

Carbohydrate–ethanol transition in cereal grains under anoxia

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Summary

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- Cereal grains differ greatly in their responses to anaerobiosis. Here, the *in vivo* conversion of carbohydrates to ethanol and CO₂ under anoxia is reported for three cereal grains.
- The conversion of glucose, fructose or sucrose to ethanol under anaerobic conditions was investigated in rice (*Oryza sativa*), barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) grains; alcohol dehydrogenase (EC 1.1.1.1) and pyruvate decarboxylase (EC 4.1.1.1) activities were also analysed under aerobic and anaerobic incubation.
- Our data suggest that rice grains are able to produce ethanol under anoxia for the whole period of anoxic treatment, whereas barley and wheat grains can produce this terminal product of fermentation only during the first days of anaerobiosis. The level of enzymes involved in the fermentation pathway increases strongly under anoxic conditions in all three cereals.
- Conversion of hexose to CO₂ is nearly unaffected by anoxia in wheat, barley and rice, whereas only rice grains are able to degrade and utilize sucrose efficiently under anoxia. By contrast, wheat and barley do not utilize sucrose efficiently under anaerobic conditions.

Key words: alcohol dehydrogenase (ADH), anoxia, fermentation, *Hordeum vulgare* (barley), *Oryza sativa* (rice), pyruvate decarboxylase (PDC), *Triticum aestivum* (wheat).

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Introduction

Cereal grains differ greatly in their responses to anaerobiosis. Rice grains represent an example of metabolic adaptation to anoxia, being able to germinate and elongate the coleoptile in an oxygen-free environment (Alpi & Beevers, 1983). By contrast, grains of barley and wheat are severely injured when imbibed under anoxia, fail to elongate coleoptile and root and cannot recover when transferred to air after a few days of anoxic treatment (Perata *et al.*, 1996).

The availability of fermentable carbohydrates appears to be one of the biochemical parameters correlating with anoxia-tolerance in cereal grains. Indeed, rice can take advantage of the starchy reserves present in the endosperm as a consequence of the successful anaerobic induction of the complete set of

starch-degrading enzymes (Perata *et al.*, 1992; Perata *et al.*, 1993; Guglielminetti *et al.*, 1995b). On the contrary, wheat and barley do not induce these enzymes, and the embryos soon suffer from sugar starvation which may account for the decline in viability (Guglielminetti *et al.*, 1995b).

The main fate of carbohydrates under anoxia is very likely to be their utilization through glycolysis and fermentation leading to ethanol as the main end product (Mayne & Kende, 1986; Bouny & Saglio, 1996). The analysis of several enzymatic activities in anaerobically germinating cereal grains revealed the absence of striking differences between the tolerant species (rice) and the intolerant species (wheat and barley). An exception was the induction of enzymes involved in the sucrose synthase pathway for sucrose utilization, which was detected in rice grains but not in wheat and barley grains, suggesting

differences between rice and the two other species in their ability to degrade sucrose under anoxia (Guglielminetti *et al.*, 1995a; Perata *et al.*, 1996; Guglielminetti *et al.*, 1997; Perata *et al.*, 1998). However, the *in vitro* enzymatic activities related to the metabolism of carbohydrates found in the nontolerant cereals may well account for considerable rates of carbohydrate degradation, but provide direct proof that the *in vivo* fluxes from the main soluble sugars to ethanol under anaerobiosis is missing.

We report here data on the *in vivo* conversion of carbohydrates (glucose, fructose, or sucrose) to ethanol and CO₂ under anoxia using cereal grains as a working model.

Materials and Methods

Plant material

Rice (*Oryza sativa* L. cv. Arborio) and barley (*Hordeum vulgare* L. cv. Himalaya) grains were obtained from the University farm (University of Pisa), while wheat (*Triticum aestivum* L. cv. Hatsuho) grains were obtained from Nagoya University (Japan). After sterilization by 2% NaClO and 0.02% Triton X-100 (30 min), the grains used for enzymatic analysis and carbohydrates/ethanol quantification were incubated in water, on filter paper, at 25°C in Petri dish under dark conditions and collected at set times. The anoxic samples were incubated in an anaerobic incubator (Forma Scientific 'anaerobic system' model 1025 Forma Scientific, Inc. Marietta, OH, USA). For evaluation of carbohydrate utilization *in vivo*, embryos were dissected from dry grains, sterilized as described above and incubated in 500 ml flasks with 20 ml of 10 mM sucrose or 20 mM glucose or 20 mM fructose containing 1 µCi of radiolabelled sugar ([U-¹⁴C]-sucrose or [U-¹⁴C]-glucose or [U-¹⁴C]-fructose, respectively), 7 µg ml⁻¹ rifampicin and 2 µg ml⁻¹ nystatin. A moistened gas stream of nitrogen (anaerobic condition) or air was passed through the flasks for ¹⁴CO₂ collection.

