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Anaerobiosis in *Echinochloa crus-galli* (Barnyard Grass) Seedlings[1]

INTERMEDIARY METABOLISM AND ETHANOL TOLERANCE

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ABSTRACT

Tolerance to ethanol and the ability to metabolize key intermediary substrates under anaerobiosis were studied in *Echinochloa crus-galli* (L.) Beauv. var *oryzicola* seeds to further characterize the mechanisms which enable it to germinate and grow without O_2.

Our results indicate that *E. crus-galli* var *oryzicola* possesses an inherently high tolerance to ethanol and is able to metabolize low levels of ethanol in the absence of O_2. Concentrations of ethanol 45-fold greater than endogenous levels did not prove toxic to germinating seeds.

Five-day anaerobically grown seedlings of *E. crus-galli* var *oryzicola* metabolized added [^{14}C]sucrose primarily to CO_2 and ethanol. Of the soluble compounds labeled, the phosphorylated intermediates of glycolysis and the oxidative pentose phosphate pathway predominated more under anaerobiosis than in air. In addition, organic acids and lipids were labeled from [^{14}C]sucrose, the latter indicating that metabolism of carbohydrate via acetyl-CoA occurred in the absence of O_2. Lipids were also labeled when seeds were supplied with [^{14}C]ethanol or [^{14}C]acetate. Labeling experiments using the above compounds plus [^{14}C]$NaHCO_3$, showed further labeling of organic acids; succinate and citrate being labeled under nitrogen, while fumarate was formed in air.

The above metabolic characteristics would allow for the maintenance of an active alcoholic fermentation system which, along with high alcohol dehydrogenase activity, would continue to recycle NAD and result in continued energy production without O_2. In addition, *Echinochloa's* ability to metabolize carbohydrate intermediates and to synthesize lipids indicates that mechanisms exist for providing the carbon intermediates for biosynthesis, particularly membrane synthesis for growth, even in the absence of O_2.

Echinochloa crus-galli var *oryzicola* (hereafter oryzicola) is a common weed of flooded rice fields. Like rice (4, 7) oryzicola can germinate and grow for long periods without O_2 (10). In the past, studies of anaerobic metabolism have concentrated mainly on the effects of flooding on plant roots with little attention directed toward germinating seeds (7). This is despite the evidence that most flood-tolerant plant roots are merely surviving temporary stress (4); whereas tolerant seeds, such as oryzicola and rice, actively metabolize under long periods of anaerobiosis (10, 13, 17).

Until recently, flood tolerance in plants was believed (3, 12) to result from decreased ethanol production dependent upon low ADH[3] activity. Part of this metabolic theory of flooding tolerance (12) was the ability to reroute glycolytic intermediates to alternate end products such as malate, lactate, and other organic acids. This theory at first appeared tenable since ethanol has frequently been cited (3, 7) as a toxic compound and presumed to be the cause of injury or death under anoxic conditions. However, research in two areas since this theory was proposed tend to dispute it. (a) Ethanol has been found to be the major product of fermentation in flood-tolerant plant roots (18) and seeds (1, 17), accompanied in both cases by large increases in ADH activity. (b) Jackson (9) has reported that ethanol is not a significant toxin in pea plants under flooded conditions even at concentrations 100-fold greater than normally found. Others (4, 20) have also found that the amounts of ethanol produced under anaerobic conditions are not the cause of cell damage. Rather, when large concentrations of ethanol are found it is more likely a consequence of anaerobic injury than a cause (4).

These results, while being in contrast to the metabolic theory of flood tolerance, do not offer an answer to how seeds or seedlings are able to synthesize structural compounds actively and maintain their energy charge and redox potential in the absence of O_2. What they do suggest is a possible physiological mechanism for tolerance of temporary anoxic conditions. With oryzicola, our objective was to determine the mechanisms which enable it to grow in the absence of O_2. We approached this in two ways. First, we wanted to estimate the inherent tolerance of oryzicola to ethanol and its ability to reduce or eliminate ethanol toxicity by metabolism of ethanol. Second, we were interested in whether oryzicola was able to metabolize key substrates, specifically sucrose, $NaHCO_3$, acetate, and ethanol under anaerobiosis. Our results indicate that in the absence of O_2, oryzicola maintains active alcoholic fermentation by tolerating high levels of ethanol. In addition, oryzicola is capable of anaerobic metabolism of carbohydrate to lipids and a number of intermediary compounds, including sugar-P, organic, and amino acids.

