Differential carbohydrate metabolism conducts morphogenesis in embryogenic callus of Hevea brasiliensis (Müll. Arg.)

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Abstract

Somatic embryogenesis in Hevea is stimulated when the embryogenesis induction medium contains maltose, rather than glucose, fructose, or sucrose, in equimolarity (Blanc et al., 1999). Kinetic analyses were carried out on various physiological and biochemical indicators over the 8 weeks that the induction phase then expression of somatic embryogenesis can take. Embryogenesis induction in the presence of glucose, fructose or sucrose revealed strong callus growth in the first 3–4 weeks, associated with a high intra- and extracellular hexose content, a high starch content and a substantial decline in protein synthesis. In the presence of maltose, callus growth was slow and only half that seen with sucrose. This morphogenetic behaviour is associated with a drop in endogenous hexose and starch contents, and an increase in protein synthesis in the first three weeks of culture. The induction of embryogenesis in the presence of maltose was uniform and twice as fast as with sucrose supply. At the end of culture, peroxidase activity, antioxidant and membrane protein contents increased in these calluses; these characteristics may be associated with somatic embryo organization and with the maintenance of effective membrane integrity within a nutrient environment that has become limiting. These new results tally with data in the literature on the roles of sugars, and provide some precise information with regard to the ‘carbohydrate deficit’ hypothesis usually put forward to explain maltose action. An analysis of these results led to the hypothesis that regulation of endogenous hexose contents at a low level, through slow maltose hydrolysis, was a key element of the biochemical signal leading this callus towards somatic embryogenesis.

Key words: Callus metabolism, carbohydrates, Hevea brasiliensis, regeneration, somatic embryogenesis.

Introduction

Somatic embryogenesis appears to be a promising technique for mass propagation of elite Hevea brasiliensis (Müll. Arg.) trees. The embryogenesis procedure, which is based on the regeneration of embryogenic, friable callus lines, has been used for some years to produce several thousand in vitro plantlets per year. The plantlets have been tested in field trials to characterize their agronomic qualities (Carron et al., 1997, 1998, 2000). A prerequisite for industrialization of the embryogenesis process is an improvement in biological yields, particularly during the embryogenesis induction phase. An earlier study showed that the carbon source in the embryogenesis induction medium affected the intensity of the phenomenon (Blanc et al., 1999). Indeed, of the four sugars tested (sucrose, maltose, glucose, and fructose), maltose proved to be significantly better than the others during this culture phase. In the presence of maltose, calluses display a radical and uniform change in cell typology within the first 2 weeks of culture; embryogenic cells are observed. Under these conditions, it gives rise to somatic embryos at a rate of 160 embryos g⁻¹ of callus. On the other hand, in the presence of sucrose, callus proliferation is greater and embryogenic cells are observed only after 4 weeks of culture. It involves a very small number of cells. A limited number of embryos is produced (20–40 embryos g⁻¹ of

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callus). Similar results are obtained with glucose and fructose.

Several authors have already reported that maltose is more effective than sucrose for embryogenesis induction in barley (Scott and Lyne, 1994), alfalfa (Strickland et al., 1987), asparagus (Kunitake et al., 1997) or fir (Norgaard, 1997). Some hypotheses have been put forward to explain the effects of maltose on somatic embryogenesis induction. Scott et al. assumed that the beneficial effects of maltose were linked to its slow hydrolysis, which limited cell carbon nutrition (Scott et al., 1995). The maltose effects might also be due to low cell uptake, leading to a drop in available carbohydrates. A drop in carbohydrate availability within the cells might create a signal that reorients the development programmes (Koch, 1996). Another hypothesis is that the maltose molecule itself has a specific effect in orienting development programmes. In this study, the biochemical effects of embryogenesis induction in the presence of maltose, sucrose, glucose or fructose were compared. The aim was to characterize the main physiological events accompanying embryogenesis induction, and attempt to explain the origin of maltose effects in relation to this development phase.

