Activities and Regulation of Enzymes of Carbohydrate Metabolism in Spruce *(Picea abies)*

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Z. Naturforsch. **46c,** 597–604 (1991); received Dezember 7, 1990/April 4, 1991

Picea abies, Cell Culture, Carbohydrate Metabolism, PP_i-phosphofructokinase, Enzyme Regulation

Activities of the glycolytic enzymes were determined in seedlings, callus cultures and cell suspension cultures of spruce *(Picea abies)* (L.) (Karst).

The rate-limiting enzymes of the pathway were the hexokinases, ATP: phosphofructokinase, fructose-1,6-bisphosphatase and pyruvate kinase. Two phosphofructokinases were found: ATP: fructose-6-phosphate 1-phosphotransferase (PFK) and pyrophosphate: fructose-6-phosphate 1-phosphotransferase (PFP). In the presence of its activator fructose-2,6-bisphosphate, PFP had a 4–5-fold higher specific activity than PFK. PFP could be activated about 20-fold by fructose-2,6-bisphosphate at saturating concentrations of the substrates (fructose-6-phosphate and pyrophosphate). The increase of V_{max} was accompanied by a strong increase in the apparent affinity of the enzyme for the substrates. K_m for fructose-6-phosphate and pyrophosphate was 0.44 mM and 24 μM, respectively. K_a for fructose-2,6-bisphosphate was 24 nM.

In seedlings, specific activity of the glycolytic enzymes was 30–300 percent higher in the hypocotyls, except for fructose-1,6-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, their activity being 100–150 percent higher in the cotyledons, This distribution remained unchanged during periods of 2–16 weeks of cultivation of the seedlings.

In callus cultures and in cell suspension cultures, grown mixotrophically with different carbohydrates, all enzymes were between 1- and 7-fold higher than in autotrophically grown seedlings. Incubation of seedlings in mineral salt mixture containing a carbohydrate resulted in a rapid coordinate increase of the activities to the levels of callus- or cell suspension cultures. This induction required a carbohydrate and oxygen. During prolonged cultivation of cell suspension cultures, when carbohydrate became limiting, activity of the enzymes slowly declined.

Introduction

Recent studies on spruce HMGCoA reductase, the rate-controlling enzyme of isoprenoid biosynthesis, indicated that during cultivation of seedlings, callus cultures and cell suspension cultures, available carbohydrate is an essential factor for the regulation of this enzyme. A number of carbohydrates, in low concentrations, increased the activity of HMGCoA reductase over 30-fold [1]. This induction was transient and could be prevented by cycloheximide [1]. Little is known about properties and capacities of the enzymes of carbohydrate metabolism in conifers. In the present communication, profile data and some regulatory features of the enzymes of glycolysis in spruce *(Picea abies)* are reported.

The interconversion of Fru-6-P and Fru-1,6-P_2 is a rate-controlling step in glycolysis and gluconeogenesis. The direction of flow of carbon through Fru-6-P is controlled by the relative activities of PFK and Fru-1,6-P_2ase. In plants, an additional enzyme is involved, which reversibly interconverts Fru-6-P/Fru-1,6-P_2 and PP_i/phosphate [2–4]. The activity of this enzyme, PFP, located in the cytoplasm [2–4], maybe equal or greater than PFK, depending on the plant tissue [2, 4, 5], thus, it might significantly contribute to sugar metabolism. Fru-2,6-P_2 strongly activates PFP [2–4, 6–8], while it inhibits Fru-1,6-P_2ase [3, 9], and it has no effect on PFK [3] (for a review see [10]),

Abbreviations: Fru-6-P, fructose-6-phosphate; Fru-1,6-P_2, fructose-1,6-bisphosphate; Fru-2,6-P_2, fructose-2,6-bisphosphate; Fru-1,6-P_2ase, fructose-1,6-bisphosphatase; PFK, ATP: fructose-6-phosphate 1-phosphotransferase; PFP, pyrophosphate: fructose-6-phosphate 1-phosphotransferase; PP_i, inorganic pyrophosphate; UDPG, uridine diphosphate glucose; PEP, phosphoenolpyruvate; HMGCoA, 3-hydroxy-3-methylglutaryl coenzyme A.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0939–5075/91/0700–0597 $ 01.30/0

PFP was also found in spruce and some kinetic data are included in this communication. Part of this work has been published in preliminary form [11].

