap Rees (23) found that ethanol was the major product of fermentation in three flood-tolerant species. Lactate was also produced, but in minor amounts. Malate did not accumulate and malic enzyme and ADH activity were present in amounts comparable to that of intolerant plants. The objective of this research was to determine changes in the end products of anaerobic metabolism, ethanol, malate, and lactate, during the germination of oryzicola under \(N_2\), and to compare these metabolites with changes in the corresponding enzymes.

**MATERIALS AND METHODS**

**Germination.** Seeds of *E. crus-galli* var. *oryzicola* were obtained from rice field populations at Biggs, Butte Co., CA. Rice (*O. sativa* cv. Colusa) seeds were obtained from the Rice Experiment Station, Biggs, CA. Pea (*Pisum sativum* cv. Alaska) seeds were supplied by the Department of Agronomy, Washington State University, Pullman, WA.

Seeds were surface-sterilized with 2.5\% NaOCl for 10 min and

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\(^3\) Abbreviations: ADH, alcohol dehydrogenase; PPFD, photosynthetic photon flux density; PEP, phosphoenolpyruvate; OAA, oxaloacetate.
washed three times with glass-distilled H₂O. One hundred seeds were imbibed on two layers of filter paper with 2.5 ml glass-distilled H₂O in 50-ml Erlenmeyer flasks. Seeds were not submersed unless indicated. Seeds were then germinated in a growth chamber with 30/20°C temperature and a 16-h photoperiod of 300 μE m⁻² s⁻¹ PPFD. Humidified air or N₂ (99.995% N₂) was passed continuously through the flasks. The same results were obtained when N₂ gas was further purified by bubbling through alkaline pyrogallol or when the seeds were vacuum-infiltrated to remove trapped O₂. Maintenance of anaerobic conditions during experiments was checked with Gas Pac O₂ indicators.

Measurement of Metabolites. After the prescribed time of imbibition, seedlings (0.5 g dry weight) were immediately frozen in liquid N₂ and ground at 4°C in 5.0 ml 5.0 M HClO₄, with a mortar and pestle. The homogenates were centrifuged at 27,000g for 30 min. The supernatant fraction was neutralized with 1.0 M K₂CO₃ and the precipitated protein removed by centrifugation. Neutralized extracts and inhibition solution were analyzed immediately. Ethanol, malate, and lactate were determined by measuring the reduction of NAD at 340 nm. The specific conditions were as follows:

**Ethanol (2).** Na-PP, buffer (pH 8.7) (71 mM), 0.57 mM NAD, 0.14 mg/ml (42 IU) ADH.

**Malate (14).** Hydrazine (0.34 mM) 0.42 mM glycine buffer (pH 9.0), 2.53 mM NAD, 42 μg/ml (46 IU) malate dehydrogenase.

**Lactate (13).** Hydrazine (0.34 mM), 0.42 mM glycine buffer (pH 9.0), 2.53 mM NAD, 42 μg/ml (23 IU) lactate dehydrogenase. Recovery experiments with known amounts of malate and ethanol added yielded 94 and 100% recovery, respectively.

Enzyme Assays. Seedlings were ground with sand in 5.0 ml extraction medium at 4°C with a mortar and pestle. The homogenates were filtered through cheesecloth and centrifuged at 15,000g for 20 min at 4°C. The supernatant fraction was analyzed immediately for ADH, malate dehydrogenase, and malic enzyme activity. The extraction medium for ADH contained 97 mM Tris-HCl (pH 8.5), 143 mM mercaptoethanol, and 2.0 mM MgCl₂. For malate dehydrogenase and malic enzyme the extraction medium was 50 mM Tris-HCl (pH 7.5), which contained 0.2 mM EDTA, 30 mM mercaptoethanol, and 16 g/l PVP-10. For the assay of PEP carboxylase, the seedlings were extracted in 195 mM K-phosphate (pH 7.0), 10 mM MgCl₂, 20 mM mercaptoethanol, 0.2 mM EDTA, and 16 g/l PVP-10. The homogenate was centrifuged for 20 min at 3000g at 4°C, and the supernatant used for the enzyme assay.

