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Anaerobic carbohydrate metabolism in wheat and barley, two anoxia-intolerant cereal seeds

Pierdomenico Perata[1], Lorenzo Guglielminetti and Amedeo Alpi

Dipartimento di Biologia delle Piante Agrarie, Sezione di Fisiologia Vegetale, Via Mariscoglio 34, University of Pisa, I-56124 Pisa, Italy

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Abstract

Cereals such as barley and wheat are unable to germinate under anoxic conditions. Data are presented on the utilization of the soluble sugars present in the dry seed of wheat and barley kept under strict anoxia, together with the status of the enzymes involved in the metabolism of carbohydrates.

The amount of glucose, fructose, and sucrose decreases during the anaerobic treatment, indicating that carbohydrate metabolism takes place in the seeds of wheat and barley kept under anoxic conditions.

The analysis of the enzymes involved in the metabolism of carbohydrates shows that the enzymatic set needed to convert sucrose, glucose, and fructose to glucose-6P is present in the anaerobic seeds. Sucrose degradation seemingly occurs through a sucrose synthase pathway with nucleoside diphosphate kinase involved in the cycling of urydilates. However, the activity of sucrose synthase, glucokinase, and fructokinase, all strongly induced in rice, an anoxia-tolerant cereal, are almost unaffected or even depressed in the two cereal seeds under investigation.

The comparison of the results obtained and described in this paper with the published data on the metabolism of carbohydrates in rice, indicate that the anoxia-intolerant species would rapidly suffer and eventually die from sugar starvation if kept under anoxia for a relatively prolonged length of time (4–8 d).

Key words: Anoxia, cereal seed, carbohydrate metabolism.

Introduction

Among cereal seeds, only rice shows an exceptional tolerance to anaerobiosis, being able to germinate and elongate the coleoptile even under complete anoxia (Alpi and Beevers, 1983). Some species of the rice field weed *Echinochloa* exhibit anaerobic germination (Kennedy *et al.*, 1992), but cereals such as barley and wheat are unable to germinate under anoxic conditions. However, the molecular and biochemical traits underlying this different behaviour are still unknown (Drew, 1990; Kennedy *et al.*, 1992; Perata and Alpi, 1993; Armstrong *et al.*, 1994; Ricard *et al.*, 1994).

In recent years, increasing attention is being paid to a possible role of carbohydrate availability and utilization in conferring anoxia tolerance (Mohanty *et al.*, 1993; Perata and Alpi, 1993; Armstrong *et al.*, 1994; Ricard *et al.*, 1994; Zhang and Greenway, 1994; Hanhijärvi and Fagerstedt, 1995). The amount of readily utilizable soluble carbohydrates is usually limited in cereal seeds, and starch represents the main storage compound. Rice seeds can take advantage of the starchy reserves even under anoxia, possessing the complete set of enzymes involved in starch degradation. Starch breakdown is hampered in barley and wheat seeds, a consequence of the failure to induce α-amylase and the other starch-degrading enzymes under anaerobic conditions (Perata *et al.*, 1992, 1993*a*; Guglielminetti *et al.*, 1995*b*). However, ethanol production can be observed in wheat seeds kept under anoxic conditions (Alpi and Beevers, 1983; Raymond *et al.*, 1985), suggesting that soluble sugars are being utilized in the anoxic wheat seed. While data are available concerning carbohydrate metabolism in anoxic rice seedlings (Alpi *et al.*, 1985; Mayne and Kende, 1986; Atwell and

[1] To whom correspondence should be addressed. Fax: +39 50 570421. E-mail: pperata@agr.unipi.it

Abbreviations: FK, fructokinase; Fru6P, fructose-6-phosphate; FW, fresh weight; GK, glucokinase; Glc1P, glucose-1-phosphate; Glc6P, glucose-6-phosphate; HK, hexokinase; NDPK, nucleoside diphosphate kinase; PGI, glucose-6-phosphate isomerase; PGM, phosphoglucomutase; PPi, inorganic pyrophosphate; SS, sucrose synthase; UDPGlc, UDP-glucose; UDPGlc-PPase, UDP-glucose pyrophosphorylase.