Experimental

Plant material

Seedlings

Seeds of *Picea abies* (L.) (Karst) were germinated and seedlings grown on humid Agriperl (plant perlite) under a normal daylight cycle at 20–22 °C and watered with tap water. Seedlings, cultivated for at least 14 days, were used for the experiments.

Callus cultures and cell suspension cultures

These were prepared and propagated as described in detail [12]. For the present experiments the cultures were grown mixotrophically (fluorescent lamps Osram L40 W/77,1000 Lux and one percent of either sucrose, glucose or fructose).

Chemicals

All biochemicals, including enzymes for coupled assays, were obtained from Sigma Chemical Co., St. Louis, U.S.A. (Deisenhofen, Germany).

Incubations

Seedlings, cut 2–3 times, were suspended in medium (3 g fresh weight/150 ml). The medium used was Murashige and Skoog basal medium (MS) (Sigma, St. Louis, U.S.A., Deisenhofen, Germany), supplemented with the phytohormones benzylaminopurine (1 mg/l) and naphthylacetic acid (3 mg/l) with or without carbohydrate (1 percent). The seedlings were incubated in 1 l fluted conical flasks under rotary shaking (60 rpm) at 27 °C in continuous white light (see above).

Enzyme isolation

Plant material (seedlings, callus cultures, cell suspension cultures) was homogenized (1 g fresh weight/10 ml buffer) with a Potter Elvehjem homogenizer in 0.05 M Tris-HCl buffer pH 7.5, containing 0.01 M $MgCl_2$, 0.02 M EDTA, 0.001 M dithioerythritol and 10 percent (w/v) polyvinylpyrrolidone 40 (soluble) at medium speed for 30 sec. The homogenate was then sonicated for 30 sec. It was first centrifuged for 10 min at $2000 \times g$, the supernatant of this centrifugation was subsequently centrifuged for 30 min at $40{,}000 \times g$ and this supernatant was used directly as the source of enzymes. All procedures were carried out at 4 °C.

Assay of enzymes

All enzymes were assayed by monitoring the change in absorbance at 340 nm in a continuous assay, in which the activity was coupled to the reduction of NAD(P) or oxidation of NAD(P)H. Assays were performed at 25 °C in a final volume of 1 or 3 ml. Activities were determined under optimized conditions with regard to substrate and pH. Blanks without substrate were used to correct for non-specific reactions when necessary. Linearity of at least 2 min was observed. Activity is expressed as $nmol \cdot min^{-1} \cdot mg\ protein^{-1}$. Protein was determined according to [13].

Assays were performed as follows: Sucrose synthase (EC 2.4.1.13) [14]; hexokinase (glucose) (EC 2.7.1.2) and fructokinase (EC 2.7.1.4) [15]; UDPG pyrophosphorylase (EC 2.7.7.9) [16]; phosphoglucomutase (EC 2.7.5.1) and phosphoglucose isomerase (EC 5.3.1.9) [17]; PFK (EC 2.7.1.11) and PFP (EC 2.7.1.90) [18]; Fru-1,6-P_2aldolase (EC 4.1.2.13) and triosephosphate isomerase (EC 5.3.1.1) [19]; glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) [20]; phosphoglycerate kinase (EC 2.7.2.3) [21]; phosphoglycerate mutase (EC 2.7.5.3) [22] (method I); enolase (EC 4.2.1.11) [23]; pyruvate kinase (EC 2.7.1.40) [20]; PEP carboxylase (EC 4.1.1.31) [24]; glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and phosphogluconate dehydrogenase (EC 1.1.1.44) [25].

Results and Comments

Enzyme isolation

Experiments on enzyme isolation, performed in order to have reliable specific activities, also for a comparison of the numerous enzymes investigated, revealed that the procedure described in the Experimental section (30 sec each of homogenization and sonication) was optimal for the isolation of the enzymes from the various plant material.

In Table I, a number of the enzymes, listed as representative activities, are shown in their re-

Table I. Effect of the isolation procedure on enzyme activity. Crude extracts were prepared as described with 3 different times of homogenization and sonication. The specific activities under optimal conditions of isolation (30 sec homogenization and 30 sec sonication) are set at 100 percent. Activity was determined within 1 h after preparation of the crude extracts. Values are means ± SEM of 3 experiments.