Materials and methods

Plant material and culture conditions

The experiment was conducted on friable callus of line 0193 (genotype PB 260) obtained from the inner integument of immature Hevea seeds (Carron et al., 1998). The line of friable callus was maintained in proliferation on a basal medium (Carron and Enjalric, 1985) containing: macronutrients salts (mM 1−1): NH4NO3, 20; KNO3, 19.8; CaCl2, 9; NaH2PO4·H2O, 2; MgSO4·7H2O, 3; micronutrients salts (μM 1−1): H3BO4, 150; MnSO4·H2O, 10; ZnSO4·7H2O, 40; CuSO4·5H2O, 1.5; Na2MoO4·2H2O, 1; KI, 5; CoCl2·6H2O, 1; FeSO4·7H2O, 100; Na2EDTA·2H2O, 100; micro-organic elements (μM 1−1): inositol, 300; nicotinic acid, 20; pyridoxine–HCl, 3; thiamine–HCl, 31.7; biotin, 0.2; n-C panthotenic acid, 1; ascorbic acid, 1; choline chloride, 1; l-cysteine HCl, 60; glycine, 5; riboflavin, 1.

Basal medium is supplemented with 30 μM AgNO3, 1.34 μM 3,4-D, 1.34 μM BAP, 0.5 μM ABA, 234 mM sucrose, and 2.3 g l−1 of phytagel at pH 5.8 (medium referred to as ENT). The calluses were transferred to fresh medium every fortnight. The cultures were grown in the dark at 27 °C, in tubes (150×25 mm) containing 13 ml of ENT medium, sealed with polycarbonate caps. The media were sterilized beforehand at 121 °C for 20 min.

At the end of the proliferation phase, 6 g of callus was suspended in 7.5 ml of liquid embryogenesis induction and expression medium (EXP medium). The EXP medium corresponded to the ENT medium with the 3,4-D and BAP concentrations reduced to 0.44 μM each, and without AgNO3. The medium contained 234 mM of sucrose, maltose, fructose or glucose. After stirring the callus suspension for 30 s, 1.5 ml of suspension (corresponding to 1 g of fresh callus) were deposited on filter paper placed beforehand on 20 ml of gel culture medium (3 g l−1 of phytagel) in a Petri dish (100×20 mm). The dishes were covered with clingfilm (filmpro, Pechiney, France). Culturing lasted for 2 months.

Biochemical analyses and enzymatic activities

All callus and culture medium analyses were carried out at the end of culturing on ENT (day zero of embryogenesis induction: D0) then after 7, 14, 21, 28, 35, 42, 49, and 56 d culture on EXP containing the sugar tested.

Dry weight measurement and callus water content: Callus fresh matter (FM) was weighed without the filter paper. The callus was then submerged in liquid nitrogen and freeze-dried. Callus dry matter (DM) was then weighed. Callus water content (WC) was expressed in g H2O g−1 DM and calculated using the formula WC = (FM − DM)/DM.

Quantification of sugars in the callus and in the media by ion chromatography: On each sampling date, the calluses were submerged in liquid nitrogen, freeze-dried and stored at −20 °C. Media samples were also stored at −20 °C. The freeze-dried callus (0.3 g) and gel media underwent extraction in 100 ml of ethanol (80%). The solution was filtered. The filtrate was evaporated in a Rotavapor (Heidolph VV 2000, Bioblock Scientific, Illkirch, France) at 45 °C until an extract of 0.15 ml was obtained. The volume was adjusted to 50 ml with deionized water, homogenized then passed through a 0.45 μm filter. Soluble sugars were analysed using this extract.

The callus residue contained starch, which was hydrolysed in 0.15 M HCl, at 110 °C for 3 h. Hydrolysis was completed by enzymatic digestion in an acid medium at 60 °C with amyloglucosidase (EC 3.2.1.3., Fluka, Hannover). The soluble sugars in the calluses and in the media, along with glucose resulting from starch hydrolysis, were analysed by ion chromatography on an anion exchange column (Carbopac, Dionex SA, Jouy en Josas, France) connected to a pulsed amperometric detector (model DPA 2, Dionex SA, Jouy en Josas, France) using the method described previously (Peschet and Giacalone, 1991).