Chemicals

All reagents were purchased from Sigma (St. Louis, MO, USA). The kit for protein quantification was purchased from Bio-Rad (Richmond, CA, USA) with bovine serum albumin as a standard. Radiolabelled carbohydrates were purchased from Amersham (Little Chalfont, Buckinghamshire, UK).

Enzyme assay

Samples (0.1–1 g f. wt) were rapidly frozen in liquid nitrogen, ground to a powder, extracted and dialysed as previously reported (Guglielminetti *et al.*, 1995a).

Alcohol dehydrogenase: samples were assayed in 1 ml assay mixture (100 mM Tris-HCl pH 9, 5 mM DTT, 5 mM MgCl₂, 0.5 mM NAD, 200 mM EtOH) monitoring spectrophotometrically the absorbance at 340 nm for 10 min.

Pyruvate decarboxylase Samples were assayed in 1 ml reaction mixture (80 mM MES pH 6, 1 mM MgCl₂, 20 mM DTT, 1 mM thiamine PPI, 10 mM pyruvate). After 3 h the reaction was stopped by adding 2 ml of dimedone reagent (330 mM ammonium acetate, 20 mM dimedone, 70 mM HCl). Samples were boiled for 20 min and, after cooling, fluorescence was measured at 460 nm (excitation at 365 nm). The fluorescence in the sample was compared to that of known amounts of acetaldehyde (50–400 nmoles) developed with the dimedone reagent.

Analysis of carbohydrates

Samples (0.1–1 g f. wt) were rapidly frozen in liquid nitrogen and ground to a powder. Samples were then extracted as described by Tobias *et al.* (1992) and assayed through coupled enzymatic assay methods (Guglielminetti *et al.*, 1995a) measuring glucose, fructose and sucrose content. The efficiency of the methods was tested by using known amounts of carbohydrates. Incubations of the samples and standards were carried out at 37°C for 30 min. The reaction mixtures (1 ml) were as follows. Glucose: 100 mM Tris-HCl, pH 7.6, 3 mM MgCl₂, 2 mM ATP, 0.6 mM NADP, 1 unit Glc6P dehydrogenase, 1 unit hexokinase the A₃₄₀ was recorded. Fructose was assayed as described for glucose plus the addition of 2 units of PGI; the increase in A₃₄₀ was recorded. Sucrose was first broken down using 85 units of invertase (in 15 mM Na acetate, pH 4.6) and the resulting glucose and fructose were assayed as described above.

Recovery experiments evaluated losses taking place during the extraction procedures. Two experiments were performed for each metabolite by adding known amounts of authentic standards to the sample before the extraction. The concentration of the standards added were similar to those estimated to be present in the tissues in preliminary experiments. The recovery ranged from 93 to 105%.

Analysis of ethanol

Samples were collected and extracted as described for carbohydrate analysis and assayed through coupled enzymatic assay methods, measuring the increase in A₃₄₀ as described by Bernt & Gutman (1974) with minor modifications. Ethanol produced by the plant material under anaerobic conditions diffuses freely into the incubation medium. Therefore the ethanol content in the incubation media was also analysed. The efficiency of the methods was tested by using known amounts of ethanol. Incubations of the samples and standards were carried out at 25°C for 70 min. The reaction mixtures (1 ml) were as follows: 72 mM Na₄P₂O₇, pH 8.7, 72 mM semicarbazide, 20 mM glycine, 0.5 mM NAD, 36 unit alcohol dehydrogenase; the A₃₄₀ was recorded and compared with the standards.

Recovery experiments evaluated losses taking place during the extraction procedures. Two experiments were performed by adding known amounts of ethanol to the sample before

the extraction. The concentration of ethanol added was similar to that estimated to be present in the tissues in preliminary experiments. The percentage recovery ranged from 90 to 110%.

