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Germination of *Echinochloa crus-galli* (Barnyard Grass) Seeds under Anaerobic Conditions[1]

RESPIRATION AND RESPONSE TO METABOLIC INHIBITORS

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ABSTRACT

Echinochloa crus-galli **L. Beauv., a rice-field weed, can germinate and grow for extended periods of time in an anaerobic environment. Compared to pea, which does not germinate under anaerobiosis, the evolution of CO_2 in *Echinochloa* and rice is lower and the peak rate of CO_2 evolution is delayed when germinated without oxygen. The plants studied also differ with respect to their respiration ratio ($[CO_2]$ N_2/$[CO_2]$ air) and metabolism used during the early stages of germination. *Echinochloa* does not increase its glycolytic rate under anaerobiosis, whereas pentose phosphate pathway activity appears to increase during the first 40 to 50 hours of germination.**

Based on its response to metabolic inhibitors (NaF, dinitrophenol, and malonate), anaerobic metabolism in *Echinochloa* proceeds primarily through glycolysis, with partial operation of the tricarboxylic acid cycle and little or no oxidative phosphorylation. Also, *Echinochloa* is sensitive to CN during aerobic germination, whereas rice appears to be able to shift to CN-insensitive electron transport. Finally, the effectiveness of cyanide and azide on inhibiting germination of *Echinochloa* in N_2, but not CO, suggests that cytochrome oxidase is not used to reoxidize pyridine nucleotides in the absence of oxygen. The possible existence of an alternate electron acceptor is discussed.

In spite of the abundance of atmospheric O_2, there are many situations where the growth and metabolism of plants is limited because of too little O_2, *e.g.* in flooded fields and natural wetlands (10). In addition, one of the serious consequences of the increased use of large-scale irrigation is saturated soils, resulting in O_2 levels which are inadequate for normal plant growth. Many native plants circumvent O_2 stress in these environments by possessing anatomical or morphological adaptations, such as aerenchyma tissue, which permit them to grow in water-logged soils by 'transport' of O_2 to the root zone (5). The only crop plant known to be able to grow in a totally O_2-free environment is rice (16). Although the exact mechanism(s) which allow rice to grow in anaerobic environments are unknown, it is becoming clear that the general hypothesis put forward (15) to explain anaerobic metabolism is untenable in many instances (18, 21).

Recently, we reported (13) that plants in the barnyard grass complex (*Echinochloa* sp.), common weeds in the rice field agroecosystem, can germinate and grow for extended periods of time in anaerobiosis. *E. crus-galli* var *oryzicola* is in fact a rice mimic (3), with growth characteristics in O_2-free environments as good as or better than those of rice. *Echinochloa* has been shown to occur in up to 81% of the rice seed lots tested (3) and may reduce rice yields by up to 40% (13). We have been interested in the metabolism of *Echinochloa* because of its ability to germinate and grow in anaerobic environments, its role as a weed in reducing rice yields worldwide, and the frequency and importance of O_2-limited soils in agricultural systems.

In previous reports, we have presented information on the structural aspects of anaerobic germination in *Echinochloa* seeds (22), seedling characteristics and cellular ultrastructure (13), chloroplast ultrastructure (23), and metabolism of anaerobic germination (18–20). This paper presents information on the respiration and response to respiratory inhibitors of anaerobically grown *Echinochloa* seedlings.

MATERIALS AND METHODS

Seed Germination. Seeds of *Echinochloa crus-galli* L. Beauv. var *crus-galli*, *E. crus-galli* L. Beauv. var *oryzicola* (hereafter, crus-galli and oryzicola, respectively), rice (*Oryza sativa* L., cv S201) pea (*Pisum sativum* L., cv Alaska), and wheat (*Triticum aestivum* L., cv Dawes) were surface-sterilized in 2.5% NaOCl for 10 min, washed three times with glass-distilled H_2O, and germinated with the appropriate N_2 (99.995% N_2) or air gas phase as before (13). No bacterial or fungal contamination was detected when an aliquot of the germination solution was incubated on agar plates containing (per liter): 15 g agar, 3 g yeast extract, and 10 g glucose (20). Data are expressed as the mean ± SE.

^{14}C-Labeling Experiments. For ^{14}C-labeling experiments, seeds were germinated as above in the presence of [U-^{14}C]glucose (275 mCi/mmol), [6-^{14}C]glucose (60.5 mCi/mmol), [1-^{14}C]glucose (58 mCi/mmol), or [U-^{14}C]sucrose (15 mCi/mmol). The $^{14}CO_2$ evolved was trapped in 1.0 M KOH and radioactivity quantified by liquid scintillation counting with PCS scintillation cocktail (Amersham). [^{14}C]Ethanol was determined by distillation and quantified as above.

For respiration rate experiments, the relative $^{14}CO_2$ evolved from [U-^{14}C]glucose under N_2 and air was identical to the endogenous $^{12}CO_2$ evolution pattern as measured by IR gas analysis.

Inhibitor Experiments. The respiration inhibitors NaF (0.1 M K-phosphate; pH 7.0), malonate (pH 5.0), and DNP[4] (pH 6.0)

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[4] Abbreviations: DNP, dinitrophenol; SHAM, salicylhydroxamic acid, ADH, alcohol dehydrogenase; PP, pentose phosphate.

were added to the germination flasks in the concentrations indicated in the figures. KCN, again in the amounts indicated, was added to the flasks (0.1 M K-phosphate or 10.0 mM Hepes; pH 7.0), flushed with N_2, stoppered, and placed in an anaerobic jar with a continuously flushing N_2 atmosphere. In other experiments, the KCN solution was changed daily, and in all cases, the seeds were continuously in contact with the KCN. SHAM and Na azide (both 2.0 mM) were added to the flasks in a flow-through system in 10.0 mM Hepes buffer (pH 7.0). KCN uptake was verified by analyzing for CN within the seeds. Uptake of the inhibitors was also verified by its concentration dependence in affecting germination, especially shoot length, and by the fact that when the seeds were removed from the inhibitor and washed, they subsequently germinated.