The quantity of carbohydrates metabolized by the callus at a given culture time corresponded to ‘the quantity of carbohydrates taken up (A0−A1) added to the difference between the initial amount of carbohydrates in the callus (C₀) and that after a given time (C_i). It was expressed in mg of carbohydrates metabolized g−1 of initial DM. The quantity of carbohydrates required to produce 1 g of dry matter was also measured at different culture stages, using the formula: [(A_i−A_{i+1})+(C_{i−C_{i+1}})]/(DM_{i+1}−DM_{i}) where i is an integer between 0 and 8, corresponding to the culture weeks; (A_{i}−A_{i+1}) is the quantity of carbohydrates absorbed by the callus during the week preceding measurement; (C_{i}−C_{i+1}) is the amount of carbohydrates disappearing from the callus; (DM_{i+1}−DM_{i}) is the gain in dry matter during the same week.

Extraction method for quantification of thiols, ascorbic acid, and peroxidase activities: Freeze-dried callus (50 mg) was crushed in a microtube using a teflon pestle in 3 ml of citrate–phosphate extraction buffer (0.3 M, pH 6). After centrifugation, the supernatant, which contained the soluble cell compounds, and the precipitate, which was resuspended in the citrate–phosphate buffer were stored at 4 °C for further analysis.

Quantification of ascorbic acid and thiols: Ascorbic acid and thiols were quantified on an aliquot of the supernatant. Okamura’s method was used to quantify ascorbic acid (Okamura, 1980). A 144 μM ascorbic acid solution was used.
Experimental plan and statistical analyses: All the results were obtained from the same experimental plan, a split-plot design. The main factor was the carbohydrate (four states: sucrose, maltose, glucose, and fructose), the subdivided factor was time (nine states from 0–8 weeks). The design comprised three experimental blocks. The data were processed by two-way analyses of variance based on an additive model. The analyses were carried out with Statbox software (Grimmer, 1997). For each parameter, the analysis result was written according to the formula \( F(\text{DF}_{\text{parameter}}, \text{DF}_{\text{error}}) = \text{value of } F; P < 0.0\). A Newman–Keuls comparison of means test (Miller, 1981) was carried out for each parameter studied.

### Results

**Changes in callus dry matter weight and water content during the expression phase**

Irrespective of the type of carbon source, the increase in dry matter was small during the first week of culture (Fig. 1A). It then varied depending on the carbon source. The fastest increase in dry matter was seen in the sucrose treatment. Callus dry weight peaked after 28 d of culture, stabilized, and then decreased day 42 onwards. The increase kinetics were similar in the hexose treatments: an increase up to day 21 of culture, then a slow and linear decline up to the end of culture. In these treatments, the total increase in dry matter during culture was only half that seen in the sucrose treatment. The slowest increase was seen in the maltose treatment, but the increase in dry weight continued up to day 35 of culture and there was no matter loss up to the end of culture. As for the hexose treatments, the increase in dry matter was only half that seen in the sucrose treatment. An analysis of variance (Table 1) revealed the reliability of the statistical design through the absence of a ‘block’ effect, whereas the ‘carbohydrate’ and ‘time’ effects were highly significant, alone and in interaction.

**Table 1. Results of the three-way analysis of variance on the dry matter weight of calluses regenerating on EXP medium with sucrose, maltose, fructose or glucose**

The values underwent log transformation prior to testing. The model chosen was blocks with two explanatory variables (time and carbohydrates). There were 15 replicates (blocks). MSD, mean squared deviation; DF, degrees of freedom; EXP, expression medium; n.s., not significant; test significance limit: ***, \( P < 0.001\); **, \( P < 0.01\); *, \( P < 0.05\).