Greenway, 1987; Perata *et al.*, 1992, 1993*a*; Guglielminetti *et al.*, 1995*a*), to our knowledge no data have been presented concerning the ability of anoxia-intolerant cereal seeds to metabolize the soluble sugars present in the dry seed. Data on the utilization of carbohydrates in wheat and barley seeds kept under anoxia, together with the status of the enzymes involved in the metabolism of carbohydrates are presented here.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L. cv. Himalaya) seeds were obtained from the University farm (University of Pisa) while wheat (*Triticum aestivum* L. cv. Hatsuho) seeds were obtained from Nagoya University (Japan). Seed germination and anoxic treatments were carried out as previously described (Perata *et al.*, 1992; Guglielminetti *et al.*, 1995*a*): a moistened gas stream of nitrogen was passed through a 500 ml flask containing the seeds, at 30 ml min^{-1} throughout the experimental period. All germination experiments were performed in the dark at 25 °C. Seedlings were collected when they reached the desired age, frozen in liquid nitrogen, and stored at −80 °C.

Preliminary experiments were performed to establish whether microbial contamination was present in the flasks containing the seedlings: no differences in terms of carbohydrate levels or enzymatic activities were found comparing control flasks with flasks with rifampicin (7 $\mu g\,ml^{-1}$) and nystatin (2 $\mu g\,ml^{-1}$) added.

Seedlings were dissected as previously described (Perata *et al.*, 1992). Recovery experiments were performed by testing the ability of seeds kept under anoxia to germinate when transferred to aerobic conditions for 10 d.

Chemicals

All the reagents used were purchased from Sigma (St Louis, MO, USA).

Analysis of carbohydrates

Samples (0.1–0.5 g FW) 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, measuring the increase in A_{340} nm. 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 $MgCl_2$, 2 mM ATP, 0.6 mM NADP, 1 unit HK, 1 unit Glc6P dehydrogenase; fructose was assayed as described for glucose plus the addition of 2 units PGI: the increase in A_{340} nm was recorded. Sucrose was first broken down using 85 units invertase (in 15 mM sodium acetate, pH 4.6) and the resulting glucose and fructose were assayed as described above. Hexose monophosphates were assayed as described by Tobias *et al.* (1992) and Mohanty *et al.* (1993) with minor modifications: 100 mM TRIS–HCl, pH 7.6, 3 mM $MgCl_2$, 0.6 mM NADP, 1 unit Glc6P dehydrogenase for Glc6P; 2 units PGI were added to the previous reaction mixture when Fru6P was measured; 4 units PGM and 20 μM glucose 1,6 bisphosphate were added for the determination of Glc1P.

Recovery experiments were performed in order to evaluate losses taking place during the extraction procedures. Two experiments were performed for each metabolite by adding known amounts of authentic standards to the samples prior to the extraction. The concentration of the standards added were closely similar to those estimated to be present in the tissues in preliminary experiments. The percentage recovery ranged between 95% and 112% for sucrose, glucose and fructose while lower recoveries were obtained for hexose monophosphates (64–79%). Data were corrected on the basis of the recovery percentages obtained for each sample.

Analysis of enzymes of carbohydrate metabolism

Samples (0.2–0.5 g FW) were extracted in 100 mM HEPES-KOH, pH 7.5 containing 1 mM EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM $NaHSO_3$. Inclusion of insoluble polyvinylpolypyrrolidone did not affect the activity of any of the enzymes under investigation and was therefore not included in the extraction buffer. Extracts were centrifuged (13 000 *g*, 15 min), the resulting pellets were washed with the extraction buffer, centrifuged again, and the resulting supernatants combined and used for the enzymatic assays. Extracts to be assayed for invertase, SS, HK, and NDPK were dialysed against the extraction buffer for 12 h at 4 °C to remove the soluble sugars that were present in the extracts and that may interfere with the assays. Assays were optimized for extracts from both aerobic and anaerobic plant material.

It was routinely tested whether mixing an extract from aerobic seeds with that of anoxic seeds could result in losses of enzymatic activities. No inactivation of the activities under investigation was found.