To determine the effects of CO, 99% CO in N_2 was continuously passed through the flasks as before (13), except all flasks were kept in the dark.

Nitrate Determinations. Nitrate was assayed by the method of Lowe and Hamilton (14) using an *E. coli* extract containing nitrate reductase.

RESULTS AND DISCUSSION

Evolution of $^{14}CO_2$ from [U-^{14}C]Glucose. Respiratory CO_2 release in pea (anaerobic intolerant species) was compared to that in rice, crus-galli, and oryzicola (tolerant plants). Based on the evolution of $^{14}CO_2$ when the seeds were imbibed and germinated in the presence of [U-^{14}C]glucose (Fig. 1), the respiratory profile in pea was virtually the same in N_2 compared to air; whereas, in the N_2-tolerant plants, the onset of germination was delayed and $^{14}CO_2$ evolution was less in N_2 *versus* air. Time to germinate also varied depending on gas phase; for example, oryzicola germinated after 45 h in air and after 63 h in nitrogen.

The difference between pea and the other seeds with respect to timing and rate of respiration is best seen in Figure 2. In air, the rate of $^{14}CO_2$ evolution was 128 cpm $^{14}CO_2$ evolved g^{-1} dry weight h^{-1} in pea, compared to 500 for rice, 1500 for crus-galli, and 1640 for oryzicola. In N_2, the rate in pea remained relatively unchanged (115 cpm g^{-1} dry weight h^{-1}), while oryzicola, rice, and crus-galli exhibited 2.3-, 3.1-, and 6.3-fold decreases in their respiration rate, respectively.

In addition to differences in their general pattern of respiration in N_2 and air, it has been proposed (15) that a second important distinction between tolerant and intolerant plants is the timing of their maximum respiration in N_2 and air. In the present studies, the peak respiration rate for the three grass species was delayed 15 to 20 h when grown in N_2 compared to air, while the respiratory profile in pea was the same in both environments. This delay was not due merely to differences in total uptake of label under N_2 since each type of seed took up equal amounts of label under the two gas phases used (Fig. 1).

Figures 1 and 2 support the concept (15) that flood-tolerant plants such as oryzicola, rice, and crus-galli delay the onset of peak respiration and reduce its rate in O_2-limiting environments. In contrast, pea, which does not germinate under anoxia, has basically the same respiratory pattern under N_2 and air. The respiratory ratio, defined here as the CO_2 evolved in N_2 compared to the CO_2 evolved under O_2 (8, 12), also differed markedly in these organisms (Fig. 3), again illustrating their differing respiratory behavior in aerobic and anaerobic environments.

If the mechanisms for tolerance of anaerobic conditions are dependent on regulation of ADH activity and ethanol production, then a lower respiratory ratio might be of adaptive significance, *i.e.* lower respiration rate, less ADH activity, and decreased ethanol production. This, combined with a delay in the onset of germination, has been suggested as the main strategy for flood tolerance among higher plants (15). Recent experimental data, however, do not support this hypothesis. We (18) have shown that oryzicola has ADH, ethanol, and malic enzyme levels similar to those attributed to flood-intolerant plants. Similar results have been found in rice (2). In addition, Smith and apRees (21) found flood-tolerant plants to possess high ADH activity and ethanol levels, while pea was recently reported (11) to be able to withstand ethanol concentrations 100-fold greater than the previously supposed toxic levels present in anaerobically grown seedlings. Thus, in the case of oryzicola, lower respiration rates and a delay in the onset of respiration do not result in low ethanol levels or ADH activity (18).

Activity of the Pentose Phosphate Pathway. The sharp difference in respiratory ratio between pea and the N_2-tolerant plants (Fig. 3), especially during the early stages of germination, sug-