![Fig. 1. Comparative changes in the morphogenetic characteristics of calluses grown on EXP medium with sucrose, maltose, fructose or glucose.](image)

**Fig. 1. Comparative changes in the morphogenetic characteristics of calluses grown on EXP medium with sucrose, maltose, fructose or glucose. (A) Dry matter weight, (B) water content. There were 15 replicates. The bars correspond to the standard deviations. EXP, expression medium; Suc, sucrose; Mlt, maltose; Fru, fructose; Glc, glucose.**

**Carbohydrate content in medium during the expression phase**

For the sucrose treatment, the initial quantity of disaccharide decreased by 53% during the first week of culture, with an accumulation of products from its hydrolysis, glucose and fructose (Fig. 2A). After a month of culture, the medium only contained fructose and glucose,
amounting to approximately 40% of the initial quantity of carbohydrates. In the hexose treatments, virtually all the carbohydrates were taken up in 42 d of culture (Fig. 2C, D). Concerning maltose treatment, uptake, which was intense between 7 d and 21 d of culture, subsequently slowed down, but remained continuous up to exhaustion of the carbon source at around day 49 of culture. Hydrolysis products did not appear in the medium. Only a very small quantity of glucose was quantified from day 21 of culture (Fig. 2B).

**Carbohydrate status in calluses**

**Soluble carbohydrate content:** The carbohydrate status in the calluses varied depending on the type of carbohydrate supplied in the medium and reflected the type of carbohydrates contained in the medium in the initial weeks of regeneration (Fig. 3).

When the medium contained sucrose, the callus sucrose content increased by a factor of 1.8 in the first week of regeneration; the amount of fructose and glucose by a factor of 2.1 (Fig. 3A). Callus sucrose and hexose contents held up during the first month of regeneration, and subsequently declined. Callus always contained less fructose than glucose. The maltose content was near to zero and remained unchanged.

In the maltose treatment, a strong and transient accumulation of maltose occurred in callus during the first week at the same time as a rapid decrease in the hexoses amount (Fig. 3B), maintaining the overall soluble carbohydrate content at its initial value. Thereafter, the maltose content declined steadily up to the fourth week of regeneration, and was close to zero in the 6th week of culture; there was no further change in the hexose content and the sucrose content remained unchanged throughout the culture period.

The carbohydrate status in calluses of hexose treatments largely varied in the same way (Fig. 3C, D). The content of hexose supplied in the medium increased temporarily, at the same time as a slight accumulation of sucrose. The sucrose remained stable up to day 21, then gradually declined and became insignificant by the end of culture.

**Quantity of carbohydrates metabolized by the calluses:** During the first 2 weeks of culture, the callus in the sucrose treatment started using the carbon source later than the calluses in the other treatments. Thereafter, the quantity of carbohydrates metabolized by this callus was similar to that of the callus in the maltose treatment, though it stood out through substantial heterogeneity.
between calluses (Fig. 4). From the third week of culture, the treatments divided into two groups: sucrose and maltose on the one hand, glucose and fructose on the other hand. The first group eventually used twice the amount of carbohydrates used by the second group. The carbohydrate metabolism measured was primarily active in the first 3 weeks (hexose treatments) to 4 weeks (disaccharide treatments) of culture.

**Quantity of carbohydrates required to produce one gram of dry matter:** The energy requirements of the callus in the maltose treatment were very high right from the beginning of culture, and higher than those in the other treatments during the first month of culture (Fig. 5). The difference was maximum with the callus in the sucrose treatment in the first 2 weeks of culture. At the end of culture, the increase in callus energy requirements in the sucrose treatment was related to their reduction in dry weight. It was more important in the fructose and glucose treatments.