MATERIALS AND METHODS

Plant Material. Seeds of *Echinochloa crus-galli* (L.) Beauv. var *oryzicola* were supplied by Dr. D. E. Seaman from rice field populations at Biggs, Butte County, CA. For routine experiments, the seeds were surface-sterilized with 2.5% NaOCl for 10 min, washed three times with glass-distilled H_2O, and germinated as previously described (17). Bacteriological controls were carried out at the start and completion of all incubations of labeled substrates by incubating an aliquot of the imbibition solution on a medium containing (in 1 L) agar, 15 g; yeast extract, 3 g; glucose,

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[3] Abbreviations: ADH, alcohol dehydrogenase; OPP, oxidative pentose phosphate.

10 g. No contaminants were found.

Chemicals. Substrates, cofactors, and enzymes were purchased from Sigma. $NaH^{14}CO_3$ (58.9 mCi/mmol), [1-^{14}C]ethanol (57 mCi/mmol), [U-^{14}C]acetate (58.6 mCi/mmol), and [U-^{14}C]sucrose (15 mCi/mmol) were all purchased from Amersham. Purity of isotopes was checked by thin-layer autoradiography.

Estimation of Ethanol Tolerance. Surface-sterilized seeds of oryzicola (125 mg dry weight) were inbibed in 125-ml Erlenmeyer flasks on two layers of filter paper with 4.0 ml of the appropriate ethanol solution, or distilled H_2O for controls. The flasks were flushed with N_2 (99.995% N_2) or air, stoppered and either left in air or placed in an anaerobic chamber. After 7 d, the per cent germination was determined and shoot lengths were measured. Using replicate samples, the imbibition solution was removed and the seeds were rinsed three times (15 s total) with distilled H_2O to remove any superficial ethanol before measuring it in the seeds. Neutralized extracts and imbibition solutions were analyzed for ethanol and ADH was extracted and assayed, as described by Rumpho and Kennedy (17).

Metabolism of ^{14}C-Labeled Compounds. Seeds of oryzicola (0.5 g dry weight) were imbibed on two layers of filter paper with 2.5 ml glass-distilled H_2O in 50-ml Erlenmeyer flasks. The flasks were continuously flushed with air or N_2. At 5 d, the imbibition solution was replaced with the appropriate ^{14}C-labeled compound. All the samples were incubated at 25°C for 3 h, termed the pulse period. For chase experiments, the seeds were labeled as above, then repeatedly rinsed of the ^{14}C label and incubated for an additional 16 h in buffer. Three treatments were used: (a) 5-d germination in N_2 followed by 3-h pulse in N_2 (designated N_2); (b) 5-d germination in N_2 followed by 3-h pulse in air (designated $N_2 \rightarrow O_2$); and (c) 5-d germination in air followed by 3-h pulse in air (designated O_2).

Incubations with [1-^{14}C]ethanol and $NaH^{14}CO_3$ were performed in a closed system, after an initial flush with N_2 or air, to prevent loss of the added label during the pulse period. Ethanol (2.28 μCi; 0.02 mM) or 130 μCi $NaH^{14}CO_3$ (1.10 mM), each in 2.0 ml 50 mM KH_2PO_4 (pH 7.5), were added to each flask separately. Aerobic flasks with $NaH^{14}CO_3$ were wrapped in aluminum foil to eliminate any photosynthetic metabolism of the label. After the 3-h pulse period, the flasks were flushed with N_2 or air and any $^{14}CO_2$ evolved was trapped in 1.0 M KOH and counted by liquid scintillation.

Labeling studies with [U-^{14}C]acetate or [U-^{14}C]sucrose were carried out in open systems in the light, with N_2 or air continuously passed through the flasks and $^{14}CO_2$ evolved, quantified as above. Acetate (11.7 μCi; 0.10 mM) or 2.0 μCi (0.067 mM) sucrose each in 2.0 ml 50 mM KH_2PO_4 (pH 5.5) were added to each sample individually.

Extraction, Separation, and Identification of Products. After labeling, the seedlings were rinsed with water three times and then killed in 80% boiling ethanol. The imbibition solution was counted separately then added to the ethanol solution. Seedlings were boiled in ethanol for 10 min ground with a mortar and pestle, extracted again with 80% ethanol and twice with water. The extracts were pooled and concentrated (11) and represent the total soluble components. Less than 5% of the total label remained in the insoluble fraction as determined by liquid scintillation counting of the entire solubilized pellet. The cpm metabolized was obtained from the total cpm recovered minus that found in the insoluble fraction and unmetabolized label. At least 90% of the nonmetabolized label was removed with the washings except for sucrose, which was measured by two-dimensional electrophoresis and chromatography as below.

