Plant Cell Reports (1999) 19:26–31

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Effects of glycerol on somatic embryogenesis in *Cichorium* leaves

Received: 30 January 1998 / Accepted after revision 25 February 1999

Abstract Direct somatic embryogenesis was induced in leaf cells of a *Cichorium* hybrid (*Cichorium intybus* L var. *sativum* × *Cichorium endivia* L. var. *latifolia*) through a two-step procedure. Leaf tissue explants were cultured for 5 days in M17 liquid medium supplemented with 30 mM sucrose and 330 mM glycerol (M17S30Gly330 medium). Synchronised divisions of embryogenic cells occurred after transfer for 7 days onto glycerol free-medium (M17S30). By doubling the sucrose concentration (60 mM) in the presence of glycerol (M17S60Gly330) during the induction step, embryogenesis increased and the length of the induction step was reduced from 5 to 4 days. Compared to sucrose, glycerol as carbon source during the induction and the expression steps had an inhibitory effect on the embryogenic response. During culture, glycerol was not detected in M17S60 medium and was at a low level in leaf fragments incubated in this medium. Initially supplied as an osmoticum, glycerol disappeared from M17S60Gly330 medium during the 4-day induction period and penetrated into the tissues where most of was metabolised. Furthermore, glycerol modified it carbohydrate metabolism, particularly during the induction period of embryogenesis. Sucrose hydrolysis was affected in the medium and sucrose and hexose contents in tissues were higher than in glycerol-free medium. The effects of glycerol as osmoticum and as a molecule itself are discussed.

Key words Carbohydrate metabolism · Chicory · Glycerol · Somatic embryogenesis

Communicated by I. Negrutui

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Abbreviations *SE* Somatic embryo

Introduction

The most commonly used carbohydrate in plant tissue or cell culture is sucrose. In somatic embryo (SE)-forming cultures of different species, a variety of carbon sources have been tested and studies have revealed sucrose to be generally the best source of carbon for embryogenesis (Verma and Dougall 1977; Gleddie et al. 1983; Eapen and George 1993), including embryogenesis in *Cichorium* (Couillerot et al. 1993). It has also been found that glycerol-pretreated cultures of selected species of *Citrus* will undergo prolific embryogenesis (Vardi et al. 1982) and in a few cases, glycerol has been reported as the preferred carbon source for this species (Ben-Hayyim and Neumann 1983).

Leaf tissues of the *Cichorium* hybrid "474" offer a new attractive model of somatic embryogenesis which is direct, rapid (less than 12 days), abundant (more than ten embryos per mm^2 of leaf), with embryos of unicellular origin (Vasseur et al. 1995). Several factors have been identified that can influence or are directly correlated with this regeneration pathway, including callose deposition in cell walls of embryogenic cells (Dubois et al. 1990), specific proteins (Hilbert et al. 1992; Helleboid et al. 1995), polyamines (Couillerot et al. 1993) and temperature (Decout et al. 1994). What makes this system attractive is that by the addition of 330 mM glycerol as osmoticum to the synthetic culture medium containing 30 mM sucrose (induction medium), the first division of embryogenic cells is synchronised after the leaf fragments are transferred to the same medium free of glycerol for embryo expression (Robatche-Claive et al. 1992).

Here, we describe improved embryogenesis in the presence of glycerol during the induction step with increased glycerol and sucrose concentrations. Glycerol effects during the two steps of the culture on embryo yield were also studied. Finally, we investigated

glycerol interaction with carbohydrate metabolism and its own utilisation by leaf tissues.

Materials and methods

Material and culture conditions

The plant material was obtained from a clone of the *Cichorium* hybrid "474" (*Cichorium intybus* L var. *sativum* × *Cichorium endivia* L. var. *latifolia*) propagated by somatic embryogenesis (Dubois et al. 1988). The plantlets grown from root embryos (Dubois et al. 1990) were subcultured on solid Heller (1953) medium containing 30 mM sucrose, 2.25 mM inositol and vitamins (Morel and Wetmore 1951), with a proton flux rate of 50 μmol s^{-1} and 12/12 h photoperiod (24 °C during the light period, 20 °C in darkness). Leaf fragments of 6-week-old plantlets were cultured for 5 days at 35 °C in darkness in 20 ml of an agitated basal M17 medium for embryo induction (Dubois et al. 1991), supplemented with 60 mM sucrose and 330 mM glycerol (M17S60Gly330). This pretreatment prevented the first division of embryogenic cells which was only observed after transfer of leaf tissues to glycerol-free medium (M17S60). The cultures were stopped after 7 days in the expression medium. In further experiments, sterilised glycerol was added only during the first step or throughout the culture.