ADH, malate dehydrogenase, and malic enzyme were assayed spectrophotometrically at 25°C by the oxidation or reduction of pyridine nucleotides at 340 nm. Reaction mixtures were as follows:

**ADH (EC 1.1.1.1) (23).** 41.5 mM Tris-HCl (pH 9.0), 0.11 mM NAD, 1.23 mM ethanol, and 25 to 50 μl enzyme extract in a final volume of 2.65 ml. The reaction was started by the addition of ethanol.

**NAD-Malate Dehydrogenase (EC 1.1.1.37) (27).** 92 mM Tris-HCl (pH 7.5), 0.18 mM NADH, 1.1 mM OAA, and 10 to 25 μl enzyme extract in a total volume of 2.72 ml. The reaction was started by the addition of OAA to the reference cuvette.

**NADP-Malic Enzyme (EC 1.1.1.40) (27).** 80 mM Tris-HCl (pH 7.5), 4.0 mM malate, 0.64 mM NADP, 8.0 mM MgCl₂, and 100 μl enzyme extract in a total volume of 1.3 ml. The reaction was started by the addition of MgCl₂.

**PEP Carboxylase (EC 4.1.1.31) (17).** PEP carboxylase was assayed by the incorporation of H¹⁴CO₃. The reaction mixture contained 25 mM Tricine (pH 8.0), 10 mM MgCl₂, 5 mM mercaptoethanol, 15 mM sodium glutamate, and 50 mM NaH¹⁴CO₃ in a final volume of 180 μl. Assays were started by the addition of 30 μl extract and run for 5 to 9 min at 27°C. Assays were terminated by the addition of 50 μl glacial acetic acid. Aliquots were sampled, dried, and quantified by liquid scintillation counting.

All enzyme activities were linear with respect to time and enzyme concentration, and were dependent upon the addition of appropriate substrates and cofactors.

**RESULTS AND DISCUSSION**

Measurements of Fermentative Activity.

**ADH Activity and Ethanol Production.** Although there was no net change in ethanol levels in oryzaicola seedlings grown for 7 days in air, when grown without O₂, total ethanol levels increased 661 μmol g⁻¹ dry weight (Fig. 1). Of the total ethanol produced under N₂, 85% (45 mM ethanol) was found in the imbibition solution. Considering the seedlings alone (inset), ethanol was 90 times greater under N₂ (11 mM ethanol) than air.

The ability of oryzaicola to vent ethanol to the external environment could be an important means of avoiding toxic ethanol accumulation within the seed itself. Bertani et al. (3) have proposed that one of the most important mechanisms of rice seedlings to withstand anaerobiosis is to couple a strong alcoholic fermentation system with the ability to excrete the ethanol produced. In their experiments, they found that 98% of the ethanol produced was located in the external medium.

The ADH activities of oryzaicola grown with and without O₂ are not significantly different until after they germinated at day 3 (Fig. 2). Between day 3 and day 7, the activity of aerobically grown seedlings decreased from 3.6 μmol g⁻¹ dry weight min⁻¹ to

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**Fig. 1.** Ethanol in E. crus-galli var. oryzaicola under aerobic and anaerobic conditions. Seedlings and imbibition solution were analyzed separately and the total values given above. Inset: ethanol concentration in seedlings alone.