Separation of products was by two-dimensional thin-layer electrophoresis and chromatography as described (11). The plates were exposed to x-ray film for 7 to 30 d to localize radioactivity. Radioactivity was then scraped from the cellulose plates, 0.5 ml water and 6 ml cocktail added, and counted as above. Products were detected by co-chromatography with radioactive standards. Amino acids were verified by spraying with 0.5% ninhydrin in 95% ethanol. Lipids represent the total ethanol extractable pool. Ethanol was separated from the imbibition solution by distillation and quantified by liquid scintillation counting.

RESULTS

Ethanol Tolerance and Metabolism. One of the major and often presumed toxic metabolites produced under anaerobiosis is ethanol. In oryzicola, there was no decrease in germination in air or N_2 at 0.3 or 1.0% ethanol concentrations (52 and 172 mM, respectively) compared to controls (Fig. 1). In 0.3% ethanol, shoot growth was also unaffected, while in 1% ethanol, shoot lengths were 75 and 80% control values for air and N_2, respectively. Three % (515 mM) ethanol inhibited germination and shoot growth

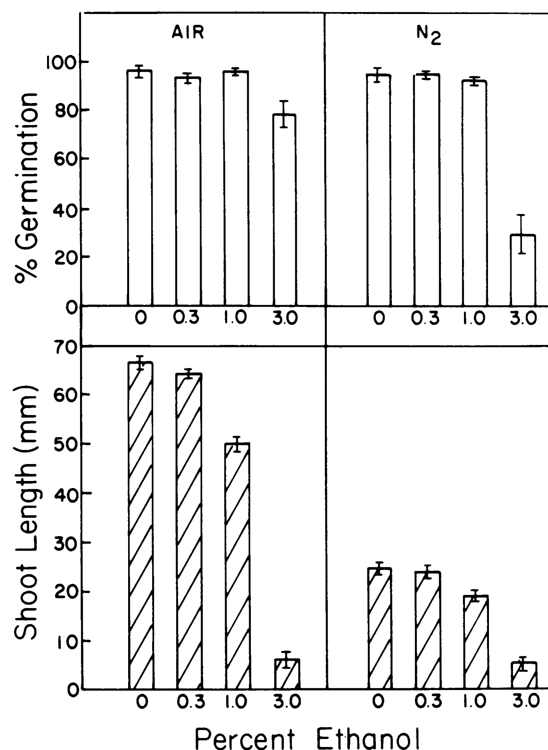

FIG. 1. Percent germination and shoot length in *E. crus-galli* var *oryzicola* imbibed for 7 d in 0, 0.3, 1.0, or 3.0% ethanol under air or N_2. Values represent the means of at least three separate experiments ± SE.

Table I. *Recovery of Ethanol from E. crus-galli var oryzicola Seedlings after 7 Days Imbibition in N_2 or Air*

Ethanol, in the concentrations given below, was added at the beginning of imbibition. After 7 d, the seedlings and imbibition solution were analyzed separately for ethanol.

Ethanol Added		Ethanol Recovered			
		N_2		Air	
		Seedlings + solution	Seedlings alone	Seedlings + solution	Seedlings alone
[% (v/v)]		*µmol mg⁻¹ dry wt*			
0		1.30	0.05	0.03	0.02
1.52	(0.3)	2.61	0.10	0.46	0.03
4.90	(1.0)	5.52	0.17	2.74	0.12
14.94	(3.0)	10.31	0.21	13.16	0.43

Table II. *ADH Activity in Seeds of E. crus-galli var oryzicola after 7 Days Imbibition in the Presence or Absence of Added Ethanol*

ADH activity was determined in dry oryzicola seeds and after 7 d imbibition with or without 3% ethanol added (14.94 μmol mg^{-1} dry weight) under both N_2 and air conditions. Values represent the means of at least three separate experiments ± SE.

Time after Imbibition and Addition of Ethanol	ADH	
	N_2	Air
d	*μmol g^{-1} dry wt min^{-1}*	
0	1.57 ± 0.12	1.57 ± 0.12
7 (without ethanol)	7.11 ± 0.15	2.63 ± 0.21
7 (with ethanol)	8.87 ± 0.70	11.75 ± 0.42

Table III. *Distribution of ^{14}C from [1-^{14}C]Ethanol Supplied to 5-Day-Old Seedlings of E. crus-galli var oryzicola*

Seedlings were supplied with [1-^{14}C]ethanol in a closed system for 3 h under N_2 or air.