Samples were assayed for the enzymatic activities at 25 °C in 0.5 ml reaction mixtures, using the following methods. α-Amylase (Doehlert *et al.*, 1982); samples, pretreated at 70 °C in the presence of 3 mM $CaCl_2$ to eliminate interference from β-amylase, were incubated with 2.5% (w/v) soluble starch in 50 mM Na-acetate, pH 5.2, 10 mM $CaCl_2$; activity of α-amylase (1 U) is defined as the amount of enzyme releasing 1 μmol glucose min^{-1}. Invertase and SS (Huber and Akazawa, 1986); UDPGlc-PPase (Sowokinos *et al.*, 1993); NDPK (method 1, described in Perata *et al.*, 1993*b*); GK and FK (50 mM HEPES-KOH, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 15 mM KCl, 2 mM ATP or UTP, 0.75 mM NADP, 4 mM glucose or fructose, 1 unit Glc6P dehydrogenase, 1 unit PGI); PGI (50 mM HEPES-KOH, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 15 mM KCl, 0.75 mM NADP, 4 mM Fru6P, 1 unit Glc6P dehydrogenase); PGM (50 mM HEPES-KOH, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 15 mM KCl, 0.75 mM NADP, 4 mM Glc1P, 20 μM glucose 1,6-bisphosphate, 1 unit Glc6P dehydrogenase). Activity of 6-phosphogluconate dehydrogenase, which may interfere with coupled enzymatic assays using NADP, is indeed present in our samples. However, no significative differences in the enzymatic assays were observed by using either NAD or NADP, ruling out the possible additional NADPH production by 6-phosphogluconate dehydrogenase activity (Huber and Akazawa, 1986).

Cycling of urydilates was assayed as described by Xu *et al.* (1989) with modifications: the occurrence of urydilates cycling through FK activity (UTP-dependent) was verified using 50 mM HEPES-KOH, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 15 mM KCl, 0.75 mM NAD, 80 mM sucrose, 8 μM UDP, 4 mM PPi, 30 μM glucose 1,6-bisphosphate, and 1 unit Glc6P dehydrogenase. The role of NDPK was tested by adding 80 μM ADP to the reaction mixture reported above.

Results

Wheat and barley seed tolerance to anoxia

A different degree of tolerance to anoxia was found in wheat and barley seeds (Table 1). While wheat seeds can withstand up to 8 d under anoxia without any serious effect on seed viability, barley seed viability dropped to 24% after only 4 d under anoxia. Therefore, all subsequent experiments were performed with an anoxic treatment up to 10 d and 6 d for wheat and barley, respectively.

Carbohydrates in aerobic and anaerobic wheat and barley seeds

Sucrose, glucose, and fructose are present in the dry seed of both wheat and barley (Tables 2, 3). Glucose and sucrose content rapidly increases as the aerobic germination proceeds, a consequence of starch degradation and concomitant sucrose synthesis. A transient decline in the fructose pool is observed in the aerobic barley and wheat seeds. Under anoxia, the carbohydrate content of the whole seeds decreases more rapidly in barley than in wheat, indicating that sugar utilization is taking place in the anaerobic seeds (Tables 2, 3).

Table 1. *Tolerance to anoxia of wheat and barley seeds*

Recovery experiments were performed by testing the ability of seeds kept under anoxia to germinate when transferred to aerobic conditions for an additional 10 d Data are the means from two separate experiments Variation width did not exceed 10% of the mean values.

		% Germination		% Recovery
		Air	Anoxia	
Wheat	0 d	0	0	100
	2 d	32	0	100
	4 d	61	0	100
	6 d	73	0	96
	8 d	98	0	85
	10 d	100	0	47
Barley	0 d	0	0	99
	2 d	31	0	99
	4 d	63	0	24
	6 d	89	0	14
	8 d	100	0	3
	10 d	100	0	0

Table 2. *Carbohydrate contents of wheat seedlings and seeds sown in air or anoxia*

Data are from two separate experiments.

		Sucrose (nmol seedling^{-1})	Glucose (nmol seedling^{-1})	Fructose (nmol seedling^{-1})
Air	0 d	2473	565	1485
		2682	612	1728
	1 d	2054	499	381
		2369	513	418
	2 d	3342	1212	564
		3728	1456	633
	3 d	7832	4371	1085
		8541	4856	1282
Anoxia	0 d	2473	565	1485
		2682	612	1728
	1 d	2319	557	1340
		2528	596	1456
	2 d	2165	569	1195
		2293	581	1235
	3 d	1695	445	951
		1802	453	989

Table 3. *Carbohydrate contents of barley seedlings and seeds sown in air or anoxia*

Data are from two separate experiments.