Estimation of embryogenesis

Leaf fragments were fixed in formaldehyde:acetic acid:ethanol solution (3.5:6.5:100, by vol) on the 12th day of culture and were stained with lugol (2% I_2 and 6% KI wt/vol). SEs were counted under a light microscope and the frequency (%) was distributed in four size classes (class 1: SE ≤ 100 μm; class 2: 100 < SE ≤ 150 μm; class 3: 150 < SE ≤ 200 μm; class 4: SE > 200 μm).

Paraffin sections

Leaves were cut into 5–10 × 5 mm pieces. Samples were dehydrated in an ethanol series and embedded in paraffin 52–54 °C (Merck). Sections 7 μm thick were stained with safranin/fast green. Observations were performed by transmitted light microscopy.

Extraction and analysis of carbohydrates and glycerol

Samples were weighed, quickly frozen in liquid nitrogen and stored at –70 °C until analysis. Frozen tissues were ground in a Potter blender. Carbohydrates and glycerol were extracted with 95% ethanol (1 ml/100 mg fresh weight) at 20 °C for 5 min. After centrifugation (30000*g*, 10 min, 20 °C), the pellet was re-extracted with 0.5 ml 95% ethanol. The two supernatants were combined and dried under vacuum. Carbohydrates and glycerol were solubilised in 2 ml 0.01 M sorbitol (used as the internal standard) and passed through a 0.45-μm Millipore filter.

Soluble carbohydrates and glycerol were analysed in 100-μl aliquots of filtered extract by HPLC. The chromatographic system consisted of a solvent degasser Spectra SYSTEM SCM400 (Spectra Physics, San Jose, Calif.), a Spectra SYSTEM P4000 pump, a column (300 × 6.5 mm) Waters Sugar-PAK I (Waters Millipore, Milfort, Mass.) maintained at 85 °C and a differential refractometer. Distilled water containing 50 mg l^{-1} calcium-EDTA was used as the solvent at a flow rate of 0.5 ml min^{-1}.

Osmolarity determination

Osmolarity was determined by an automatic osmometer (Roebling, Berlin, Germany) which used the freezing point of aqueous solutions. A 300-mOsmol sucrose solution was used as standard.

Data collection and statistical analysis

SEs induced per square millimetre of each explant were counted 12 days after culture initiation. A total of 90 areas (10 areas from three replicates of three explants per treatment) were analysed by ranking of treatment means to determine whether the results were statistically significant using the least-significant-difference test.

Results and discussion

Improvement of embryogenesis by increased sucrose and glycerol concentrations

Previous studies demonstrated that somatic embryogenesis in *Cichorium* is sensitive to the carbohydrate source in which it is incubated (Couillerot et al . 1993; Vasseur et al. 1995). Sucrose was found to be the most efficient carbon source for the induction of SEs. Leaf tissues were usually induced for 5 days in M17 medium containing 330 mM glycerol and then transferred to M17 medium for 7 days. By doubling the initial sucrose concentration from 30 to 60 mM, the yield of SEs at day 12 was significantly ($P = 0.05$) and considerably improved (14.71 ± 0.28 against 9.58 ± 0.54 SEs mm^{-2}). The absence of first division of the embryogenic cells described by Robatche-Claive et al. (1992) until the 5th day was only maintained until the 4th day in M17S60Gly330 (Fig. 1B) because embryogenic cells coexisted at day 5 with pluricellular SEs (Fig. 1A). Furthermore, higher concentrations of glycerol [495 mM (Fig. 2A) and 660 mM (Fig. 2B)] did not block the initial mitosis of the embryogenic cells; above 660 mM, tissues were necrosed. However, an increase in glycerol concentration delayed the first division of the reactivated cell. Moreover, the embryogenic cell and SEs were observed closer to the vascular bundle. Our results are in agreement with those observed during somatic embryogenesis of coconut (Buffard-Morel et al. 1991) and carrot (Guzzo et al. 1994, 1995). In leaf tissues incubated for 4 days and transferred for 8 days in M17S60 without glycerol, the yield of embryos was not significantly higher (15.5 ± 0.27 SEs mm^{-2}), but no synchronisation of the first division of the embryogenic cells was obtained. Following these data, the embryogenesis induction step was henceforth reduced to 4 days.