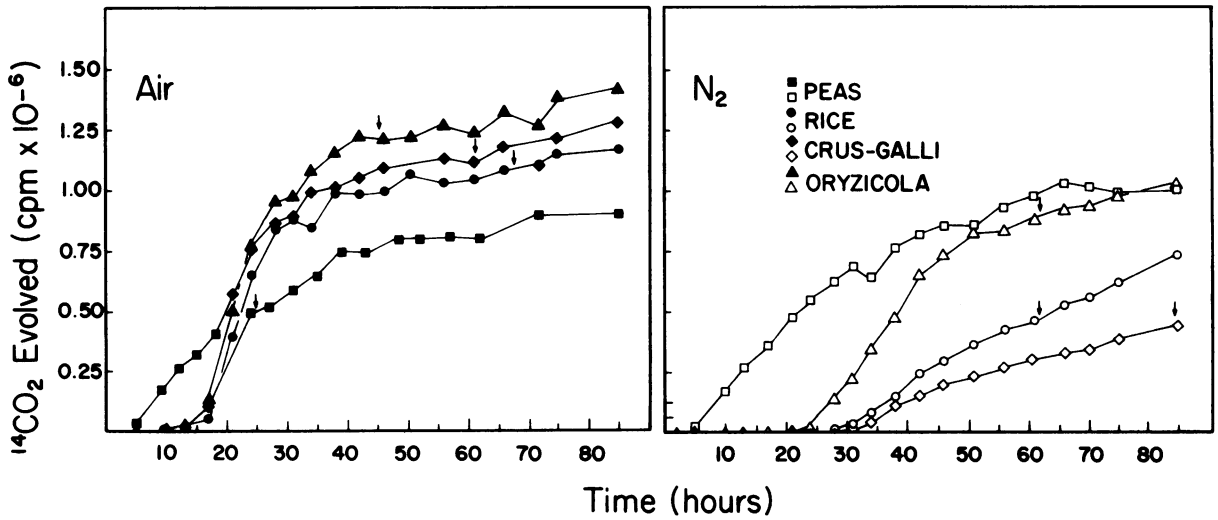

FIG. 1. Evolution of $^{14}CO_2$ during germination of pea (intolerant), rice, crus-galli, and oryzicola seeds (tolerant) grown in the presence of [U-^{14}C] glucose in air (left) or N_2 (right). Amount of plant material was 1.3 g dry weight for peas; 0.7 g dry weight for rice, and 0.25 g dry weight for crus-galli and oryzicola. Equal amounts of glucose were taken up by the seeds in either N_2 or air, measured by radioactivity localized in replicate seed lots at various periods throughout the time course, with approximately one-half of the radioactivity (1.5×10^6 cpm) added per flask evolved as $^{14}CO_2$. Leakage of metabolites back into the imbibition medium was not found to be significant, being less than 1% of the label present at any given time period. Arrows indicate when seed germination was first observed for each species under the two regimes studied.

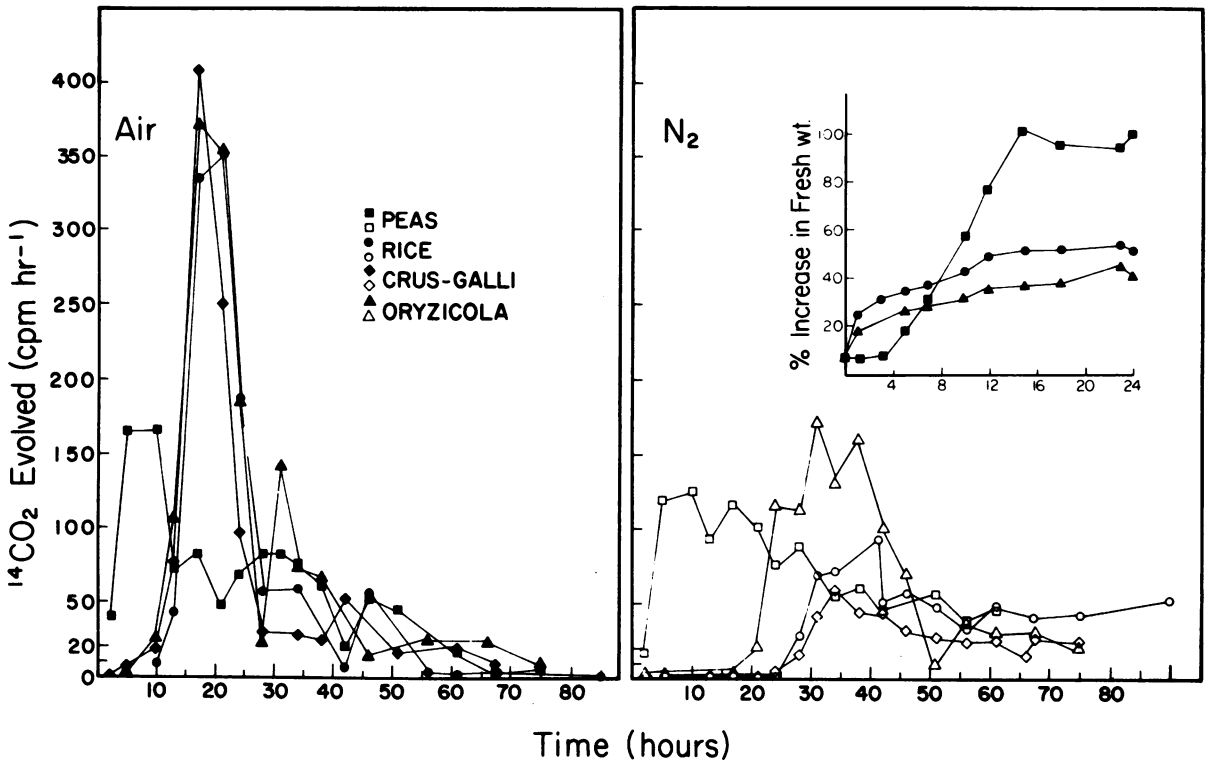

FIG. 2. Rate of $^{14}CO_2$ evolution from seeds germinated on [U-^{14}C]glucose under air and N_2 atmospheres. Seed weights and label uptake as given in Figure 1. Inset, Time course of imbibition (percent increase in fresh weight).