	Percent specific activity			
	15 sec homogenization +15 sec sonication		120 sec homogenization +120 sec sonication	
	A Seedlings	B Callus cultures Suspension cultures	C Seedlings	D Callus cultures Suspension cultures
Sucrose synthase	75	80	86	91
Hexokinase	70	82	100	96
UDPG pyrophosphorylase	65	75	92	100
Phosphoglucomutase	61	74	100	92
PFK	68	80	91	90
PFP	58	74	97	95
Triosephosphate isomerase	63	79	105	110
Pyruvate kinase	57	69	85	90
PEP carboxylase	62	78	83	90
Glucose-6-P-DH	78	90	100	105

sponse to varying times of homogenization and sonication. In this Table, the specific activities obtained with an isolation procedure of 30 sec each of homogenization and sonication were set at 100 percent. It can be seen that with shorter periods (15 sec each), specific activity of the enzymes was 20–30 percent lower, extraction, thus was not yet at optimum (A and B). While treatments of 60 sec (not shown) resulted in specific activities identical to those of 30 sec, prolonged homogenization and sonication (120 sec each) slightly inactivated most of the enzymes (C and D). It can also be seen from Table I that, due to the different nature of the plant material, extraction with short time periods was less effective in seedlings than in the cell cultures (compare A and B).

The presence of phenolic compounds in plant material is known to be very critical for the activity of enzymes, as phenols, contained in the vacuoles, form insoluble complexes with proteins (enzymes) during extraction of the plant material, whereby inactivating the soluble enzymes. Polyvinylpyrrolidone (PVP) is able to bind phenols and thus protect the enzymes from being inactivated.

It therefore had to be excluded that phenolic compounds interfere with enzyme activity in spruce. This is shown in Table II. Four representative activities of the investigated enzymes are

Table II. Inactivation of the enzymes by phenolic compounds and protection of the activity by polyvinylpyrrolidone (PVP). A, callus cultures and cell suspension cultures; B, seedlings. Crude extracts were prepared as follows (see Experimental section): I, preparation with PVP; II, preparation without PVP; III, preparation without PVP and with a homogenate of needles (cytoplasmatic fraction) (see [1]) used as extraction medium. The homogenate was prepared without PVP. IV, preparation as in III, but the needle homogenate was prepared with PVP (as in [1]). Specific activities in III and IV are corrected for the activity of the needle homogenate. Values are means of 2 identical experiments. For SEM 5–15% (not shown; see Table III).

	Specific activity							
	I		II		III		IV	
	A	B	A	B	A	B	A	B
Phosphoglucomutase	1725	680	1685	560	680	270	1690	690
PFP	278	98	290	73	150	40	295	100
Phosphoglycerate kinase	2205	395	2190	260	850	120	2400	390
PEP carboxylase	220	44	225	30	86	13	220	39

shown here. It can be seen that callus- and cell suspension cultures (which exhibit very similar levels of activity (see Table III)), if at all, did not contain phenols in quantities that would inactivate the enzymes as no decreased activity was observed when PVP was omitted during the extraction of the plant material (compare I A and II A). In contrast, spruce seedlings did contain phenolic substances and PVP was necessary during extraction to protect the enzymes (compare I B and II B). When homogenates of needles, which are known to contain significant amounts of phenols, were present during the extraction of cell cultures or seedlings, enzyme activities were considerably lower (compare II A and III A and also II B and III B). When the needle homogenate used was prepared with PVP to bind the phenols, the enzymes were no longer inactivated (compare I A and IV A). In addition, the PVP in the needle homogenate preparations also protected the enzymes in the seedlings from becoming inactivated. Thus, the PVP in the standard isolation procedure (see Experimental section) protected the enzymes completely from a possible inactivation by phenols.

Enzymes of carbohydrate metabolism in cell cultures and seedlings

Cytoplasmatic fractions of seedlings, callus cultures and cell suspension cultures of spruce contained all the enzymes which are required for the conversion of sucrose, glucose or fructose to pyruvate and to oxalacetate *via* the glycolytic pathway. The pentose phosphate enzymes glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase were also present.