Glycerol and sucrose are important in the induction phase of embryogenesis

We screened the effects of glycerol applied during the induction and/or the expression steps of *Cichorium*

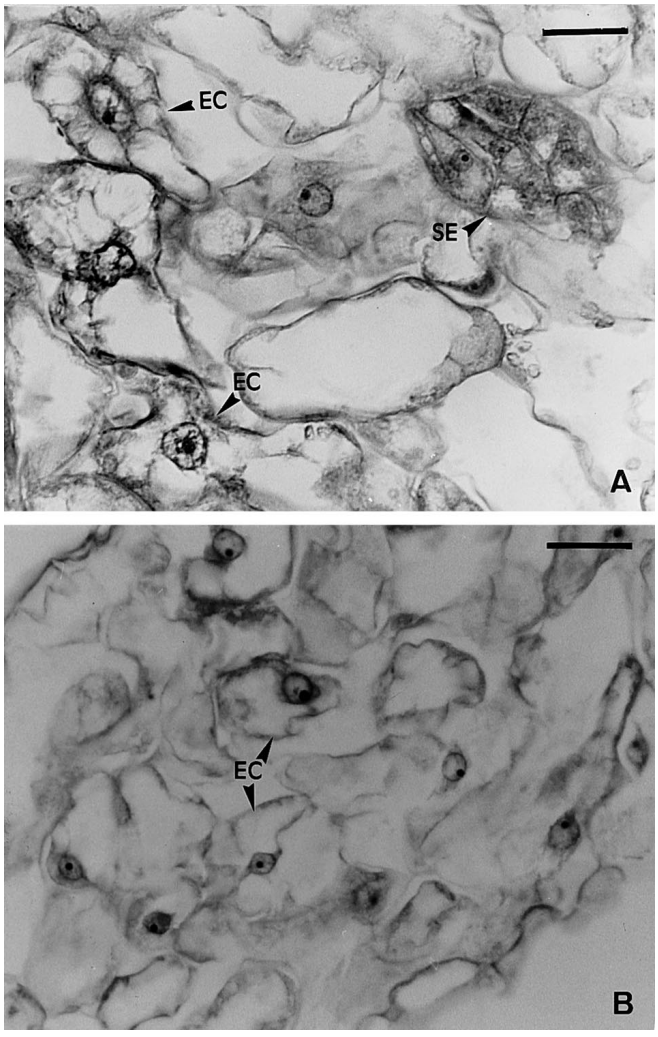

Fig. 1 Effect of glycerol on embryogenic cell first division. Transversal 7-μm-thick sections of *Cichorium* leaf fragments showing early stages of embryogenesis in M17S60 medium supplemented with 330 mM glycerol at day 5 (**A**), and at day 4 (**B**). Sections were stained with safranin-fast green (**EC** embryogenic cell, *SE*, somatic embryo, *bar* 20 μm)