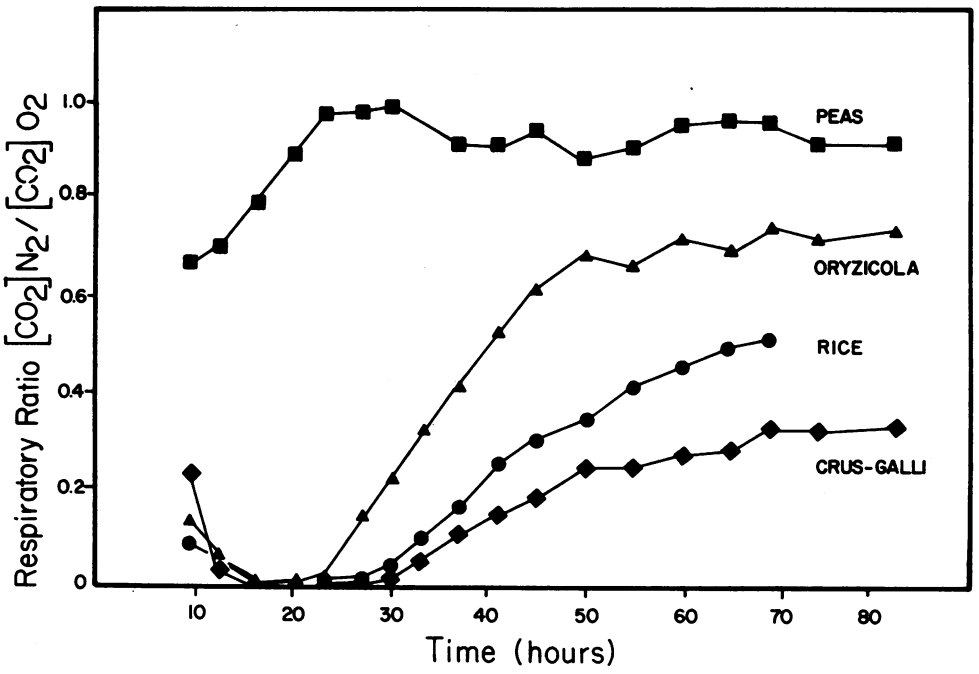

FIG. 3. Respiratory ratio $[CO_2]N_2/[CO_2]O_2$ (8, 12) for pea, rice, crus-galli, and oryzicola seeds imbibed with [U-^{14}C]sucrose. Once imbibed, the respiration ratio for pea was generally around 1.0; whereas, the N_2/O_2 ratio in the N_2-tolerant seeds decreased to less than 0.1 in the first 30 h of germination.

gested to us that major differences in respiratory pathways may exist in the seeds, depending on the presence of O_2. We were particularly interested in the relative activity of the PP pathway since the latter has been suggested to be of prime importance in germinating seeds before O_2 concentrations within the seed reach ambient levels (17). Replicate seed lots were fed [6-^{14}C]glucose and [1-^{14}C]glucose, the $^{14}CO_2$ evolved was quantified and the C-6/C-1 ratio determined to assess the activity of glycolysis (C-6/C-1 ratio near unity) *versus* the PP pathway (C-6/C-1 ratio generally well below 1) (Fig. 4). Although there are difficulties in using C-6/C-1 ratios quantitatively (1), the large differences in the ratios observed here clearly indicate qualitative differences in operation of the two pathways. In air, rice consistently had C-6/C-1 ratios of 1.0 or greater throughout the time course surveyed, while pea,

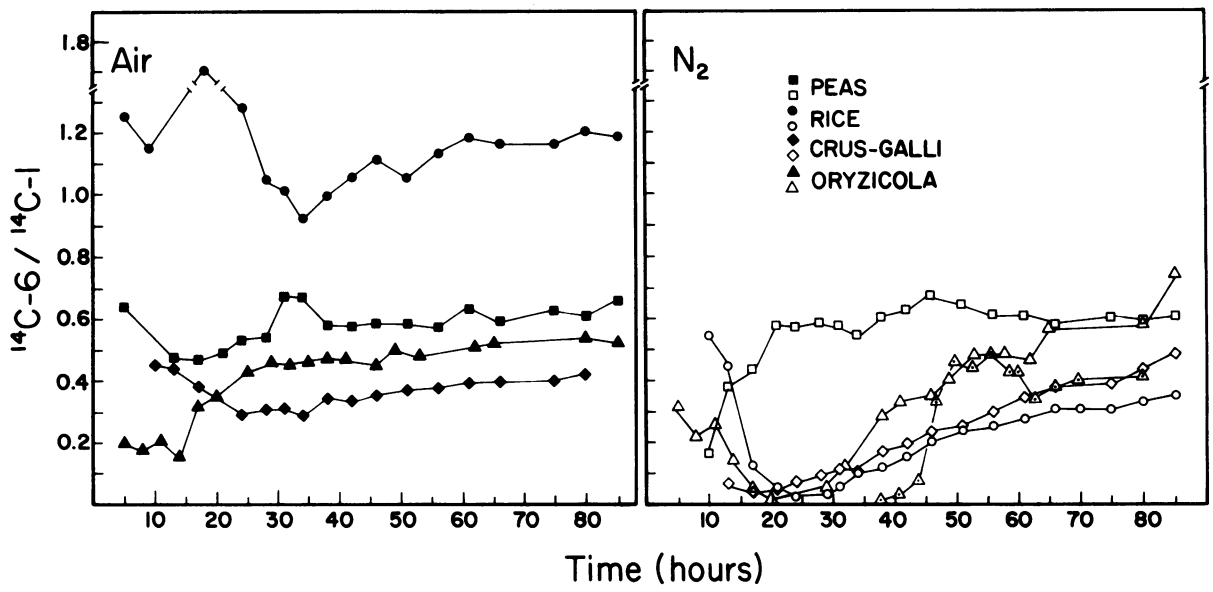

FIG. 4. C-6/C-1 ratios of peas, rice, oryzicola, and crus-galli. Equal amounts of [6-^{14}C]glucose and [1-^{14}C]glucose were added to separate samples of each seed and allowed to germinate as given in "Materials and Methods." $^{14}CO_2$ evolved was trapped, counted, and the ratio of C-6/C-1 determined to give an estimate of glycolysis (C-6/C-1 generally about 1.0) *versus* the pentose phosphate pathway (C-6/C-1 generally <0.1). For oryzicola, a second curve is given under N_2 (Δ). This line indicates the C-6/C-1 ratio when the labeled glucose was added during a time course of germination to eliminate an artificial increase in the ratio caused by randomization of label. Note the sharp change in C-6/C-1 ratio after approximately 30 h, suggesting differences in the pathway of carbohydrate utilization during the first 30 h of germination compared to later stages (*cf.* Fig. 3).

oryzicola, and crus-galli had values around 0.4. In an anaerobic environment, the most noticeable differences were seen. Starting with ratios of 0.4 to 0.6, the C-6/C-1 ratios in the N_2-tolerant species decreased simultaneously during the first 20 h germination time, reaching values of less than 0.1. These values indicate a predominance of the PP pathway over glycolysis. Thereafter, the C-6/C-1 ratios increased to approximately 0.4 to 0.6 after 60 h of germination. During the first 20 h, the ratio in pea, on the other hand, increased from an initial value of about 0.2 to 0.6 before stabilizing.