In vivo carbohydrates utilization

CO₂ derived from sugar utilization was trapped in 10 ml 14% KOH solution and radioactivity was monitored. A moistened gas stream of nitrogen (anaerobic condition) or air was passed through the flasks, finally bubbling in a test tube containing the KOH solution for ¹⁴CO₂ collection. To exclude micro-organism contamination a flask containing the medium without embryo (for each sugar) was also monitored under aerobic and anaerobic conditions and a sample of the KOH solution was measured, but significant levels of ¹⁴CO₂ were not detected.

Table 1 Effect of anoxia on alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC) activity. Data are mean ± SE of three replicates

	Air			Anoxia	
	0 day	4 days	8 days	4 days	8 days
PDC Activity (nmol min ⁻¹ mg ⁻¹ protein)					
Rice					
Endosperm	41 ± 17	18 ± 5	49.5 ± 22.5	56.5 ± 9.5	73 ± 21
Embryo	45 ± 0.5	69.5 ± 4.5	70.5 ± 3.5	124.5 ± 7.5	261 ± 41
Coleoptile	–	13.5 ± 4.5	17.5 ± 0.5	53.5 ± 5.5	104.5 ± 15.5
Root	–	8.5 ± 2.5	11.5 ± 1.5	–	–
Wheat					
Endosperm	8 ± 1	11 ± 1	8 ± 0.5	14.5 ± 2.5	10 ± 2
Embryo	38 ± 5	49 ± 3	35.5 ± 8.5	76 ± 3	111.5 ± 2.5
Coleoptile	–	5 ± 1	6 ± 1	–	–
Root	–	13.5 ± 1.5	13 ± 2	–	–
Barley					
Endosperm	4 ± 0.5	3 ± 1	9 ± 2	5.5 ± 0.5	6 ± 1
Embryo	12 ± 1	11 ± 2	9.5 ± 1.5	59.5 ± 3.5	63 ± 11
coleoptile	–	3.5 ± 0.5	4 ± 1	–	–
Root	–	8.5 ± 1.5	11 ± 4	–	–
ADH Activity (mU mg ⁻¹ protein)					
Rice					
Endosperm	146.5 ± 5.5	26 ± 3	172 ± 52	88.5 ± 2.5	94.5 ± 3.5
Embryo	491.5 ± 65.5	61.5 ± 21.5	225.5 ± 110.5	900 ± 18	834.5 ± 18.5
Coleoptile	–	36 ± 9	57.5 ± 18.5	685 ± 62	866.5 ± 85.5
Root	–	n.d.	n.d.	–	–
Wheat					
Endosperm	74.5 ± 14.5	56.5 ± 20.5	55.5 ± 2.5	48 ± 1	26 ± 3
Embryo	46.5 ± 12.5	62.5 ± 1.5	25.5 ± 1.5	106 ± 8	130.5 ± 22.5
Coleoptile	–	7 ± 0.5	6.5 ± 2.5	–	–
Root	–	n.d.	n.d.	–	–
Barley					
Endosperm	76.5 ± 1.5	37.5 ± 11.5	24.5 ± 5.5	30 ± 2	31 ± 11
Embryo	53 ± 5	13.5 ± 0.5	6.5 ± 3.5	93 ± 4	122 ± 21
Coleoptile	–	9 ± 2	n.d.	–	–
Root	–	n.d.	n.d.	–	–

n.d., not detectable.

Results

Alcohol dehydrogenase and pyruvate decarboxylase in aerobic and anaerobic cereal seedlings

We assayed the activity of the two enzymes involved in alcoholic fermentation, namely alcohol dehydrogenase (ADH) and pyruvate decarboxylase (PDC). The data reported in Table 1 indicate that both enzymes are present in the dry endosperm and in the dry embryo of the three species under analysis. The PDC activity of the dry endosperm is similar to that of the dry embryo in rice. Higher PDC activity was instead found in wheat and barley dry embryos when compared to the dry endosperm activity value. ADH activity was very high in the dry rice embryo (10 times that found in wheat and barley) as well as in the rice endosperm (two times that found in the other species). During aerobic germination

the activity of PDC remained relatively stable in all cases. PDC activity was also detectable in the roots and shoots of the three cereals under investigation. In air ADH activity did not increase in the endosperm of the three cereals under investigation. The ADH activity of aerobic embryos decreased in all three cereals. During anaerobic incubation, the activity of both enzymes increased strongly in the embryo of the three cereals under investigation, while ADH activity decreased in the endosperm and PDC activity remained stable in the same tissue. In the rice coleoptile (the only species under investigation able to germinate under anoxia) the ADH and PDC activities were present at high levels.