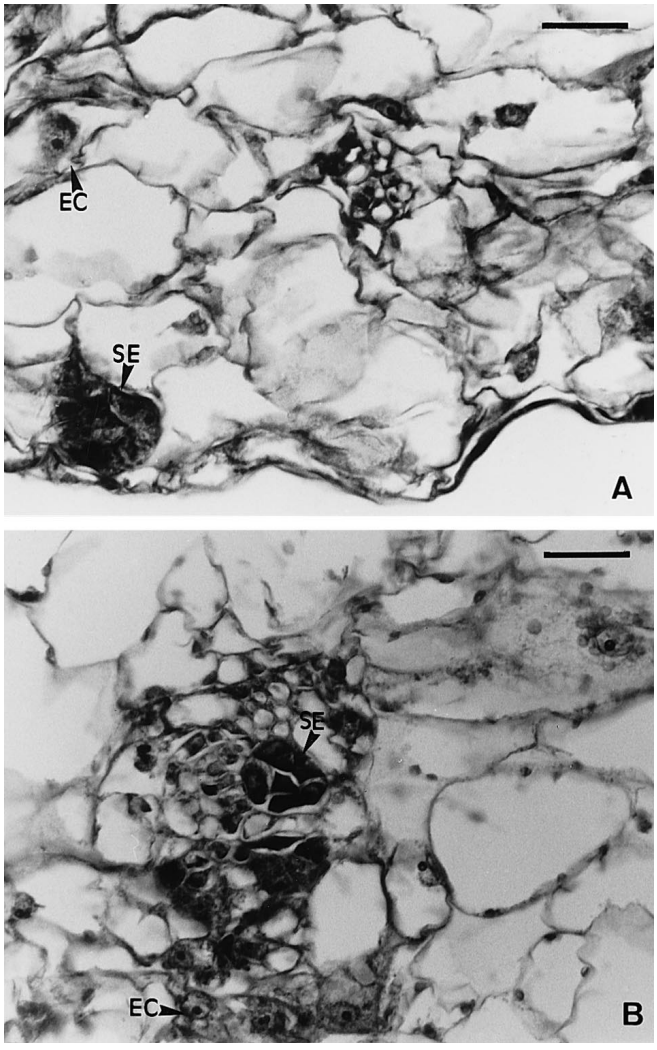

Fig. 2 Stimulation by glycerol of somatic embryogenesis of *Cichorium* leaf. Transversal 7-μm-thick sections of *Cichorium* leaf fragments incubated for 5 days in M17S60 medium supplemented with 495 (**A**) and 660 mM (**B**) glycerol. Sections were stained with safranin-fast green (**EC** embryogenic cell, *SE* somatic embryo, *bar* 20 μm)

embryogenesis. Through the induction period, glycerol was tested at 330 mM supplemented with 60 mM sucrose or with 60 mM glycerol in place of sucrose (Fig. 3). During the 8 days of the embryogenesis expression step, different combinations of sucrose and glycerol were tested. The analysis of variance revealed a significant effect ($P<0.001$) of glycerol concentration on embryo number. Results show that 60 mM sucrose supplemented with 330 mM glycerol during 4 days induction and transfer to an expression medium containing 60 mM sucrose gave the highest embryo yields (Fig. 3A). Most embryos had a diameter >100 μm (size classes 2–4). When 330 mM glycerol was present in M17S60 medium during the 12 days of culture with a transfer, after day 4 (Fig. 3B), the number of embryos was appreciably less. Without transfer, the number of embryos was even lower, probably because the carbon source and the inorganic nutrients were limiting (Fig. 3C). Furthermore, under those conditions, about 70% of the embryos had a diameter <100 μm (size class 1). Embryos were rare in leaf tissues incubated in transfer medium in which 60 or 330 + 60 mM glycerol was the only carbon source (Fig. 3D, E). The presence of glycerol during the expression step dramatically reduced embryo yield and affected their development. Babbar and Gupta (1986) reported similar results during haploid embryo production from *Datura metel* microspores cultured on glycerol-containing medium.

Glycerol (60 mM) tested as a replacement for sucrose in the induction medium supplemented by 330 mM glycerol as osmoticum had a drastic inhibitory influence on embryogenic response despite transfer to the sucrose-containing expression medium (Fig. 3F, G).