		Sucrose (nmol seedling^{-1})	Glucose (nmol seedling^{-1})	Fructose (nmol seedling^{-1})
Air	0 d	2345	1264	4947
		2748	1352	5621
	1 d	3338	1535	877
		3965	1728	956
	2 d	6150	3060	1140
		6893	3548	1326
	3 d	9706	5029	1410
		11264	5861	1733
Anoxia	0 d	2345	1264	4947
		2748	1352	5621
	1 d	1306	944	4099
		1524	1027	4584
	2 d	267	624	3250
		312	745	3457
	3 d	254	492	2218
		296	546	2561

The anaerobic metabolism of carbohydrates was further investigated by analysing the presence of the three carbohydrates in tissues isolated from seeds kept under anoxic conditions for a longer period of time (Fig. 1).

The content of sucrose, glucose and fructose declined in both the embryo and endosperm. The decline was particularly rapid in barley seeds: a 10-fold reduction in the sucrose content can be observed after only 2 d under anoxia. The availability of soluble sugars is reduced to about 0.2–0.1 times the initial value after 6 d and 4 d under anoxia in wheat and barley, respectively. When considering the single seed tissues, the amount of soluble sugars is very low in the embryo after a few days under anoxic conditions (Fig. 1). Anoxic tissues from cereal seedlings become very leaky to metabolites, including carbohydrates (Alpi *et al.*, 1985). However, soluble carbohydrates were not detected in the seed incubation media which, instead, contained a prominent amount of ethanol, thus accounting for the decline of the carbohydrate content of the seeds which is, therefore, due to anaerobic metabolism (data not shown).

The amount of hexose monophosphates in the anoxic seed tissues was always below the detection limit of the method used (100 nmol g^{-1} FW, data not shown). The clear increase in the concentration of glucose-6-phosphate previously observed in anoxic rice seeds (Guglielminetti *et al.*, 1995*a*) is absent in these anoxia-intolerant cereal seeds.

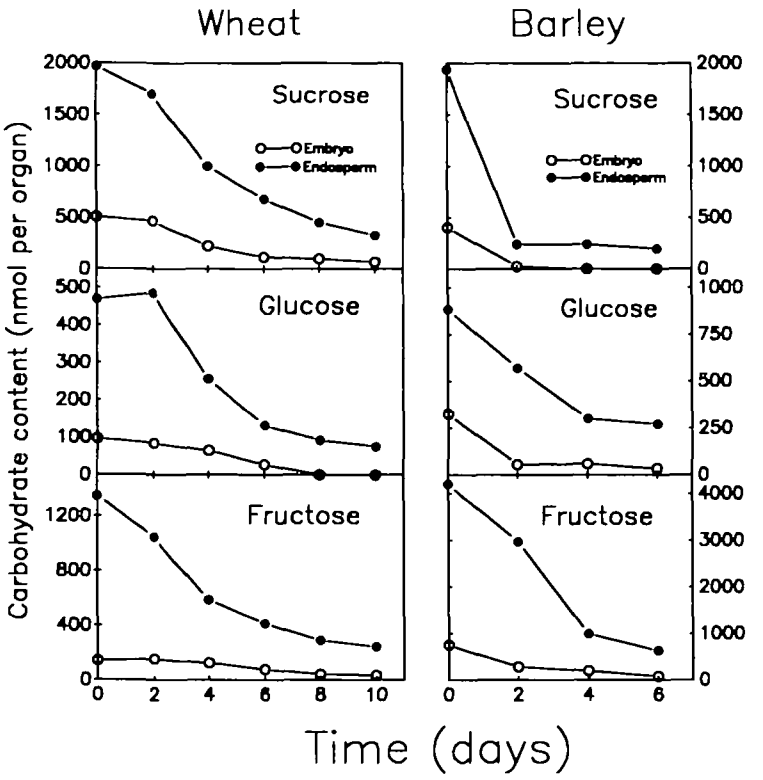

Fig. 1. Content of sucrose, glucose, and fructose in wheat and barley seeds under anoxic conditions Data are mean of three replicates (±SE did not exceed 15% of the given values).

Enzymes of carbohydrate metabolism in aerobic and anoxic wheat and barley seeds

The continuous decline in the soluble sugars content observed in the anoxic cereal seeds under investigation, together with the absence of the phase of increased sugar availability consequent to starch breakdown, can be easily explained on the basis of the recent results showing the inability of wheat and barley seeds to produce α-amylase under anoxic conditions (Perata *et al.*, 1992, 1993*a*; Guglielminetti *et al.*, 1995*b*). Indeed, α-amylase activity is high in the aerobic seedlings, but undetectable in the anoxic seeds (Tables 4, 5).