**Variation in starch reserves:** An analysis of variance revealed that starch reserves were highly significantly affected by the carbohydrate in the medium (Fig. 6) \[F(3; 60) = 16.68; P < 0.001\], the time factor \[F(7; 60) = 6.28; P < 0.001\], and ‘time–carbohydrate’ interaction

![Fig. 3. Soluble carbohydrate contents in the callus on EXP medium with sucrose, maltose, fructose or glucose, during regeneration. (A) Sucrose treatment, (B) maltose treatment, (C) fructose treatment, (D) glucose treatment. There were three replicates. The bars correspond to the standard deviations. EXP, expression medium; Suc, sucrose; Mlt, maltose; Fru, fructose; Glc, glucose.](image)

![Fig. 4. Effect of the type of carbohydrate in the EXP medium on the quantity of sugars metabolized by the calluses. The quantity of carbohydrates metabolized corresponds to the quantity of carbohydrates taken up \(\frac{A_0 - A_i}{C_0}\) added to the difference between the initial quantity of carbohydrates in the callus \(C_0\) and that after a given time \(C_i\) based on the following formula: \([A_0 - A_i] + (C_0 - C_i)\) in mg of metabolized carbohydrates g\(^{-1}\) DM\(_0\) (= dry matter set to regenerate on day 0 of the experiment). There were three replicates. The bars correspond to the standard deviations. EXP, expression medium; Suc, sucrose; Mlt, maltose; Fru, fructose; Glc, glucose.](image)
starch in the calluses in the last week of culture. However, in the callus of the maltose treatment, the starch content fell sharply in the first week of regeneration and remained low for the rest of the culture period.

Antioxidant contents and soluble peroxidase activity in the calluses

Ascorbic acid and thiol contents: The ascorbic acid content, which was under 1 μmol g⁻¹ of DM in the callus at the end of the proliferation phase, did not vary for the first 4 weeks of culture, irrespective of the type of carbon source (Fig. 7). The same applied for the thiol content, which remained below 5 μmol g⁻¹ of DM for all the treatments in the first month of culture (Fig. 8). Thereafter, the ascorbic acid and thiol contents increased by a factor of 13 and 4.5, respectively, in the callus of the maltose treatment, whereas they remained very low for the sucrose and hexose treatments. Anova revealed that these parameters were highly significantly affected by time [ascorbic acid: F(8; 70) = 4.23; P < 0.001; and thiols: F(8; 70) = 12.21; P < 0.001], by the carbohydrate in the medium [ascorbic acid: F(3; 70) = 27.88; P < 0.001 and thiols: F(3; 70) = 91.82; P < 0.001] and by ‘time–carbohydrate’ interaction [ascorbic acid: F(24; 70) = 5.41; P < 0.001; and thiols: F(24; 70) = 16.47; P < 0.001].

Peroxidase activity: Anova showed that soluble peroxidases were highly significantly affected by the time factor [F(8; 35) = 28.73; P < 0.001], by the carbohydrate in the medium [F(3; 35) = 18.80; P < 0.001] and by ‘time–carbohydrate’ interaction [F(24; 35) = 4.41; P < 0.001]. The specific activity of soluble peroxidases, which was near to zero in the first 4 weeks of culture, subsequently increased up to the end of the culture period (Fig. 9). This significant increase was recorded for all treatments, but it was most marked in the callus of the maltose treatment, where it was multiplied by 20 between day 28 and day 42 of culture.
Carbohydrates conduct Hevea regeneration

Fig. 9. Specific activity of soluble peroxidases in callus grown on the EXP media with sucrose, maltose, fructose or glucose. The specific activity is expressed in oxidized tetraguaiacol mg\(^{-1}\) protein h\(^{-1}\). There were three replicates. The bars correspond to the standard deviations. The multiple comparison of means test revealed that the data for the 'maltose' treatment were significantly greater than those of the other treatments from day 42 of regeneration. EXP, expression medium; Suc, sucrose; Mlt, maltose; Fru, fructose; Glc, glucose.

**Discussion**

The type of carbohydrate in the embryogenesis expression medium was an environmental change that had a profound effect on somatic embryo production (Blanc et al., 1999). The effects on a cellular scale were clearly visible from the second week by histological examination of the calluses. Monitoring of the kinetics of a certain number of biochemical and physiological indicators, described here, revealed two carbon source groups that had a clearly typical effect on the metabolic orientation of Hevea calli: sucrose, fructose and glucose on the one hand, and maltose on the other hand.