One of the most serious criticisms with this methodology for determining activity of the pentose phosphate pathway concerns the randomization of the label that may occur during experimentation (1). While this is a valid criticism, randomization would, if anything, result in over-estimating glycolytic activity compared to PP pathway activity. The major difference shown in Figure 4 is a decrease in the C-6/C-1 ratio in the grass seeds during the first 30 h of germination in N_2; the opposite of what you would expect if randomization had occurred. Additional verification of the C-6/C-1 ratios was obtained by addition of label to the seeds at 36, 48, and 58 h after imbibition and counting the $^{14}CO_2$ evolved every 2 h thereafter. When randomization of label was minimized by this technique, the low C-6/C-1 ratio (<0.1) of the N_2-tolerant seeds was maintained for the first 40 h of germination, again indicating the importance of the PP pathway during this phase of anaerobic germination.

It is interesting to note that the decrease in the C-6/C-1 ratio for the N_2-tolerant seeds occurred at the same time that the respiratory ratio decreased. This also suggests operation of the pentose phosphate pathway inasmuch as the lower CO_2 evolution by the PP pathway, if it were the predominant route of carbohydrate oxidation during that period, would result in a low respiration ratio. Recent experiments (19, 20) also support the role of the PP pathway during anaerobic germination of these seeds. When metabolites from seeds grown on [U-^{14}C]sucrose in air are compared to those grown in N_2, more radioactivity is localized in PP pathway intermediates under N_2 than air (20). Also, relative to growth rates in air, N_2-grown seeds have high glucose-6-P dehydrogenase activity, the first enzyme of the PP pathway (19).

Finally, relative rates of glycolysis *versus* the PP pathway in oryzicola can be judged by comparing the CO_2 evolved and ethanol produced under N_2 and air. Conventional interpretations of anaerobic metabolism in plants suggest that intolerant species have a faster rate of carbohydrate breakdown under low O_2 than in air, *i.e.* they have a Pasteur effect (8). In oryzicola, carbon flow was in fact less under N_2 than in air, based on cpm evolved as $^{14}CO_2$ or accumulated in ethanol after labeling with [U-^{14}C]sucrose (Table I). Nearly twice as much ethanol was produced under N_2 compared to air. Yet under N_2, the CO_2 production was greater than that of ethanol and still resulted in only two-thirds the amount of label respired as CO_2 in air. The ratio of CO_2/ethanol (<0.1) is consistent with partial operation of the tricarboxylic acid cycle and PP pathway contributing to the excess CO_2 produced (19). Combined, the amount of carbon lost under anoxia as CO_2 plus ethanol was 83% of that in air. These results strongly support a conclusion that, as classically defined, oryzicola does not exhibit a Pasteur effect under anoxia, as also reported by Effer and Ranson in buckwheat (8).

Metabolic Inhibitors. A different approach to determine which metabolic pathways were important during anaerobic germination was the use of inhibitors. When oryzicola seeds were germinated in air or N_2 in the presence of malonate (an inhibitor of succinate dehydrogenase), germination percentage was unaffected in either gas phase, even at 100 mM malonate (Fig. 5a). The same concentration of inhibitor decreased shoot length under aerobic condi-

Table I. *Distribution of $^{14}CO_2$ from [U-^{14}C]Sucrose-Labeled Oryzicola Seedlings after 5 Days Germination in N_2 or Air*

Compound	Radioactivity in Seedlings	
	N_2	Air
	cpm (× 10^{-3})	
Ethanol	156 ± 11	66 ± 2
$^{14}CO_2$	667 ± 3	922 ± 6
Total	823	988