Specific activities of the enzymes are summarized in Table III. It can be seen that the rate-limiting activities were the hexokinases, PFK, Fru-1,6-P_2ase and pyruvate kinase. Very high specific activities were found for phosphoglucomutase, phosphoglucose isomerase, triosephosphate isomerase and phosphoglycerate kinase. Data from other plant sources (soybean, cauliflower) show similar profiles for the glycolytic enzymes [19, 20]. The findings with *Picea abies*, being a representative of the gymnosperms, might indicate that the patterns of the capacities of glycolysis are similar in angiosperms and in gymnosperms.

Table III. Specific activity of enzymes of carbohydrate metabolism in seedlings (A) and in callus cultures and cell suspension cultures (B) of spruce. Values are means ± SEM of 6–8 experiments.

Enzyme	Specific activity	
	A Seedlings	B Callus cultures Cell suspension cultures
Sucrose synthase	30 ± 4	78 ± 8.5
Hexokinase	15 ± 1.7	46 ± 6.1
Fructokinase	18 ± 1.9	54 ± 4.9
UDPG pyrophosphorylase	140 ± 12	335 ± 30
Phosphoglucomutase	700 ± 61	1750 ± 180
Phosphoglucose isomerase	350 ± 40	525 ± 51
PFK	20 ± 1.8	46 ± 5.3
PFP	95 ± 9.8	282 ± 28
Fru-1,6-P_2ase	13 ± 1.1	17 ± 2.1
Fru-1,6-P_2aldolase	100 ± 9.2	170 ± 16
Triosephosphate isomerase	800 ± 76	2880 ± 300
Glyceraldehyde-3-P dehydrogenase	140 ± 16	582 ± 51
Phosphoglycerate kinase	400 ± 38	2200 ± 210
Phosphoglycerate mutase	190 ± 21	608 ± 64
Enolase	150 ± 16	300 ± 31
Pyruvate kinase	18 ± 2.4	128 ± 11
PEP carboxylase	40 ± 3.8	224 ± 23
Glucose-6-P dehydrogenase	190 ± 16	422 ± 48
Phosphogluconate dehydrogenase	150 ± 14	330 ± 31

Specific activity was considerably higher in mixotrophically grown callus cultures and cell suspension cultures as compared with autotrophically grown seedlings. This is also shown in Table III (B). Specific activity was, on an average, increased 2.5-fold, phosphoglycerate kinase, pyruvate kinase and PEP carboxylase, however, were up to 7-fold higher. Fru-1,6-P_2ase was only slightly increased. Levels of activity were very similar in callus cultures and in cell suspension cultures and also when the cultures were grown with either sucrose, glucose or fructose.

Distribution of enzyme activities in seedlings

The enzymes depicted in Table III were not uniformly distributed within the spruce seedlings. As can be seen from Table IV there were pronounced differences in the activity between cotyledons and hypocotyls. Levels of activity of most enzymes were higher in the hypocotyls. Phosphoglucomutase, phosphoglucose isomerase, Fru-1,6-P_2ase and enolase were only slightly higher, while the activity of the phosphofructokinases (PFK and PFP), of pyruvate kinase and PEP carboxylase was considerably higher in the hypocotyls. In contrast, Fru-1,6-P_2aldolase, glyceraldehyde-3-P dehydrogenase and phosphoglycerate kinase were significantly higher in the cotyledons. This distribution remained unchanged during a 4 months cultivation period tested.

A similar non-uniformal distribution of activity has already been found with two regulatory enzymes of spruce: HMGCoA reductase, the rate-controlling enzyme of isoprenoid synthesis, which consists of a microsomal (105,000 × *g* pellet) and a mitochondrial (18,000 × *g* pellet) activity, had a much higher specific activity in the hypocotyls of the seedlings [1]. Likewise, the activity of phenylalanine ammonia-lyase, a cytoplasmatic enzyme which is the rate-determining activity in the synthesis of phenolic compounds, was considerably higher in the hypocotyls [34].

Properties of spruce phosphofructokinase (PFP)

As shown for a number of other plant systems, seedlings, callus- and cell suspension cultures of spruce also contained two phosphofructokinases, PFK and PFP. The latter enzyme was partially characterized, using the cell suspension culture.