The clear utilization of carbohydrates observed in the anoxic seeds indicates that the enzymes involved in the channelling of non-phosphorylated sugars to glycolysis are present under anoxia.

GK activity, needed to phosphorylate glucose to Glc6P is present in the aerobic seedling and anoxia slightly depresses its activity in both wheat and barley embryos (Tables 4, 5). The same applies to FK, whose activity is lower in the anoxic embryo. Under anoxia, the activity of GK is higher in the endosperm of both species, while the activity of FK is higher in barley endosperm but lower in wheat endosperm (Tables 4, 5).

The conversion of Glc6P to Fru6P, a step needed for entering Glc6P in the glycolytic flux, is catalysed by the enzyme PGI, whose activity is high under both aerobic and anaerobic conditions. Again, a slightly lower activity was detected in anoxic wheat and barley embryos (Tables 4, 5). In both wheat and barley endosperm, PGI activity remains at the same level found in the dry seed endosperm when the seeds were kept under anoxia, while the activity decreases considerably under aerobic conditions (Tables 4, 5).

Sucrose metabolizing enzymes involved in the cleavage of sucrose are alkaline invertase and sucrose synthase (ap Rees, 1992). Sucrose is rapidly utilized under anoxia (Fig. 1) and both the sucrose metabolizing enzymes are present in the anoxic seeds (Tables 4, 5). However, the anoxic treatment considerably depressed the activity of invertase, while the SS activity remains at the same level as under aerobic conditions or increases slightly in both anoxic wheat and barley (Tables 4, 5).

The different effect of anoxia on the two sucrose-degrading enzymes may suggest that, as shown recently for rice seeds (Guglielminetti *et al.*, 1995*a*), a SS pathway (Huber and Akazawa, 1986; Black *et al.*, 1987) may preferentially operate in the anaerobic seeds.

The activity of FK, UDPGlc-PPase and PGM needed for the operation of the SS pathway (Huber and Akazawa, 1986; Black *et al.*, 1987) was investigated. All these activities are almost unaffected by anoxia when

Table 4. *Effects of anoxia on enzymes of carbohydrate metabolism of wheat seeds*

Data (mU mg^{-1} protein) are mean of three replicates ±SE. n.d = not detectable

	Air			Anoxia		
	0 d	4 d	8 d	0 d	4 d	8 d
α-Amylase						
Endosperm	n.d.	2061±111	2778±528	n d.	n.d.	n.d.
Embryo	n.d.	498±205	1243±445	n.d.	n.d	n.d.
Shoot	–	n.d.	n.d.	–	–	–
Root	–	n.d.	n.d.	–	–	–
GK						
Endosperm	14.5±0.5	12±1	8.5±1.5	14.5±0.5	12.5±0.5	10±1
Embryo	13±3	32.5±0.5	23.5±5.5	13±3	13±1	19.5±3.5
Shoot	–	17±3	23.5±4.5	–	–	–
Root	–	38.5±5.5	30±3	–	–	–
FK						
Endosperm	8.5±0.5	13.5±1.5	15±4	8.5±0.5	8.5±0 5	5±1
Embryo	13±1	37±4	35.5±8.5	13±1	23±2	22.5±4.5
Shoot	–	22.5±2.5	28±5	–	–	–
Root	–	58.5±3 5	48.5±3.5	–	–	–
Invertase						
Endosperm	4.5±0.5	10.5±1.5	12.5±1.5	4.5±0.5	3±0.5	3±0.5
Embryo	5.5±0.5	25±2	15.5±2.5	5.5±0 5	3.5±0.5	5.5±0.5
Shoot	–	56±3	27.5±1.5	–	–	–
Root	–	41±3	51±1	–	–	–
SS						
Endosperm	28.5±1.5	6±1	n.d.	28.5±1.5	22±4	14.5±1.5
Embryo	4±1	32±5	20±2	4±1	25±2	21.5±1.5
Shoot	–	20.5±6 5	15 5±3.5	–	–	–
Root	–	20±2	45±3	–	–	–
PGI						
Endosperm	443±53	246±16	214±42	443±53	447±5	386±32
Embryo	350±57	350±70	418±46	350±57	360±12	320±22
Shoot	–	144±17	236±61	–	–	–
Root	–	214±41	294±24	–	–	–
PGM						
Endosperm	704±42	460±1	429±60	704±42	506±41	365±24
Embryo	686±162	995±220	1128±129	686±162	949±138	780±98
Shoot	–	505±52	621±127	–	–	–
Root	–	716±83	560±214	–	–	–
UDPGlc-PPase						
Endosperm	850±20	1020±130	630±20	850±20	820±70	660±30
Embryo	1570±590	3260±720	4530±580	1570±590	1110±10	1110±30
Shoot	–	1140±100	1190±460	–	–	–
Root	–	1570±250	1510±120	–	–	–
NDPK						
Endosperm	264±12	153±2	150±44	264±12	259±49	192±27
Embryo	247±50	434±24	243±22	247±50	339±41	365±26
Shoot	–	544±86	505±37	–	–	–
Root	–	499±62	463±152	–	–	–