Carbohydrate-ethanol transition

Soluble carbohydrates are utilized when cereal grains are placed under anoxia (Fig. 1a). Indeed, during the first days of incubation, the level of fermentable sugars decreases. Subsequently, the level of soluble carbohydrates in the rice anoxic seedlings begins to increase – probably a consequence of starch degradation. In barley and wheat grains (unable to degrade starch under anoxia; Guglielminetti *et al.*, 1995b) the level of free sugars does not increase. Analysis of the external incubation media allowed us to exclude the possible leakage of carbohydrates (data not shown).

During anaerobiosis, the level of ethanol increases as a consequence of fermentative metabolism (Fig. 1b). Lactate is present only in trace amounts (data not shown). During the first days of anoxic incubation the rate of ethanol production is comparable in the three species under investigation, but after 3–4 d fermentation proceeds in rice seedlings only, while ethanol production is stopped in both wheat and barley grains.

We calculated the apparent ratio between the rate of ethanol production and hexose-equivalent utilization (Fig. 1c). The data indicate that, during the first days of anoxic incubation, the rate in rice seedlings is close to 2 (complete conversion of carbohydrate to ethanol, since 2 moles of ethanol are produced per mole of hexose); after that the ratio increases rapidly to values that are not compatible with changes in grain content of soluble carbohydrates. This high ratio is however, easily explained since α -amylase induction has already taken place during the second day of incubation (not shown), allowing the utilization of the glucose units deriving from starch degradation. The ratio of ethanol produced to carbohydrates utilized for wheat and barley is, on the contrary, lower than 2 during the period under analysis.

In vivo carbohydrates utilization

The activity of several enzymes involved in the metabolism of carbohydrates under both aerobic and anaerobic conditions have been described (Guglielminetti *et al.*, 1995a; Perata *et al.*, 1996). The results suggest that all the three cereal species under