Experimental Conditions	Label Metabolized	Total Label Added	^{14}C Distribution (Label Metabolized)		
			$^{14}CO_2$	Insoluble	Soluble
	cpm	*%*		*%*	
N_2	6.5×10^4	(2.3)	38.2	1.4	60.4
O_2	1.1×10^6	(80.4)	52.4	1.7	45.8

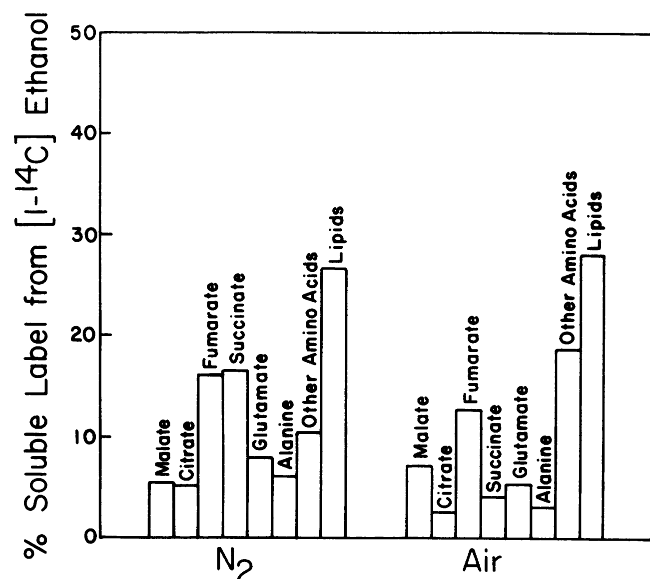

FIG. 2. Changes which occur in metabolites of *E. crus-galli* var *oryzicola*, germinated for 5 d under N_2 or air, then supplied with [1-^{14}C]ethanol for 3 h. Values are expressed as percent of total soluble label.

initially, but never proved toxic to the seedlings.

Ethanol metabolism and adapative mechanisms for ethanol tolerance in oryzicola are suggested by the data in Table I. When seeds were germinated in the presence of exogenously added ethanol, after 7 d 95% of the recovered ethanol was found in the imbibition solution. In air or N_2, the seedlings never contained more than 5% of the total ethanol added. Some of the ethanol added to the seeds in air was probably metabolized and may explain the decrease in total ethanol content of the external solution plus seedlings. Even without O_2, ethanol appeared to be metabolized, considering the exogenous ethanol would have been supplemented by inherent fermentative metabolism. Specifically, after 7 d in N_2, 1.25 μmol ethanol mg^{-1} dry weight was metabolically produced and recovered in the imbibition medium compared to 0.03 in air.

The ability to metabolize ethanol under air or N_2 is further supported by the increase in ADH activity (Table II), and from [1-^{14}C]ethanol labeling studies (Table III). Previously, we (17) reported that under anaerobic conditions ADH activity continually increased in oryzicola seedlings during the first 7 d. On the other hand, in air, ADH levels initially increased and then decreased to levels similar to those of dry seeds. In the present experiments, however, when the seeds were germinated in air plus 3% ethanol, ADH activity increased about 4.5-fold over control values (Table II).

Seedlings supplied with [1-^{14}C]ethanol metabolized 80% of the added isotope under aerobic conditions *versus* 2.3% under N_2 (Table III). In air, over 50% of the label was evolved as CO_2. Under anaerobic conditions, 38% of the radioactivity was given off as CO_2 with 60.4% located in total soluble compounds. Within the latter category, 43% of the ^{14}C was located in organic acids under anaerobic conditions (Fig. 2). Several intermediates of the tricarboxylic acid cycle were labeled under N_2 and air, with succinate, fumarate, and citrate being greater under a N_2 environment. Lipids were the major labeled compounds under both aerobic and anaerobic environments, comprising 28 and 26.5%, respectively, of the total soluble components.

Incorporation of $NaH^{14}CO_3$. Distribution of metabolites from dark fixation of $H^{14}CO_3$ was measured in 5-d-old oryzicola seedlings to determine if they were capable of fixing CO_2, and if the malate produced was metabolized during a chase period. As expected, malate was the major product under all conditions and accounted for 60, 65, and 35% of the total soluble metabolites under N_2, $N_2 \rightarrow O_2$, and O_2, respectively (Fig. 3; Table IV). The role of malate as a metabolite is seen in the chase period. Under all three conditions, the percentage of label in malate declined during the chase period (designated by arrows), and even in the absence of O_2, malate was metabolized.