considering the endosperm tissue. However, when considering the embryo, where the amount of energy needed to cope with the needs of the germinating embryo is higher, both FK and PGM activities are slightly depressed under anoxia (Tables 4, 5), and a stronger negative effect can be observed in the activity of UDPGlc-PPase, catalysing the conversion of UDPGlc to Glc1P and UTP. An opposite effect was observed in the activity of NDPK, which is higher in the anoxic samples (Tables 4, 5).

Cycling of urydilates

If sucrose degradation takes place through SS and UDPGlc-PPase, a mechanism for recycling UTP to UDP is needed. The UTP formed by UDPGlc-PPase could be used by HK, leading to cycling of urydilates needed for the SS pathway (Huber and Akazawa, 1986; Xu *et al.*, 1989). However, these results indicate that both GK and FK can not take advantage of UTP as an efficient phosphate donor under either aerobic or anaerobic conditions (data not shown; Guglielminetti *et al.*, 1995*a*). An increased UTP-dependent activity of GK and FK was observed in anoxic rice seedlings (Guglielminetti *et al.*, 1995*a*). This increase could not be observed in anoxic wheat and barley seeds (data not shown).

NDPK activity may allow the cycling of urydilates through the ADP-mediated conversion of UTP to UDP

Table 5. *Effects of anoxia on enzymes of carbohydrate metabolism of barley seeds*

Data (mU mg^{-1} protein) are mean of three replicates ±SE. n d. = not detectable

	Air			Anoxia		
	0 d	3 d	6 d	0 d	3 d	6 d
α-Amylase						
Endosperm	n.d.	8566±58	14350±1371	n.d	n.d.	n.d.
Embryo	n d	3340±926	4648±440	n.d.	n.d	n d.
Shoot	–	n.d.	n.d.	–	–	–
Root	–	612±138	1115±176	–	–	–
GK						
Endosperm	13±3	6±3	6.5±3.5	13±3	21.5±5 5	22.5±1.5
Embryo	17±4	43.5±0.5	42.5±5.5	17±4	21.5±0.5	22.5±1 5
Shoot	–	20±3	13±3	–	–	–
Root	–	21.5±1.5	21±3	–	–	–
FK						
Endosperm	14±2	7±4	8.5±1.5	14±2	15.5±7 5	9.5±1.5
Embryo	19.5±0.5	43±6	44±6	19.5±0.5	30±4	30±6
Shoot	–	31.5±2.5	23±3	–	–	–
Root	–	38±3	21±3	–	–	–
Invertase						
Endosperm	5±1	8.5±2.5	8 5±3.5	5±1	5±2	3±1
Embryo	4.5±0.5	10.5±1 5	10±4	4.5±0.5	2.5±0.5	4.5±0 5
Shoot	–	50 5±4.5	46±9	–	–	–
Root	–	54.5±6.5	28.5±7.5	–	–	–
SS						
Endosperm	74±14	2±2	n d.	74.5±14	16.5±6.5	8.5±1.5
Embryo	6.5±0.5	22±2	16.5±3 5	6.5±0.5	13.5±1.5	20.5±0 5
Shoot	–	15±1	11±1	–	–	–
Root	–	33±2	27.5±3.5	–	–	–
PGI						
Endosperm	234±39	170±35	223±32	234±39	281±35	229±58
Embryo	341±47	516±128	643±28	341±47	469±25	404±23
Shoot	–	226±18	224±10	–	–	–
Root	–	163±8	228±60	–	–	–
PGM						
Endosperm	901±210	596±83	547±88	901±210	1360±64	1032±225
Embryo	1276±37	1533±414	1419±145	1276±37	1400±59	1214±227
Shoot	–	1137±3	1006±138	–	–	–
Root	–	912±32	611±86	–	–	–
UDPGlc-PPase						
Endosperm	2180±530	1080±110	1070±150	2180±530	1470±300	890±210
Embryo	2760±40	8410±1880	6910±60	2760±40	3190±60	2650±550
Shoot	–	2340±260	2130±130	–	–	–
Root	–	2350±220	1840±260	–	–	–
NDPK						
Endosperm	370±95	100±28	157±35	370±95	675±122	418±81
Embryo	362±26	291±32	387±33	362±26	523±62	492±10
Shoot	–	568±43	490±21	–	–	
Root	–	378±31	354±65	–	–	