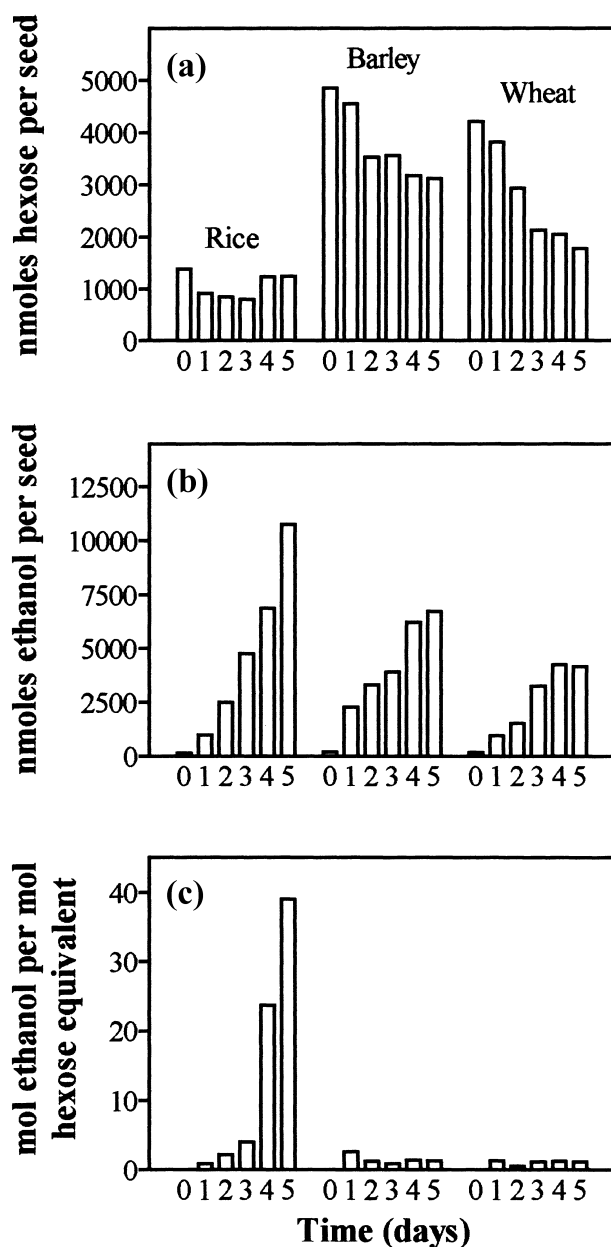


Fig. 1 Carbohydrate/ethanol transition in rice, barley and wheat grains under anoxia. (a) Carbohydrate content expressed as nmoles hexose-equivalents per grain. (b) Ethanol content expressed as nmoles per grain. (c) Ratio between ethanol production and changes in soluble carbohydrate content. Data are means of two replicates. Range of variation did not exceed 14% of the given values.

anoxia contain enough enzyme activities to sustain carbohydrate metabolism (from sucrose cleavage to hexose phosphorylation). In rice several enzyme activities involved in the sucrose synthase pathway for the anaerobic utilization of sucrose (essentially sucrose synthase and fructokinase) were found to be induced by the anaerobic treatment; on the other hand, no induction was observed in wheat and barley grains under anoxia

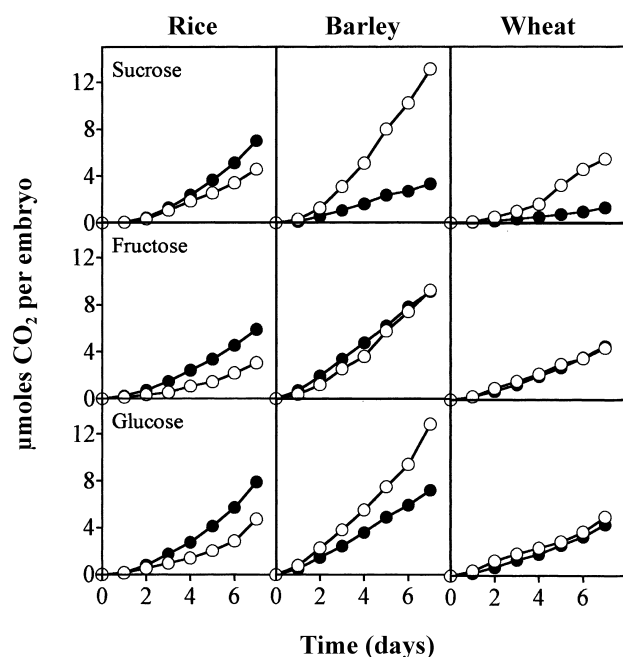


Fig. 2 *In vivo* carbohydrate utilization in rice, barley and wheat under aerobic and anaerobic conditions. CO₂ production is monitored from dissected embryos of rice, barley and wheat incubated, under air (open circles) or anoxia (closed circles), in 10 mM sucrose and 1 µCi of [¹⁴C]-sucrose or 20 mM fructose and 1 µCi of [¹⁴C]-fructose or 20 mM glucose and 1 µCi of [¹⁴C]-glucose. Data are means of two replicates. Range of variation did not exceed 12% of the given values.

(Guglielminetti *et al.*, 1995a; Perata *et al.*, 1996). In order to obtain convincing evidence of the ability of cereal grains to degrade sucrose under anoxia, we performed experiments with radiolabelled sucrose fed to endosperm-less embryos from the three species under study. The results reported in Fig. 2 show that only rice is able to convert sucrose to CO₂ under anoxia with efficiency comparable to, or higher than, that of the embryos incubated in air. On the contrary, the rate of sucrose utilization to CO₂ in wheat and barley embryos, is much lower (by a factor of five) under anaerobic conditions than under aerobiosis (Fig. 2). This could result from difficulties in degrading sucrose, but also from an inefficient utilization of glucose and fructose units resulting from sucrose degradation. To clarify this point we performed experiments utilizing radiolabelled glucose and fructose. The results reported in Fig. 2 show that wheat and barley embryos are able to utilize fructose with the same efficiency under aerobic and anaerobic condition, while rice utilizes fructose with higher efficiency (two times) under anaerobic conditions than under aerobiosis. The results reported in Fig. 2 show that rice also utilizes glucose with higher efficiency (two times) under anaerobic conditions than under aerobiosis. Wheat utilizes glucose equally well under air or anoxia, while barley utilizes glucose better under normoxia than under anoxia.