Metabolism of malate is suggested in both air and N_2 by changes in per cent label of the intermediates, citrate, succinate, and, especially in air, fumarate (Fig. 3). During the chase period, citrate decreased in N_2, but increased in $N_2 \rightarrow O_2$ and O_2, while fumarate increased in all three labeling experiments. In the air chase, the decrease in label in malate was offset by a similar increase in fumarate, whereas in the N_2 chase, the decrease in malate was mainly offset by an increase in $^{14}CO_2$ evolution. In the $N_2 \rightarrow O_2$ chase, increases in citrate, fumarate, and CO_2 accounted for the 40% decrease in malate. Glutamate was also formed under all three experimental conditions and decreased during the chase except under N_2. Aspartate accounted for 9.8% of the 'other amino acid' fraction in O_2 and 3.0 and 3.1% in N_2 and $N_2 \rightarrow O_2$, respectively. During the chase period, aspartate decreased to about 1% in all three cases.

Metabolism of [U-^{14}C]Acetate. Oryzicola seedlings were labeled with [U-^{14}C]acetate to measure the extent of conversion into organic acids and lipids under anaerobic *versus* aerobic conditions. Seedlings grown under aerobic conditions metabolized about 6 times more label than seedlings imbibed under N_2 (Table V). Upon transfer from N_2 to O_2, there was only a slight increase (0.9%) in the amount of label metabolized compared to N_2. There were also major qualitative differences between air and N_2; 68% of the label metabolized in air was evolved as $^{14}CO_2$, compared to 50% under N_2 and 33% in $N_2 \rightarrow O_2$. Under N_2, the distribution of label in the total soluble fraction was quite uniformly distributed, with the basic fraction containing slightly more ^{14}C due to the labeling of glutamate (Fig. 4). Less radioactivity was found in the acid fractions under N_2 relative to aerobic levels. Under O_2, tricarboxylic acid cycle intermediates, especially malate and fu-

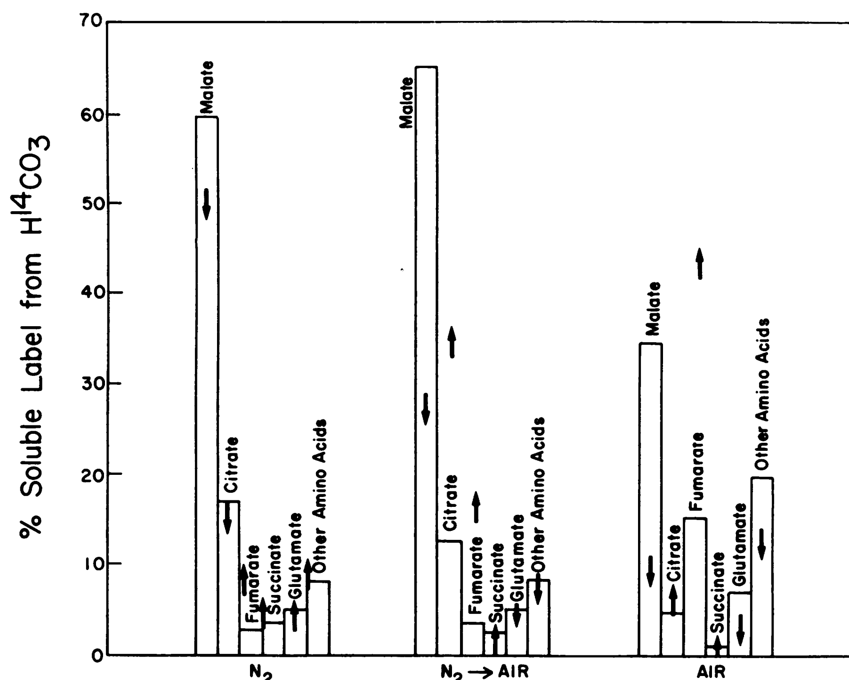

FIG. 3. ^{14}C-Labeled metabolites of *E. crus-galli* var *oryzicola* pulsed with $NaH^{14}CO_3$ for 3 h in the dark. Seedlings were germinated for 5 d in either N_2 or air and then pulsed with $NaH^{14}CO_3$ for 3 h under N_2, air, or after transfer from N_2 to air. The arrows indicate the changes in metabolite levels after a 16-h chase in the absence of $NaH^{14}CO_3$. Values represent the percent of total soluble compounds labeled.

Table IV. *Distribution of* ^{14}*C in Seedlings of E. crus-galli var oryzicola Supplied with* $NaH^{14}CO_3$ *under* N_2 *or Air*

Seeds were germinated for 5 d in N_2 or air and then pulsed for 3 h with $NaH^{14}CO_3$ in N_2, air, or after transfer from N_2 to air (designated $N_2 \rightarrow O_2$).