Activity was determined in the forward (PP_i-consuming) direction. The possible presence of inhibitors of enzyme activity in the enzyme preparation was checked for by addition of extracts (in amounts for determining the activity) to an assay mixture which contained commercial PFP. No effect of the extracts on the activity was found. In

Table IV. Distribution of enzyme activity between hypocotyls and cotyledons in seedlings of spruce. A, enzyme activity higher in hypocotyls than in cotyledons; B, enzyme activity higher in cotyledons than in hypocotyls. Values are means ± SEM of 4–6 experiments.

A	Percent higher than in cotyledons	B	Percent higher than in hypocotyls
Sucrose synthase	60	Fru-1,6-P_2aldolase	110
Hexokinase	80	Glyceraldehyde-3-P dehydrogenase	140
Fructokinase	60		
UDPG pyrophosphorylase	100	Phosphoglycerate kinase	165
Phosphoglucomutase	30		
Phosphoglucose isomerase	50		
PFK	200		
PFP	185		
Fru-1,6-P_2ase	20		
Triosephosphate isomerase	100		
Phosphoglycerate mutase	90		
Enolase	30		
Pyruvate kinase	300		
PEP carboxylase	200		
Glucose-6-P dehydrogenase	140		
Phosphogluconate dehydrogenase	130		

the presence of its activator Fru-2,6-P_2, specific activity of PFP was more than 4-fold higher than PFK (see Table III). This relation was identical in all the spruce material tested. In most of the plant systems assayed for PFK and PFP, specific activity of the latter enzyme was either identical with or higher than PFK.

Activity of PFP was strongly dependent on the presence of Fru-2,6-P_2, it was very low in its absence (see Fig. 1). Activation by Fru-2,6-P_2 was around 20-fold at saturating concentrations of the substrates Fru-6-P and PP_i. Activation was caused by lowering the K_m for Fru-6-P from 12.5 mM to 0.44 mM and for PP_i from 133 µM to 24 µM. In both the absence or presence of Fru-2,6-P_2 hyperbolic kinetics were found for Fru-6-P and PP_i. The K_a for Fru-2,6-P_2, as calculated from Fig. 1, is 24 nM. PFP from spruce was highly specific for Fru-6-P. It exhibited maximum activity at pH 7.5 and had a strong requirement for Mg^{2+}.

Thus, PFP of spruce resembled the characteristics of this enzyme found in other plant sources including potato tubers [7, 26], leaves of spinach [3, 27] and pineapple [28], seeds of pea [29] and *Phaseolus* [30], seedlings of wheat [31] and castor bean [4] and cell cultures of soybean [32], *Catharanthus roseus* [18] and *Chenopodium* [33].

In the autotrophically grown spruce seedlings Fru-1,6-P_2ase is present in very low activity (see Table III). This activity could be inhibited by addition of Fru-2,6-P_2, it thus represented Fru-1,6-P_2ase. Spruce seedlings contained Fru-2,6-P_2. Efforts to demonstrate a stimulation of Fru-1,6-P_2ase in response to decreasing Fru-2,6-P_2 levels revealed that when cytosolic fractions, in order to remove Fru-2,6-P_2, were either dialyzed (against 100 volumes of extraction buffer) or were passed through a Sephadex G-25 column, the activity of Fru-1,6-P_2ase increased about 50 percent. At the same time, PFP decreased and became scarcely detectable. Therefore, the PFP measured without added Fru-2,6-P_2 (see Fig. 1) might represent the level of activity in the presence of the Fru-2,6-P_2 available in the cell.

Thus, in spruce a regulation of Fru-1,6-P_2ase by Fru-2,6-P_2 is very likely. As for the capacity of the Fru-1,6-P_2ase reaction in sucrose synthesis, it is not possible to attribute this enzyme a major role under the described conditions of cultivation of the seedlings. However, as Fru-2,6-P_2 activates PFP in the glycolytic- as well as in the gluconeogenic direction [8, 10], this might possibly represent an alternative for the formation of Fru-6-P for the synthesis of sucrose in the spruce system.