**Activation of cell growth by sucrose, glucose or fructose**

Sucrose, fructose and glucose treatments stimulated callus growth. Sucrose treatment multiplied the dry matter 7-fold in 4 weeks; hexose treatments increased the dry matter by a half compared to the former. Although the growth steps did not have the same duration, the growth kinetics followed similar steps: one-week latency followed by an increase in dry matter, a stationary step and a loss of weight. Supplying the medium with sucrose or glucose at the same concentration of equivalent hexoses eliminated the differences in dry matter weight, as well as in duration step (unpublished results). These carbohydrates were called 'callogenic sugars' (El Maataoui et al., 1998).

In Albizzia, they triggered the synthesis and accumulation of starch reserves in all the cells of the callus, which were followed by the differentiation process: elongation and vacuolation. In Hevea calli, sucrose, fructose and glucose stimulated disorganized growth: the proportion of parenchymatous like cells largely increased during the growth compared to the proportion of undifferentiated meristematic like cells (Blanc et al., 1999). A drop in the protein content also characterized the differentiation process. The formation of embryonic cells occurred when the growth stopped, after 4 and 3 weeks, respectively, in sucrose and hexose treatments. At the same time, starch reserves decreased sharply in the callus.

The calluses turned brown and cells accumulated polyphenols. It was shown that high sucrose or hexose contents triggered metabolic reactions typical of plant tissues in hypoxia in barley microspores (Scott et al., 1995): low incorporation of \(^{14}\)C in the insoluble compound fraction and of glutamine and, on the other hand, high incorporation of alanine, combined with a drop in the adenyl energy load (ATP + ADP), and significant ethanol accumulation. An initial substantial drop in soluble protein contents, an increase in mineral elements contained in the culture media and a drop in membrane protein contents at the end of culture were observed on Hevea callus (G Blanc, L Lardet, A Martin, JL Jacob, MP Carron, unpublished results). This confirmed that the senescence process had been triggered, along with a loss in membrane integrity and leakage of cell contents, due to inefficient protection systems. Further, the contents of ascorbic acid and thiols, which are powerful reducing agents involved in cell defence mechanisms against active oxygen species (AOS; Noctor and Foyer, 1998), remained unchanged.

Senescence could be linked to the poor nutrient and carbohydrate contents in the medium as well as the low initial growth regulator content insufficient to maintain the proliferation of callus. However, most of these changes are not observed in callus from the maltose treatment. As in barley microspores, the morphological and biochemical changes reflected a specific physiological state as a consequence of the type of carbohydrate supplied in the medium.

Monitoring of the culture medium composition revealed that the sucrose content decreased sharply right from the first week of culture, with an accumulation of its hydrolytic products (Fig. 2). Sucrose hydrolysis in the medium has been reported for a long time (Obata-Sasamoto and Thorpe, 1983; Amino and Tazawa, 1988). Invertase activity is measurable before callus proliferation initiates growth, and enables an accumulation of directly assimilatable carbohydrates in the medium. It is combined with acidification of the medium too. In this work, rapid and substantial hydrolysis of sucrose may have led to a drop in the osmotic potential of the medium, as reported previously (Find et al., 1998). The concomitant increase in the hexose content of the callus also decreased the osmotic potential and maintained the water content.

Concerning the hexose treatments, the water potential of the medium might have gradually increased, while the water content of the callus increased. When calluses were involved in a growth process, they were sensitive to the osmotic pressure of the medium.
**Induction of somatic embryogenesis by maltose**