Experimental Conditions	Label Metabolized	Total Label Added	^{14}C Distribution (Label Metabolized)		
			$^{14}CO_2$[a]	Insoluble	Soluble
	cpm	*%*		*%*	
N_2	3.9×10^6	(17.6)	82.3	0.3	17.4
$N_2 \rightarrow O_2$	2.8×10^6	(12.7)	69.5	0.6	30.0
O_2	6.5×10^6	(29.4)	92.2	0.3	7.5

[a] These values include any $^{14}CO_2$ that was evolved including that from volatilization of the added $NaH^{14}CO_3$.

Table V. *Distribution of* ^{14}*C in Seedlings of E. crus-galli var oryzicola when Supplied with [U-*^{14}*C]Acetate*

Seeds were germinated for 5 d in N_2 or air and then pulsed for 3 h with [U-^{14}C]acetate in N_2, air, or after transfer from N_2 to an aerobic atmosphere.

Experimental Conditions	Label Metabolized	Total Label Added	^{14}C Distribution (Label Metabolized)		
			$^{14}CO_2$	Insoluble	Soluble
	cpm	*%*		*%*	
N_2	9.7×10^5	(6.2)	50.4	1.5	45.8
$N_2 \rightarrow O_2$	1.1×10^6	(7.1)	32.9	2.6	64.5
O_2	5.5×10^6	(35.3)	67.7	6.4	25.9

marate, were heaviest labeled in air. Per cent label in glutamate was less than one-half that under N_2.

If the seedlings were imbibed for 5 d in N_2, then transferred to air for the 3-h pulse period ($N_2 \rightarrow O_2$), most of the ^{14}C label was located in citrate, with smaller amounts in glutamate and malate. Acetate was metabolized to lipids (Fig. 4) under all three conditions. ^{14}C-Lipids were greatest under N_2, increasing further during the chase period. The increase in labeling of succinate under anaerobiosis (*versus* fumarate in air) is well demonstrated under all three conditions.

Metabolism of [U-^{14}C]Sucrose. The effect of anaerobiosis on the metabolism of carbohydrates (the major seed reserve) was determined by supplying oryzicola seeds with [^{14}C]sucrose and analyzing the compounds labeled. Anaerobiosis resulted in a 23% decrease in the amount of [^{14}C]sucrose metabolized relative to aerobic metabolism (Table VI). Under both conditions, most of the label was recovered as $^{14}CO_2$, representing 9.2×10^5 cpm in air *versus* 6.7×10^5 cpm in N_2. The next most heavily labeled compound under N_2 was ethanol, accounting for 14.7% or 1.6×10^5 cpm.

Of the ^{14}C in soluble compounds, most was recovered as acidic compounds under both N_2 and air (Fig. 5). Acidic components accounted for 61% of the total soluble cpm in N_2 compared to 47% in air. Heavier labeling of phosphorylated sugars without O_2 made up the difference. Finally, anaerobiosis led to an increase in labeling of succinate and a decrease in fumarate and aspartate, and in both air and N_2 a substantial percentage of the label was recovered in the lipid fraction.

DISCUSSION

E. crus-galli var *oryzicola* is well adapted to anaerobiosis; it maintains an active alcoholic fermentation system (17) and is capable of additional intermediary carbohydrate metabolism. ADH activity and ethanol production are high in anaerobically germinated seeds, while malate, lactate, or other organic acids do not accumulate as major alternatives to ethanol production (17). Active alcoholic fermentation in oryzicola is maintained by a combination of: (a) an ability to vent most of the ethanol produced to the external medium (17); (b) the capability to metabolize at least some of the remaining ethanol; and (c) the possession of a high inherent tolerance to increasing ethanol concentrations.

Although the real mechanism of action of ethanol is not known, nor why responses to it differ (15), it should not be surprising that some plants are more tolerant of higher ethanol concentrations