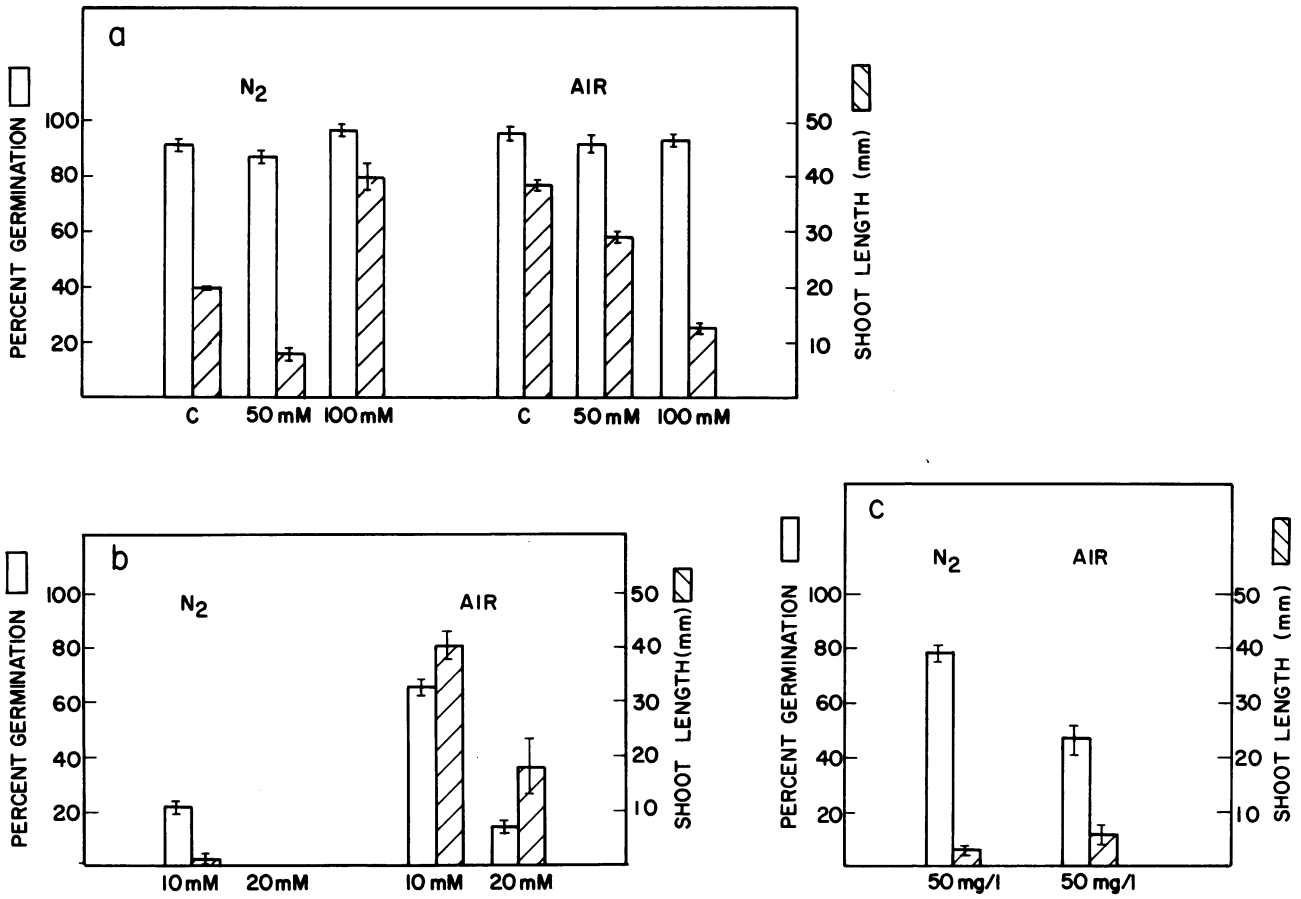

FIG. 5. Effects of malonate (a), NaF (b), and DNP (c) on germination percentage and shoot length of oryzicola during a 7-d germination period. Control values for germination and shoot length under N_2 and air are given only in (a).

tions, but stimulated it under anaerobiosis. These results are consistent with the observation that, under anaerobiosis succinate often accumulates (5, 10) (presumably because of decreased activity of succinate dehydrogenase). Thus, one may not expect a major effect of malonate during anaerobic metabolism. DNP, an uncoupler of electron transport, inhibited shoot length and percent germination markedly in air-grown seedlings, but had less of an effect on either growth characteristic when grown in N_2 (Fig. 5c). In contrast to the two inhibitors above, NaF, an inhibitor of the glycolytic enzyme enolase, has its greatest effect and at lower concentrations on anaerobically grown seedlings (Fig. 5b). Compared to their respective controls, NaF at 10 mM reduced germination percentage and shoot length 4- to 10-fold in N_2. At higher concentrations, NaF totally inhibited the germination and shoot growth of oryzicola. Thus, malonate and DNP, inhibitors of the tricarboxylic acid cycle and oxidative phosphorylation, respectively, had little effect on anaerobically grown oryzicola seedlings, but reduced the growth of those grown in the air. NaF had the reverse effect, illustrating the probable importance of glycolytic reactions and substrate level phosphorylation during anaerobic metabolism.

Electron Transport Inhibitors. The last aspect studied was the differing response of rice and oryzicola to electron transport inhibitors (Fig. 6). In the presence of 2 mM KCN in N_2, the germination of both flood-tolerant seeds was drastically reduced; zero germination in the case of oryzicola. The most interesting difference, however, is in their response in air. Germination of rice in air is unaffected by the presence of KCN. Apparently, rice shifts to a CN-insensitive pathway since SHAM + cyanide (inhibitors of the alternate and conventional mitochondrial electron transport, respectively) (6) reduced rice germination drastically when both were included in the imbibition medium. Aerobic germination in oryzicola, on the other hand, was reduced to less than 20% in the presence of cyanide, with little further inhibition by the addition of SHAM. Incidently, azide (data not shown) had the same effect on oryzicola germination that KCN did, while SHAM alone had no effect. These results indicate that, although oryzicola and rice are similar in many aspects of their anaerobic metabolism, under aerobic conditions, there is a major difference in the presence or absence of the CN-insensitive alternate electron pathway found in many plant tissues. The extent of these differences are presently being studied.

The effects of cyanide and azide on inhibiting germination of oryzicola suggested to us (13) that oryzicola may use a terminal electron acceptor other than O_2 when germinated under anaerobic conditions (so-called 'alternate electron acceptor') (7). The reasoning was that since cyanide and azide inhibit Cyt oxidase even in the absence of O_2, Cyt oxidase could mediate electron transfer to some other oxidant during anaerobic germination. In earlier studies, Wang (24) showed that Cyt oxidase was present at 30% of the activity of aerobic controls. To check this hypothesis, oryzicola seeds were germinated in the presence of carbon monoxide, a more specific inhibitor of Cyt oxidase. In this inhibitor, however, the seeds germinated as well as the air-dark (Fig. 7) or N_2-dark (13) controls. Shoot fresh and dry weight and seedling length were very similar to that obtained under N_2. And, as also occurs under anaerobic germination, no root growth took place. These results indicate that, although cyanide and azide may affect some other alternate electron acceptor, it is unlikely that their inhibiting effect on anaerobic germination in oryzicola is through Cyt oxidase. It is curious to note that nitrate reductase activity is inhibited by cyanide and azide, but not by CO, exactly the results observed