A very different metabolic situation was seen in callus grown on the medium containing maltose. Callus growth kinetics were different from the outset: the increase in dry matter was only half that seen with sucrose. This slow growth was associated with the acquisition of embryonic characteristics in all the cells of callus (Blanc et al., 1999). This homogenous cellular state was probably responsible for the stabilization in callus water content, whilst changes in the osmotic potential of the medium may have been similar to those seen in the hexose treatments. The callus (grown on maltose) was undergoing somatic embryogenesis and was less sensitive to the osmotic pressure of the culture medium: embryonic cells have a reduced vacuolar size, which exerted smaller suction than in parenchymatous cells, which have a larger vacuoles. Callus of the maltose treatment grew slowly compared to other treatments. However, callus took up maltose and used it, as its amount in the medium, and later in the callus, decreased. Moreover, callus of the maltose treatment metabolized similar amounts of sugars compared to callus of the sucrose treatment during the culture (Fig. 4). So, the slow growth observed was not due to a carbon nutrition deficit, but rather to a specific physiological state due to the cells entering the embryonic pathway. Actually, in the first weeks of culture, the quantity of carbohydrates metabolized to produce 1 g of dry matter (Fig. 5) revealed the importance of catabolic pathways and the high energy use by these calluses. Replacing sucrose with maltose in the somatic embryogenesis expression medium for *Hevea* increased cell energy requirements, as did replacing sucrose with galactose in *Citrus deliciosa* cultures (Cabasson, 1993). These high energy requirements may have supported the strong protein biosynthesis and numerous mitoses observed in the callus (Blanc et al., 1999).

The amount of maltose in the medium decreased during the culture, following similar kinetics as the amount of fructose and glucose in the hexose treatment. It seems that maltose uptake by the cells was not a limiting factor; although in the first days of culture the endogenous hexose content as well as starch reserves decreased (Figs 3B, 6). The slow hydrolysis of maltose could be responsible for the drop in sugar content in the callus. Maltose hydrolysis is 20 times slower than sucrose hydrolysis in barley anther cultures (Roberts-Oehlschlager et al., 1990), to such a point that some authors recommend sterilization of the media by autoclaving, rather than filtration, to encourage the production of a small amount of glucose by hydrolysis (Redenbaugh, 1993), or even using a maltose–glucose mixture (Kinnersley and Henderson, 1988). The drop in endogenous hexose content could be a key element in the reorientation of cell metabolism. As early as 1974, Kochba and Button concluded that sugar deprivation led to culture synchronization towards somatic embryo development, whereas a sucrose-rich medium stimulated proembryo proliferation (Kochba and Button, 1974). This approach was soon to be taken for date palm (Veramendi and Navarro, 1996) and for tobacco (Touraev et al., 1996). In a review reporting the

![Diagram](image-url)  
**Fig. 10.** Diagrammatic representation of the biochemical data recorded and of the hypotheses put forward concerning the action of different types of sugars on callus cell metabolism.
expression of genes controlled by carbohydrates, Graham estimated that hexoses, rather than their intermediate metabolites, acted as the first signal for the induction of gene expression (Graham, 1996), which tallied with the results obtained by Strickland et al., who concluded that glucose-1-phosphate and glucose-6-phosphate, metabolic intermediaries of maltose, were ineffectual in promoting somatic embryogenesis in alfalfa (Strickland et al., 1987).

One of the known effects of low endogenous hexose content is alpha-amylase synthesis and stimulation of starch catabolism (Yu et al., 1991), which numerous authors agree is a prerequisite for organ formation and somatic embry differentiation (Thorpe and Meier, 1972; Wurtele et al., 1988). A group of 46 kDa alpha-amylases that were strongly influenced by a sucrose deficit were identified, along with a constitutively expressed 44 kDa group (Chen et al., 1994). The expression of alpha-amylase genes was found to be 5–7 times greater in certain cellular loci, such as undifferentiated cell nodules, that have not been affected by senescence.

As a conclusion, these data shed light on the biochemical ways by which maltose stimulates somatic embryogenesis induction. These data show that neither absorption nor consumption of the maltose by cells is a limiting factor. So the hypothesis currently invoked, that in maltose-fed callus carbohydrates are not available enough and lead to a limited cell carbon nutrition (Scott et al., 1995), is disproved. The regulation of the endogenous content of hexoses at a low level seems to be the basis for metabolism reorientation.

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