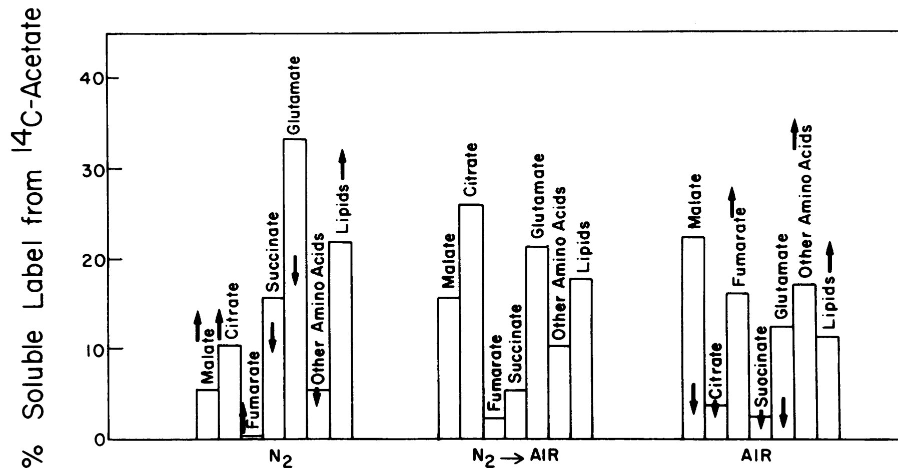

FIG. 4. Distribution of label in soluble metabolites of *E. crus-galli* var *oryzicola* germinated for 5 d under N_2 or air. Seeds were imbibed for 3 h with [U-^{14}C]acetate under N_2 or air as shown. Arrows indicate changes in ^{14}C-labeled products after a 16-h chase in the absence of [U-^{14}C]acetate.

Table VI. *Distribution of ^{14}C in Seedlings of E. crus-galli var oryzicola when Supplied with [U-^{14}C]Sucrose*

Seeds were germinated for 5 d in N_2 or air and then pulsed for 3 h with [U-^{14}C]sucrose in N_2 or air.

Experimental Conditions	Label Metabolized	Total Label Added	^{14}C Distribution (Label Metabolized)			
			$^{14}CO_2$	[^{14}C]Ethanol	Insoluble	Soluble
	cpm	*%*		*%*		
N_2	1.06×10^6	(26.5)	63.0	14.7	0.9	21.7
O_2	1.38×10^6	(34.5)	66.9	4.8	0.3	28.5

than others. In a survey of the literature, Jackson (9) reported endogenous ethanol concentrations under flooded conditions varied from 0.04 mM in wheat seedlings to 46.5 mM in pea seeds. Growth was inhibited by minimum concentrations of externally supplied ethanol ranging from 3.9 to 173.6 mM. Lethal conditions developed at levels of 100 mM ethanol in willow roots and wheat coleoptiles. We (17) measured 11 mM ethanol in oryzicola seeds after 7 d anaerobiosis and 45 mM in the imbibition solution. As shown here (Fig. 1), external concentrations as high as 515 mM were not lethal to germinating oryzicola seeds.

Several ideas have been proposed to account for the differing sensitivities of plants and animals to ethanol (1, 2, 9, 15, 17). Among these are the synthesis of specific isozymes of ADH (8, 15) and the increased ability to metabolize ethanol (2, 7). In oryzicola, ADH activity increased significantly under N_2. ADH activity also increased markedly if ethanol was included in the imbibition medium (Table II). In nature, particularly under low O_2 in plants, the widespread occurrence of ADH may be less important than the ability to synthesize or activate a specific isozyme during periods of stress as earlier studies (15) suggest.

In oryzicola, two possible functions of ADH could be: (a) to allow for a greater turnover of the glycolytic cycle and, hence, increased ATP synthesis by substrate-level phosphorylations; and/or (b) to metabolize the ethanol produced under stress. Results from the [1-^{14}C]ethanol labeling studies (Table III) suggest that