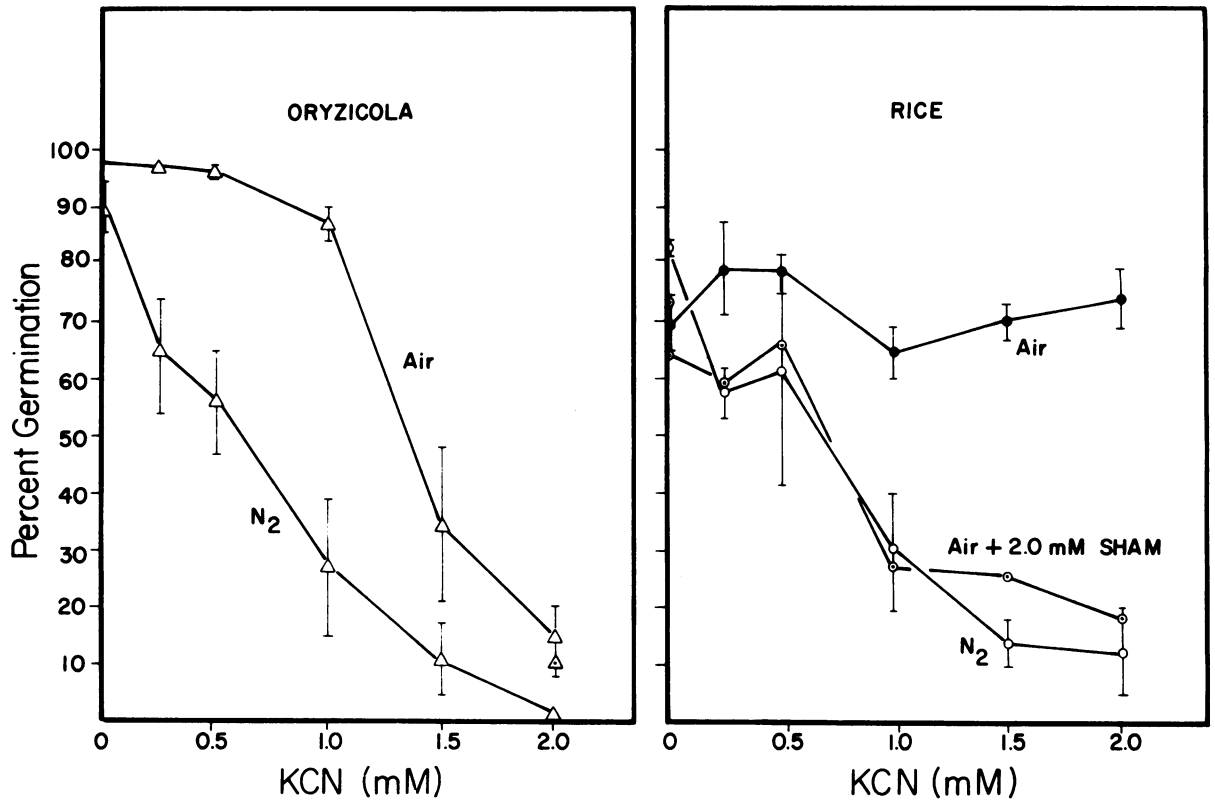

FIG. 6. Effect of varying KCN concentrations and 2.0 mM SHAM on germination in rice and oryzicola. Symbols with dots indicate effect of KCN plus SHAM. Seeds were imbibed in the presence of the inhibitor, as given in "Materials and Methods," and germination recorded after 7 d. The inhibiting effect of KCN on the seeds was verified by germination of the seeds after KCN removal from the system.

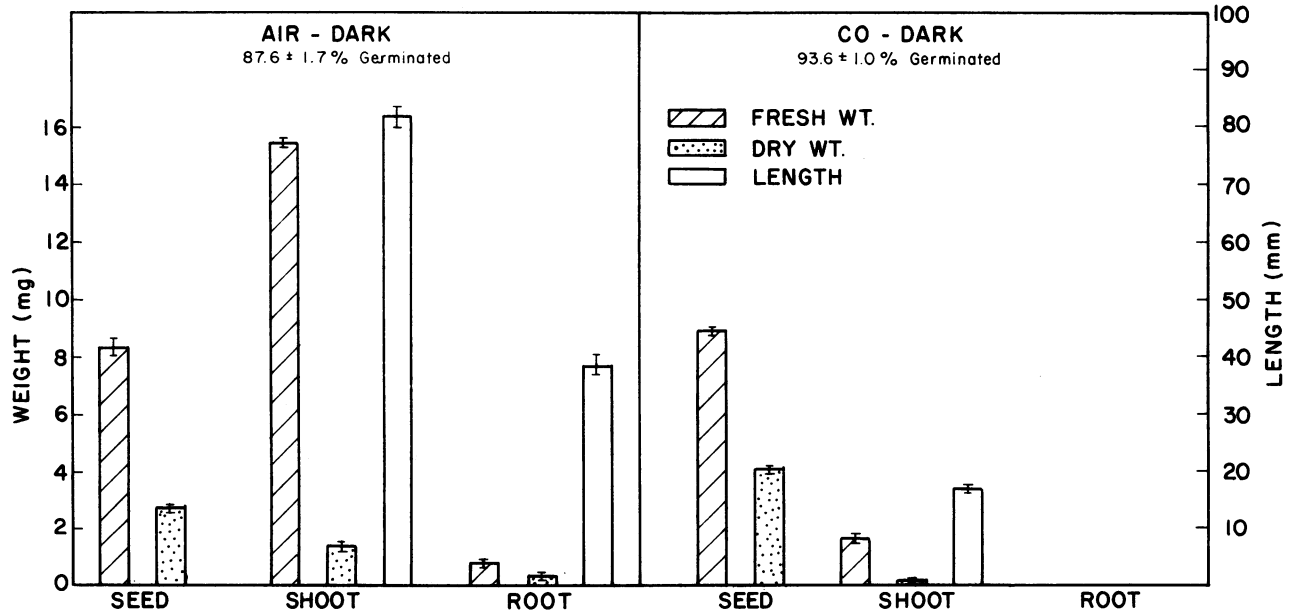

FIG. 7. Percent germination and shoot length of oryzicola grown for 7 d in air-dark or in the presence of CO (99.0% CO).