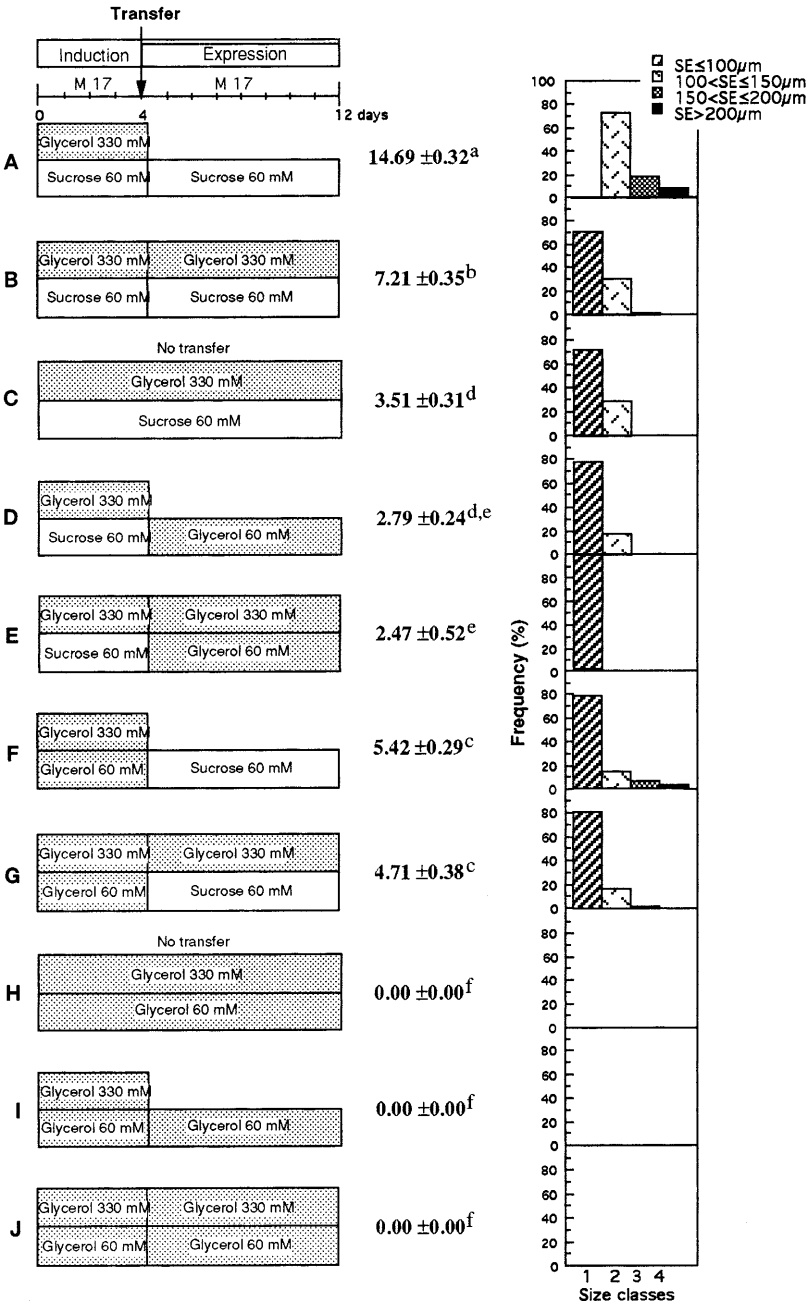

Fig. 3 Screening of the glycerol effect on somatic embryogenesis. Tissues were incubated for 4 days in M17 medium supplemented with 60 mM sucrose and 330 mM glycerol (M17S60Gly330) and then cultivated for 8 days in various media: M17S60 (**A**), M17S60Gly330 (**B**), M17S60Gly330 without transfer (**C**), M17Gly60 (**D**), M17Gly330+60 (**E**), or incubated for 4 days in M17 medium supplemented with 330+60 mM glycerol (M17Gly330+60) and then cultivated for 8 days in various media: M17S60 (**F**), M17S60Gly330 (**G**), M17Gly330+60 (**H**), without transfer, M17Gly60 (**I**), M17Gly330+60 (**J**). The number of somatic embryos (*SE*) mm^{-2} of leaf was counted at day 12 (mean ± SE, $n=270$) and distributed in size classes. Means with the same letters are not significantly different ($P=0.05$) as determined by F-LSD test

Moreover, embryo sizes suggested induction of embryogenic cells after transfer. When glycerol was the only carbon source in the expression medium in the absence (Fig. 3I) or in the presence of 330 mM glycerol with (Fig. 3J) or without transfer at day 4 (Fig. 3H), no embryo was observed at day 12. Our results agree with those obtained by Strickland et al. (1987) with embryogenic *Medicago sativa* cells in culture, in which glycerol did not improve embryogenesis compared to sucrose. No embryos were observed in *Cichorium* "474" leaf fragments incubated in glycerol as the only carbon source, while it might be preferred for the *Citrus* embryogenesis response (Ben-Hayyim and Kochba 1982; Ben-Hayyim and Neumann 1983; Vu et al. 1993). It was concluded that glycerol and sucrose were both important in the induction phase. Used to synchronise embryogenic cell division, glycerol alone did not stimulate embryogenesis. We investigated if glycerol effects on somatic embryogenesis were due to the molecule glycerol as such, to its utilisation, to the osmotic stress it may cause or to its interaction with carbohydrate metabolism.