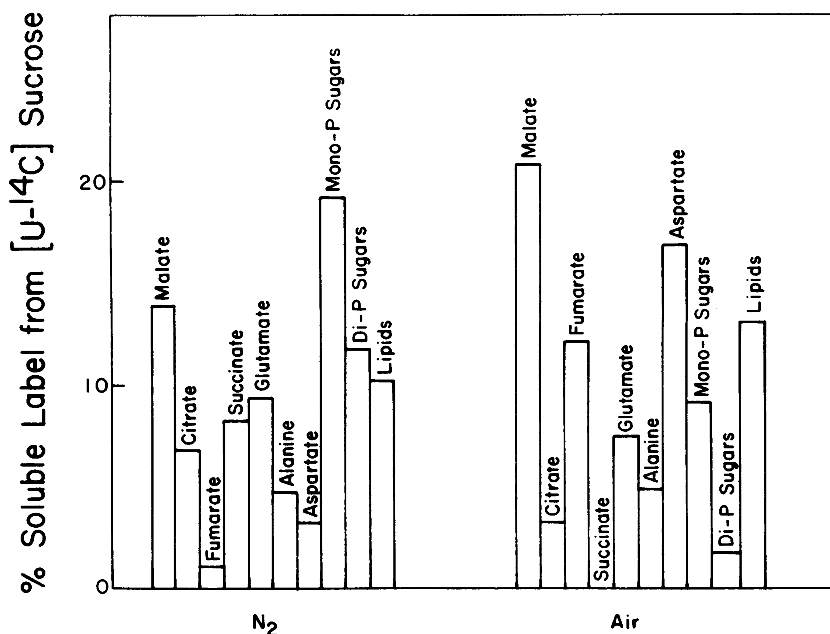

FIG. 5. ^{14}C-Labeled metabolites of *E. crus-galli* var *oryzicola* from [U-^{14}C]sucrose feedings. Seeds were germinated for 5 d in either N_2 or air and then labeled with sucrose for 3 h in N_2 or air. Values represent the percent of total soluble compounds labeled.

very little ethanol is actually metabolized without O_2 (2.3% under N_2 *versus* 80.4% in air), indicating that ADH may function as indicated in 'a' above. However, it is difficult to quantify the amount metabolized under anaerobiosis due to dilution of added label by endogenous ethanol. In addition, external ethanol concentrations would have to exceed that found normally in solution after 7 d under N_2 (45 mM) (17) and that recovered internally (11 mM) by at least 11- and 45-fold, respectively, before becoming lethal (>500 mM) (Fig. 1). Hence, it is unlikely that a substantial amount of ethanol would need to be metabolized to prevent toxicity under anaerobiosis. Metabolism of ethanol would, however, conserve carbohydrate carbon for biosynthetic purposes. At the same time, it is possible that increased production of ethanol induces ADH for metabolism of the accumulated ethanol once more favorable conditions return.

As previously stated, alcoholic fermentation is the major route of metabolism in anaerobically germinated seeds of oryzicola. This is to be expected since glycolysis is the main source of energy without O_2. Oryzicola is able to maintain a high ATP level with an energy charge of at least 0.8 in the absence of O_2 (Rumpho, Kennedy, Pradet, unpublished data). However, in addition to energy, seeds germinated under anaerobic conditions require additional mechanisms to recycle pyridine nucleotides (both NAD and NADP), while carbon intermediates are essential for the biosynthesis of membranes and cellular components (10, 19). Both the OPP pathway and tricarboxylic acid cycle may produce carbon intermediates, while the OPP pathway has been shown to provide the NADPH needed for lignin (14) and lipid (7) synthesis. For oryzicola, [1-^{14}C]ethanol, [U-^{14}C]acetate, and [U-^{14}C]sucrose studies indicate that lipid synthesis occurs under both N_2 and air (Figs. 2 and 4 to 5) and could provide a mechanism for reoxidizing NADPH. Electron micrographs of 5-d anaerobically germinated oryzicola seedlings show an accumulation of lipids in the cytoplasm of the primary leaves and coleoptiles, and extensive proplastid and mitochondrial membrane synthesis (19). Metabolism of sucrose into lipids indicates that, in the absence of O_2, carbohydrate can be broken down to acetyl-CoA for use in lipid synthesis. Earlier, we (10) presented evidence for the operation of the OPP pathway in anaerobically germinated oryzicola seeds based on glucose labeling experiments. Further studies (16; M. E. Rumpho and R. A. Kennedy, unpublished data) of the OPP pathway enzyme glucose-6-P dehydrogenase and specific labeling of CO_2, ethanol, and intermediate compounds from [6-^{14}C] *versus* [1-^{14}C]glucose labelings support the operation of the OPP pathway under anaerobic conditions. Additional evidence is shown here (Fig. 5) inasmuch as more label was recovered in phosphorylated sugars (produced by the OPP pathway) under N_2 than air.

Conclusive evidence for tricarboxylic acid cycle metabolism under anaerobic conditions is lacking. This study, however, does show labeling of key intermediates as a result of feeding with various precursors of the glycolytic and tricarboxylic acid cycles. In particular, we have shown sucrose breakdown to organic acids, the metabolism of acetate, and production of glutamate and other amino acids, presumably by transamination of oxoglutarate. The increase in labeling of succinate in oryzicola under anaerobiosis (Figs. 2–5) has also been reported in other species (4–6, 21), but the pathway of formation has not been proven.

Thus, anaerobic growth in oryzicola is characterized by certain metabolic features, including a high fermentative activity and ethanol production. In addition, oryzicola may possess additional adaptive strategies such as (a) a greater tolerance for high ethanol levels; (b) the ability to metabolize ethanol; and (c) additional intermediary metabolism (other than fermentation) in order to metabolize carbohydrate reserves and to provide for biosynthetic puposes.

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