here. Nitrate, through nitrate reductase, is a common alternate electron acceptor in microbial systems (9) and could function in that capacity in oryzicola seeds. Arguments against this hypothesis, however, would be that whatever the alternate acceptor is, it has to be endogenous inasmuch as the seeds are routinely germinated in the laboratory with distilled H_2O only. Also, in anaerobic soils virtually all nitrate is quickly reduced to ammonia (4) and most nitrogen within the seed would be expected to be in the reduced form. Conversely, support for the alternate electron acceptor hypothesis comes from the observation that oryzicola seeds have significant amounts of nitrate which could serve as a terminal electron acceptor under anaerobic conditions. Oryzicola seeds contain 4 times more nitrate than rice seeds and greater than 10 times more than wheat, a flood-intolerant grass seed (Fig. 8). Moreover, the NO_3^- level drops to one-fourth of its original value within the first 24 h of imbibition, indicating that the NO_3^- could

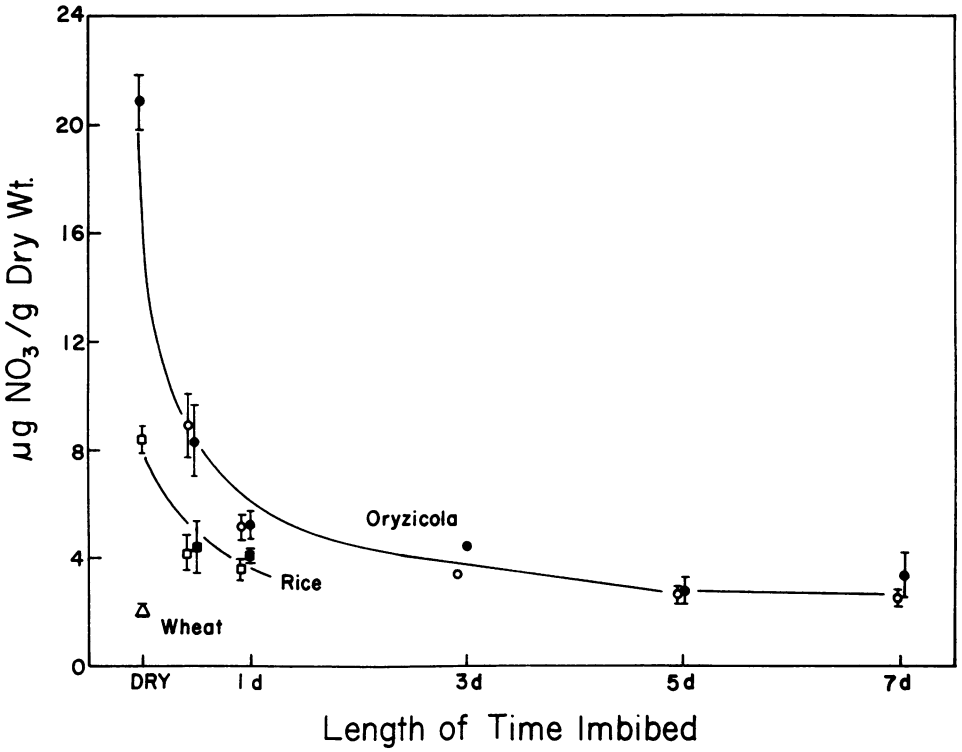

FIG. 8. Nitrate content of wheat, rice, and oryzicola seeds and changes in nitrate levels during germination of rice and oryzicola.

serve as an alternate electron acceptor in the absence of O_2, as commonly occurs in microbes (9). Although the initial seed NO_3^- value is lower in rice, it too decreases during germination (Fig. 8).

CONCLUSIONS

Comparing respiratory behavior and responses to metabolic inhibitors in oryzicola, rice, and pea, we found the following.

(a) In contrast to pea, the respiratory activity of flood-tolerant plants was greater in air than under anaerobic conditions, where it was suppressed and its onset delayed. This may be a mechanism to achieve a sustained level of energy production (by substrate level phosphorylation through glycolysis) until more favorable growth conditions resume.

(b) *Echinochloa* and rice had respiration ratios ($[CO_2]N_2/[CO_2]O_2$) near zero during the early stages of germination. During this same period, activity of the pentose phosphate pathway under anaerobiosis was suggested by C-6/C-1 ratios of less than 0.1. In *Echinochloa*, CO_2 and ethanol produced under N_2 was less than that produced in air.

(c) Based on the differential effects of malonate, DNP, and NaF, anaerobic metabolism in oryzicola is less dependent on full operation of the tricarboxylic acid cycle and oxidative phosphorylation than on glycolytic activity.

(d) Although cyanide and azide block germination of oryzicola under N_2 suggesting the possibility of an alternate electron acceptor, operation of Cyt oxidase is ruled out because of the ineffectiveness of CO in inhibiting anaerobic germination.

(e) Finally, there appears to be a major difference in the response to added KCN between rice and *Echinochloa*. In air, germination in rice was unaffected by KCN but inhibited by SHAM (apparently switching to a SHAM-sensitive pathway), while germination of oryzicola was inhibited by KCN with or without SHAM.

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