Regulation of the enzyme of carbohydrate metabolism in callus- and cell suspension cultures

As mentioned above, the specific activity of all enzymes was higher in mixotrophically grown callus cultures and cell suspension cultures than in

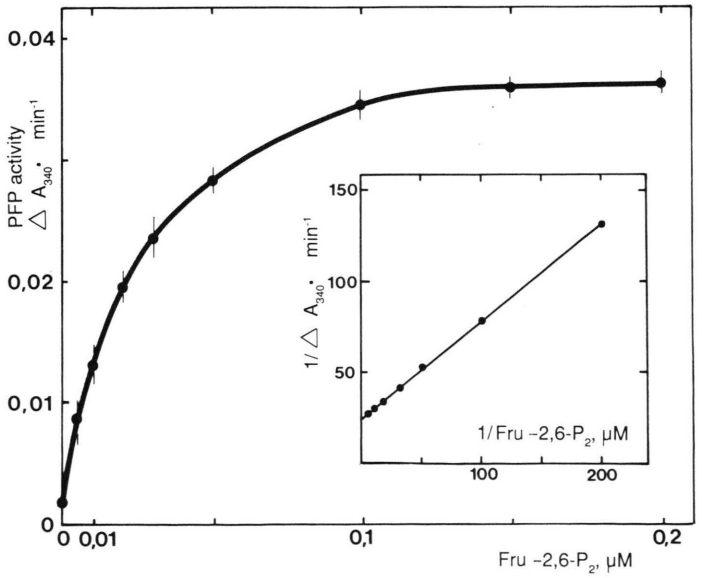

Fig. 1. Activation of pyrophosphate: fructose-6-phosphate 1-phosphotransferase from cell suspension culture of spruce by fructose-2,6-bisphosphate. The assay mixture contained 2.0 mM Fru-6-P, 0.5 mM PP_i and Fru-2,6-P_2 as indicated. The reaction was initiated by addition of 40 µg of enzyme preparation. K_a calculated from the inset is 24 nM. Bars represent standard deviations. Values are means of 4 replicate experiments.

autotrophically grown seedlings (Table III). When seedlings were incubated in medium (see Experimental section), containing a carbohydrate, a coordinate induction of the enzymes occurred. Within 48 h, activities had increased to the levels of callus- or cell suspension cultures. For example, specific activity of pyruvate kinase increased from 18 ± 2 (see Table III, A) to 115 ± 11 in 12 h and to the maximal level of 135 ± 14 after 24 h of incubation of the seedlings. This induction required the presence of a carbohydrate (see below) and also of oxygen.

The induction was not transient as with the spruce HMGCoA reductase [1] or with phenylalanine ammonia-lyase [34], but the induced enzyme levels in incubated seedlings were stable for at least 3 days. (After this time, carbohydrate-containing seedling incubations tended to become unsterile.) The extent of induction of each enzyme was restricted to the levels of the cell cultures, in no case a higher specific activity was observed. The induction was identical with either sucrose, glucose or fructose used as inducing carbohydrate.

Thus, the lower levels of activity of an autotrophically grown system can become adapted to the higher enzyme niveau of the mixotrophically grown system by carbohydrate.

In callus- and in cell suspension cultures, which are cultivated with sucrose, sucrose synthase, UDPG pyrophosphorylase and PFP were present (see Table III). Thus, a break-down of sucrose *via* sucrose synthase, UDPG pyrophosphorylase, which feed glucose-1-phosphate into glycolysis [8, 10, 14, 15], plus the further degradation to the levels of triose phosphates, involving PFP, which is activated by Fru-2,6-P_2 (see [35]) would be possible. When callus cultures or cell suspension cultures are being cultivated with glucose or fructose, the hexokinases would be active. Being about twofold higher in the cell cultures than in the seedlings, these levels probably are a sufficient activity for the degradation of the sugars.

During prolonged cultivation of cell suspension cultures, the medium becomes deprived of carbohydrate. Under these conditions the activity of the investigated enzymes decreased. The decline, measured 3 days after carbohydrate had completely disappeared from the medium, was 10–25 percent. Thus, a lack of sugar will gradually reduce the activity of the enzymes. This result, together with the observed increase of the activities of seedlings in the presence of a sugar (see above) make a sensitive regulation of the enzymes of glycolysis in spruce by carbohydrate appear to be likely.

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