with ATP production (Renz and Stitt, 1993). The results here show that a high NDPK activity is present under both aerobic and anaerobic conditions (Tables 4, 5).

The cycling of urydilates was tested using dialysed extracts of anaerobic wheat and barley seeds in order to verify if urydilates can be cycled during the breakdown of sucrose through the coupled activity of SS, UDPGlc-PPase and PGM present in the extracts (Guglielminetti *et al.*, 1995*a*). The stoichiometry of the conversion of sucrose to Glc1P using a limiting amount of UDP allows one to verify if cycling of uridylates is taking place through the action of FK (UTP dependent). The ratio between the NADH produced (through the oxidation of Glc6P catalysed by the addition of Glc6P dehydrogenase from *Leuconostoc mesenteroides*) and the UDP added for the activity of UDPGlc-PPase should be higher than 1 if FK participates in the urydilates cycling, as shown by Xu *et al.* (1989) using potato extracts. The results indicate that FK plays no role in the cycling of uridylates, but addition of ADP to the reaction mixture results in a ratio of NADH/UDP higher than 1 in the presence of ADP (Fig. 2), indicating that the NDPK-mediated conversion of UTP to UDP is taking place, despite the depressing effect of anoxia on some of the enzymes involved in the SS pathway (Tables 4, 5).

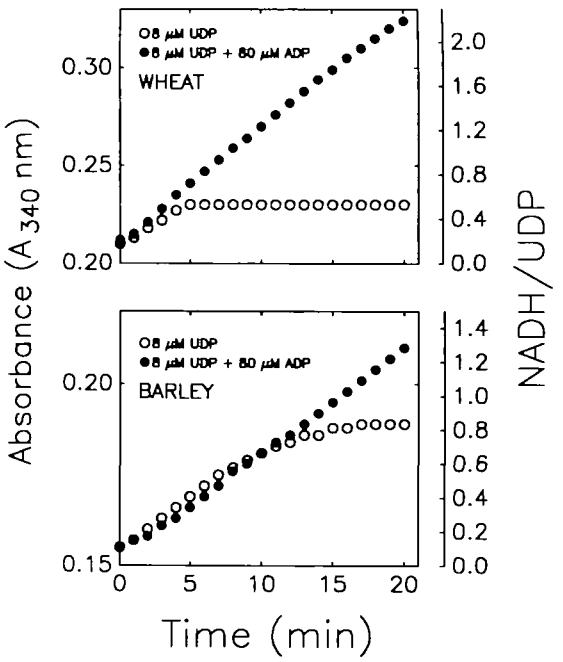

Fig. 2. Urydilates cycling via NDPK in dialysed extract from 6- and 4-d-old anoxic wheat and barley seedlings, respectively. Sucrose conversion to Glc6P via the SS pathway (involving the activity of SS, UDPGlc-PPase and PGM) was measured by recording NADH formation by *Leuconostoc* Glc6P dehydrogenase. NADH production was dependent on the presence of both PPi and UDP (not shown). In the absence of ADP the reaction stopped when UDP was consumed (open symbols) leading to the NADH/UDP ratio shown in figure. Cycling of urydilates was observed if ADP was added to the reaction mixture (filled symbols) since the reaction continued even when virtually all the UDP added was consumed.