**Fig. 2.** Alcohol dehydrogenase activity in seedlings of E. crus-galli var. oryzaicola germinating under aerobic and anaerobic conditions. Under both conditions germination was evident after 3 days imbibition. The specific activity of ADH at day 7 for N₂ and aerobically grown seedlings was 1.34 and 0.15 μmol mg⁻¹ protein min⁻¹, respectively.
2.6, while that of N<sub>2</sub>-grown seedlings increased to 7.1 μmol g<sup>-1</sup> dry weight min<sup>-1</sup>. For comparison, ADH activities for peas, a flooding-intolerant plant, and rice, a flooding-tolerant plant, are given in Table I. Rice showed little difference in ADH activity and ethanol production under N<sub>2</sub> compared to air. Peas and oryzicola (Figs. 1 and 2), on the other hand, exhibited a marked increase in both ADH activity and ethanol production under N<sub>2</sub> relative to air. In peas and oryzicola, the anaerobic levels of ADH were 2.8 times greater than aerobic values and there was a 5-fold increase in ethanol production under N<sub>2</sub> versus air at day 7.

In a related experiment, we have found that when seeds of oryzicola, rice, and peas were imbibed in 0.3% and 3.0% ethanol solutions for 7 days in air, all species germinated normally (greater than 80%) at 0.3%, whereas only oryzicola germinated normally at 3.0%. In anaerobic conditions, peas never germinated, as would be expected, while oryzicola germinated 80% or better at both ethanol concentrations. Rice, on the other hand, germinated about 50% at 0.3% ethanol and 11% at 3.0% ethanol. At the higher ethanol concentration, the shoot growth of rice was greatly inhibited. These results suggest that different tolerance levels to ethanol exist in each of these species, beyond which germination and growth is inhibited even in anaerobiosis-tolerant species. There have also been reports that ethanol is metabolized when aerobic conditions are re-established (5, 6). Initial results (unpublished data) indicate that oryzicola can metabolize ethanol to various products including sugars, organic acids, amino acids, and lipids.

**Measurements of Possible Alternate End Products.**

**Organic Acids.** It has been reported (7, 10) that lactate and malate may be formed as alternate end products to ethanol under anaerobic conditions. In our studies, until day 3 (when the oryzicola seeds germinated) there was no significant difference in the levels of malate or lactate (Fig. 3) under either aerobic or anaerobic conditions. After germination, however, there was a large increase in both acids under anaerobic conditions. In the presence of O<sub>2</sub>, malate and lactate remain relatively unchanged throughout the germination, with a small increase in lactate at day 7. As shown in the inset, 90% of the total malate was found in the seedlings after 7 days; whereas only 29% of the total lactate was contained in the seedlings.

Relative to the quantities of ethanol produced, the amounts of malate and lactate were not appreciable and they did not accumulate until well after ethanol production was significant. Their role in prolonging the period before ethanol accumulates, as suggested by Davies et al. (10) and Crawford (7), is not substantiated here. Aldosoro and Nicolas (1) reported that chick pea seeds (Cicer arietinum) are in an intermediate position between intolerant and tolerant to anoxia. They found that, similar to oryzicola, ethanol, malate, and lactate all increase under anaerobic conditions, but that the maximum concentrations were reached at the same point, with ethanol being only 4 times that of lactate and twice that of malate.

**Enzyme Activities.** According to Crawford’s (21) theory for flooding tolerance, the formation and accumulation of malate would require the activity of PEP carboxylase or PEP carboxykinase and malate dehydrogenase. In addition, malic enzyme, which would decarboxylate malate if present, must be absent or repressed. In oryzicola, the activity of malic enzyme under N<sub>2</sub> (Table II) increased 5-fold in 7 days, comparable to the increase we measured in air/dark-grown seedlings. Malate dehydrogenase increased 2-fold under N<sub>2</sub> and 4-fold under air. After 7 days, the malate dehydrogenase activity of seedlings grown without O<sub>2</sub> was 23% that of air/dark-grown plants.

The pattern of changes we found in malic enzyme and malate dehydrogenase are in agreement with the change in malate concentration (Fig. 3) under both aerobic and anaerobic conditions. The utilization of malate via the citric acid cycle is suggested by the aerobic rate of malate dehydrogenase and the constant malate concentration over 7 days. Under N<sub>2</sub>, the increase in malate dehydrogenase activity is less. This may be important in the relatively small accumulation of malate.