Discussion

The metabolic adaptations of higher plants to anaerobic conditions are complex. Several pathways have been proposed to operate for the re-oxidation of NADH, but the fermentative pathway is believed to play a predominant role in the anaerobic recycling of pyrimidine nucleotides. (Perata & Alpi, 1993; Drew, 1997). In higher plants, the fermentation end-products are mainly ethanol, lactic acid and alanine (Perata & Alpi, 1993; Drew, 1997). After an initial production of lactic acid (catalysed by lactate dehydrogenase, allowing the re-oxidation of NADH to NAD⁺), there is a massive production of ethanol derived from the decarboxylation of pyruvate to acetaldehyde by PDC and a subsequent reduction of acetaldehyde by ADH with concomitant reoxidation of NADH to NAD⁺ (Perata *et al.*, 1998). The enzymes involved in the two-step alcoholic fermentation mechanism and the genes encoding them were all shown to be up-regulated during low oxygen stress conditions (Hagemann & Flesher, 1960; Laszlo & St. Lawrence, 1983). Several studies investigated the role of different plant PDC and ADH genes and related proteins under anoxia. In several plant species (maize, rice, and wheat) there is evidence of at least two different subunits of these enzymes, presumably encoded by different genes (Lee & Langston-Unkefer, 1985; Zehender & Ullrich, 1985; Zehender *et al.*, 1987; Rivoal *et al.*, 1990). A differential induction of pyruvate decarboxylase subunits and transcripts occurs in anoxic rice seedlings (Rivoal *et al.*, 1997; Huq *et al.*, 1999), as well as in *Arabidopsis* seedlings (Dolferus *et al.*, 1997). A similar differential induction is demonstrated for ADH in different plant systems (Dolferus *et al.*, 1994; Dolferus *et al.*, 1997). Supported by the high levels of fermentative enzymes under anoxia, a strong production of ethanol is observable when plant material is subjected to low oxygen concentration (Alpi & Beevers, 1983; Raymond *et al.*, 1985; Atwell & Greenway, 1987).

We have analysed the total activity of the fermentative enzymes PDC and ADH and confirmed, for the three species under investigation, a strong induction in both activities when the tissues were subjected to oxygen deprivation. We observed considerable ethanol production for all the cereals under investigation during the first days of germination, but only rice maintained the ability to fermentate under a long-term anoxic treatment. Wheat and barley soon suffered sugar starvation (Perata *et al.*, 1998) due to their inability to induce α -amylase under anoxia; this explains the strong increase of ethanol : hexose ratio observable in rice but not in the other two cereals.

The critical role of sucrose synthase (SS) for anoxic tolerance in several plant species has been reported (Germain *et al.*, 1997; Guglielminetti *et al.*, 1997; Ricard *et al.*, 1998; Zeng *et al.*, 1998). It has been proposed that an anaerobic SS pathway operates in rice seedlings (SS is strongly induced under anoxia, while invertase is repressed) that is potentially able to produce 6 moles of ATP from the fermentation of 1 mole of sucrose, provided that a source of high energy bonds is

available from PPI (see Guglielminetti *et al.*, 1995a). On the contrary, in the anoxic-intolerant cereal species (wheat and barley), SS is not induced under anoxia (Perata *et al.*, 1996). Remarkably, it has been demonstrated recently that sugar and oxygen signals for the regulation of SS genes overlap (Koch *et al.*, 2000). It would be interesting to investigate differences in the ability to sense sugars under low oxygen in anoxia-tolerant vs anoxia-intolerant species.

Using radiolabelled glucose as an exogenous carbohydrate source, Mayne & Kende (1986) and Atwell & Greenway (1987) demonstrated the conversion of this sugar mainly to ethanol under anoxic conditions in different rice tissues. We performed similar experiments confirming these results in the three cereal species under investigation. Rice, barley and wheat were able efficiently to convert exogenous glucose and fructose to ethanol under anoxia. However a difference is observable when the exogenous carbohydrate source is sucrose; only rice is able to utilize this sugar efficiently under anoxia (at twice the rate under aerobic condition), while barley and wheat utilize sucrose five times better under air than under anoxia, supporting the hypothesis that an anaerobic sucrose-degradation pathway likely involving SS is peculiar to rice.

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