Glycerol interaction with carbohydrate metabolism in media and leaf tissues

The glycerol influence on carbohydrate metabolism was studied by incubating leaf fragments in M17S60Gly330 or in M17S60 media and transferring

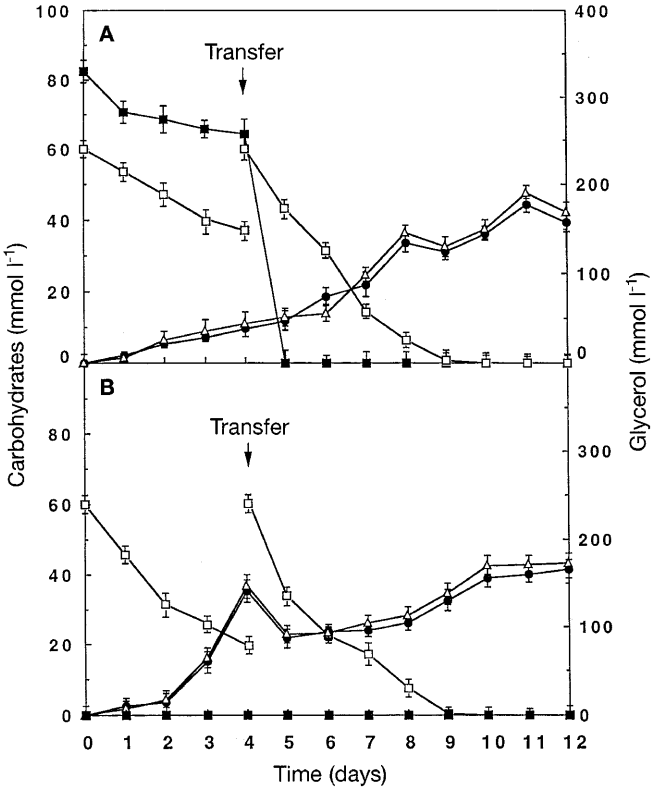

Fig. 4 Levels of sucrose (□), glucose (●), fructose (△) and glycerol (■) in the media for *Cichorium* leaf tissues incubated for 4 days in M17S60Gly330 (**A**) or in M17S60 (**B**) and transferred for 8 days to M17S60. Mean (±*SE*) of three cultures of three explants for each culture with three replications

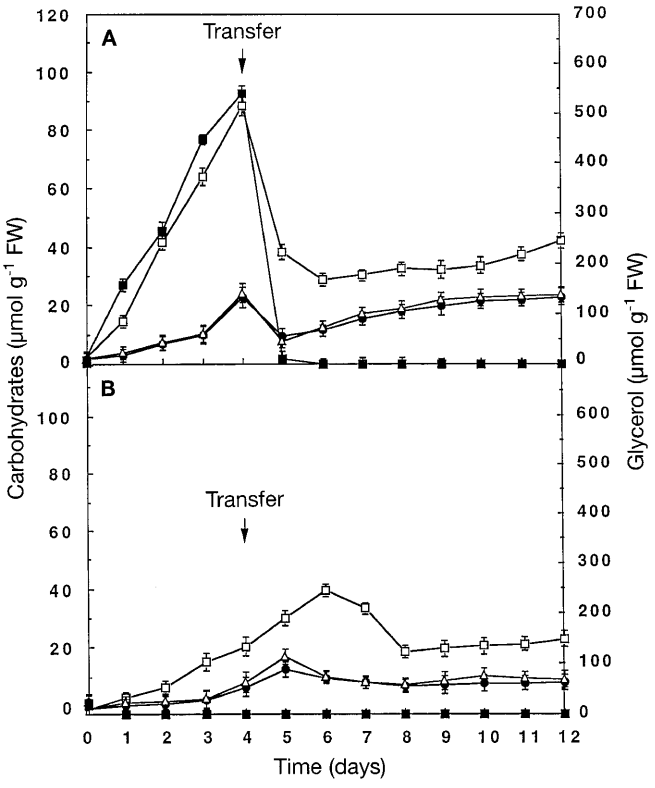

Fig. 5 Levels of sucrose (□), glucose (●), fructose (△) and glycerol (■) in *Cichorium* leaf tissues incubated for 4 days in M17S60Gly330 (**A**) or M17S60 (**B**) and transferred for 8 days to M17S60. Mean (±*SE*) of three cultures of three explants for each culture with three replications (*FW* fresh weight)