The ability of oryzicola to carboxylate PEP to malate under aerobic and anaerobic conditions was determined by measuring the activity of PEP carboxylase and PEP carboxykinase. Over 7 days, PEP carboxylase activity increased 2-fold under N<sub>2</sub> conditions and contained 13% of the PEP carboxylase activity of seeds grown under air in the dark (Fig. 4). Under air/light, seedlings showed a 25-fold increase in PEP carboxylase activity after 7 days. PEP carboxykinase activity was undetectable in oryzicola seeds

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**Table I. Alcohol Dehydrogenase Activities in Germinating Seeds of Rice Peas and under Aerobic and Anaerobic Conditions**

Values represent the means of two separate experiments. Corresponding values of ethanol released in solution in rice ranged from 9.4 μmol g<sup>-1</sup> dry weight at day 1 to 468.2 at day 7 under N<sub>2</sub> and from 8.2 to 358.0 under air. The values for peas on days 1 and 7 are 97.6 to 149.1 under N<sub>2</sub> and 98.6 to 257.7 under air.

<table>
<thead>
<tr>
<th>Time after Imbibition</th>
<th>Rice</th>
<th>Peas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Air</td>
</tr>
<tr>
<td><strong>μmol g&lt;sup&gt;-1&lt;/sup&gt; dry wt min&lt;sup&gt;-1&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.55</td>
<td>1.38</td>
</tr>
<tr>
<td>7</td>
<td>2.83</td>
<td>2.03</td>
</tr>
</tbody>
</table>

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**Table II. Malic Enzyme and Malate Dehydrogenase Activities in E. crus-galli var. oryzicola during 7-Day Time Course under Aerobic and Anaerobic Conditions**

Values represent the means of at least three separate experiments ± SE.

<table>
<thead>
<tr>
<th>Time after Imbibition</th>
<th>NADP-Malic Enzyme</th>
<th>NAD-Malate Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Air</td>
</tr>
<tr>
<td><strong>μmol min&lt;sup&gt;-1&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>1</td>
<td>0.06 ± 0.01</td>
<td>0.06 ± 0.004</td>
</tr>
<tr>
<td>3</td>
<td>0.13 ± 0.03</td>
<td>0.13 ± 0.04</td>
</tr>
<tr>
<td>5</td>
<td>0.17 ± 0.02</td>
<td>1.08 ± 0.07</td>
</tr>
<tr>
<td>7</td>
<td>0.21 ± 0.04</td>
<td>1.80 ± 0.35</td>
</tr>
<tr>
<td>(dk)</td>
<td>0.25 ± 0.01</td>
<td>0.25 ± 0.01</td>
</tr>
</tbody>
</table>

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**FIG. 3. Malate and lactate in E. crus-galli var. oryzicola seedlings and imbibition solution under aerobic and anaerobic conditions. Inset: malate and lactate concentrations in seedlings alone.**
under all conditions. These results are inconsistent with the proposal (21) that flood-tolerant species synthesize malate (via PEP carboxylase and malate dehydrogenase) and lack malic enzyme, and therefore the ability to convert malate back to pyruvate and hence ethanol. Lactate dehydrogenase activity was also not detectable in aerated or un aerated seeds. When a known amount of pure lactate dehydrogenase was added to the extraction medium, all of the activity was recovered.

Our results suggest that one general theory for flooding tolerance cannot explain the adaptive mechanisms of all species. In particular, oryza cola, which is a highly adapted, tolerant, rice-field weed, had ADH, ethanol, and malic enzyme levels similar to those attributed to flood-intolerant plants, but synthesized malate and lactate in amounts comparable to flood-tolerant plants. In addition, the anaerobic germination of oryza cola is characterized by an ability to vent most of the ethanol produced to the external environment, high tolerance for ethanol, lack of a Pasteur effect under anaerobic conditions, and operation of the oxidative pentose phosphate pathway during the early stages of germination (18). The extent to which these and other adaptations are important to flood tolerance in oryza cola is being further investigated.

LITERATURE CITED


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