Discussion

Wheat and barley, anoxia-intolerant seeds, are unable to utilize starch under anoxia (Perata *et al.*, 1992, 1993*a*; Guglielminetti *et al.*, 1995*b*). However, the presence of soluble sugars in the endosperm may allow the operation of anaerobic pathways for the utilization of carbohydrates under a condition of energy shortage (Drew, 1990; Kennedy *et al.*, 1992; Perata and Alpi, 1993; Armstrong *et al.*, 1994; Ricard *et al.*, 1994). No information is available concerning the metabolism of the soluble carbohydrates present in the dry seed of wheat and barley.

The data presented in this paper clearly show that anaerobic wheat and barley seeds rapidly utilize the pool of soluble carbohydrates present in the dry seed. This probably results in sugar starvation of the embryo. It is worth noting that the longer survival of wheat seeds when compared with barley correlates with the amount of carbohydrates remaining in the seed, particularly in the embryo. Raymond *et al.* (1985) reported data indicating that only in rice can continuous ethanol production be observed under anaerobic conditions, while the rate of fermentation decreases in wheat. This is probably the consequence of the large amount of fermentable carbohydrates available in the anaerobic rice seedlings deriving from the anaerobic starch degradation (Perata *et al.*, 1992; Guglielminetti *et al.*, 1995*b*), while in wheat the availability of readily utilizable carbohydrates rapidly declines during the anaerobic treatment.

Vartapetian *et al.* (1976) proposed that rice coleoptiles are tolerant to anoxia as a consequence of their ability to transport organic compounds from the seed to the anaerobic coleoptile, in agreement with the report showing the ability of rice to utilize starch under anoxic conditions (Perata *et al.*, 1992).

The availability of readily fermentable sugars could, therefore, play a role in anoxia tolerance. Some authors reported data indicating that carbohydrates supplied exogenously enhance anoxia tolerance of plant tissues (Webb and Armstrong, 1983; Andreev *et al.*, 1991; Waters *et al.*, 1991*a*, *b*; Perata *et al.*, 1992; Zhang *et al.*, 1992; Zhang and Greenway, 1994), while others proposed an opposite view (Roberts *et al.*, 1985; Malki *et al.*, 1989).

The anoxic treatment had different effects on the enzymatic activities tested. While the effect on α-amylase is very well defined, with no enzyme produced under anoxia, the other enzymes were differently affected (Tables 4, 5). In the embryo of both wheat and barley, all the activities except NDPK were generally lower under anoxia than under aerobic conditions. This behaviour is very different from the pattern of enzymatic activities observed in rice (Guglielminetti *et al.*, 1995*a*). In rice, α-amylase is produced under anoxia and the activity of GK, FK, and SS are much higher under anoxia than in the aerobic seedling. The other enzyme activities (PGI, PGM, UDPGlc-PPase, and NDPK) were almost unaffected by the anaerobic treatment of rice. Only invertase activity was considerably depressed in the anoxic rice seedling (Guglielminetti *et al.*, 1995*a*). This may suggest that the anaerobic pathway for sucrose degradation proposed to be operative in the anoxic rice seedling may not operate efficiently in the anoxic wheat and barley seeds. However, when the enzymatic activities were calculated on a biological unit basis (U/seeds, data not shown) it was evident that the activities present in the seeds, including the anoxic ones, could well account for the rate of carbohydrate degradation observed in the anoxic cereal seeds studied. Moreover, the activities present can support the cycling of urydilates previously proposed (Guglielminetti *et al.*, 1995*a*), even if the data obtained from *in vitro* experiments can hardly be considered as clear evidence of the occurrence of the cycling *in vivo*.

Strategies to understand and improve stress tolerance have been recently reviewed by Bartels and Nelson (1994): the limiting factor, in any approach, is the identification of genes and regulatory elements which positively contribute to stress tolerance improvement.

For anaerobic stress, a wide range of information is available concerning the induction of enzymatic activities (Drew, 1990; Kennedy *et al.*, 1992; Perata and Alpi, 1993; Armstrong *et al.*, 1994; Ricard *et al.*, 1994), but only a

very limited number of papers deal with a comparison between tolerant and non-tolerant species in terms of induction of anaerobic polypeptides (Mujer *et al.*, 1993). This would help in the efforts to identify the proteins that contribute to adaptation to anaerobic conditions in tolerant species. The identification of α-amylase, GK, FK, and SS as enzymes differentially modulated by anoxia in cereal seeds showing a varying tolerance to anaerobic treatments represents a step ahead for a molecular approach to manipulate anaerobic stress tolerance.

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