them at day 4 into M17S60 medium devoid of glycerol for 8 days. Addition of glycerol to the medium induced a notable rise in osmolarity (384 against 62 mOsmol in glycerol-free medium). HPLC analyses of the neutral sugars and glycerol in the media are shown in Fig. 4. The glycerol concentration in the medium declined from 330 to 257 mM during the induction step (Fig. 4A), while it was never detected in glycerol-free medium (Fig. 4B). After transfer to M17S60 medium, glycerol in the medium disappeared (Fig. 4A). The presence of glycerol in the medium modified sucrose metabolism (Fig. 4A, B). About 65% of the sucrose disappeared from the glycerol-free induction medium against about 35% in medium containing glycerol. Glucose and fructose levels increased slowly and concurrently to reach about 10 mM at day 4 in M17S60Gly330 whereas they reached about 35 mM in M17S60. The importance of hexose levels in the medium without glycerol resulted from sucrose hydrolysis through a higher invertase activity in leaf tissues (data not shown). After transfer, similar sucrose and hexose profiles were observed. Sucrose content decreased steadily in the media from day 5 to disappear by day 9, while glucose and fructose levels rose gradually to reach about 45 mM at day 12.

Carbohydrate and glycerol were extracted daily from leaf tissues cultured as previously (Fig. 5). No significant levels of endogenous water-soluble carbohydrate or glycerol (less than 1.5 µmol g^{-1} fresh weight) were recovered from *Cichorium* leaves before incubation. In leaf fragments cultured for 4 days in M17S60 induction medium, glycerol remained slightly detectable (about 2 µmol g^{-1} fresh weight) while the sucrose content rose gradually (22 µmol g^{-1} fresh weight at day 5, Fig. 5B). Elevated levels of glycerol and sucrose were recovered from leaf fragments incubated in M17S60Gly330 medium, especially between days 2 and 4 (539 and 88 µmol g^{-1} fresh weight, respectively; Fig. 5A). During the initial 4 days, glucose and fructose levels were twofold higher in M17S60Gly330 than in the glycerol-free condition (Fig. 5A, B). Such glycerol and carbohydrate levels in tissues incubated in the presence of glycerol reflected the reaction to osmotic stress and the inhibition of invertase activity in tissues (data not shown), the hexose/sucrose ratio remaining lower than in the glycerol-free condition (52% and 86% at day 4, respectively). After transfer, the carbohydrate profiles of the leaf fragments in both culture types were similar, but in glycerol-pretreated tissues, the carbohydrate fall coincided with the synchronised resumption of cell division.

When comparing the concentration of the glycerol in the medium and in the explants through the culture period, it can be concluded that most of the imported glycerol was metabolised by tissues. In suspension cultures of *Glycine max*, added [^{14}C]glycerol was not only absorbed for lipid synthesis. MacDonald and ApRees (1983) reported the labelling of free sugars, starch, structural polysaccharides, organic acids and proteins, and some of the [^{14}C] entered the respiratory pathways. These aspects are now under investigation in *Cichorium* leaf tissues.

In a previous study, Robatche-Claive et al. (1992) demonstrated that sucrose and non-absorbed mannitol at the same concentration as used here with glycerol (330 mM) were unable to block the first mitosis of embryogenic cells. Even if osmotic stress may encourage embryogenesis, as described for *Daucus carota* by Wetherell (1984) and for *Helianthus annuus* by Jeannin et al. (1993), the lack of first division shown in *Cichorium* was not a result of increased osmolarity caused by glycerol but due to the presence of the molecule itself. Though initially supplied to the induction medium as an osmoticum, glycerol modified carbohydrate metabolism, was also transported into the tissues, and can provide carbon and energy for leaf tissues undergoing embryogenesis.

Acknowledgements The authors thank Dr. Théo Hendriks (Agricultural University Wageningen, Netherlands) for critical reading of the manuscript.

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