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THE EFFECT OF CARBOHYDRATE SOURCE ON THE DEVELOPMENT OF *BRASSICA NAPUS* L. IMMATURE EMBRYOS IN VITRO

HALINA ŚLESAK AND LESLAW PRZYWARA*

Department of Plant Cytology and Embryology, Jagiellonian University, ul. Grodzka 52, 31-044 Cracow, Poland

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The effect of fructose, glucose, maltose and sucrose at various concentrations on *Brassica napus* embryos was studied. The morphogenetic reaction depended on the type and concentration of sugar in the medium. The frequency of developing embryos was highest on sucrose, followed by maltose and glucose. Fructose did not stimulate embryo growth. Spectrophotometry of autoclaved fructose showed an absorbance rise between 260–320 nm that could correspond to the formation of furfural derivatives. Autoclaving-induced toxicity probably inhibited embryo growth; heart-shaped stage embryos developed on filter-sterilized fructose. The frequency of developing embryos increased with sugar concentration, but normal embryogenesis occurred only on 1% sucrose and maltose; at higher concentrations callus and/or shoots were formed. On media with 6% sucrose and 12% maltose, shoots and somatic embryos were produced.

Key words: *Brassica napus*, carbohydrates, embryo culture, organogenesis, somatic embryogenesis.

INTRODUCTION

Globular stage embryos preceding cotyledon initiation are referred to as proembryos. Proembryos are heterotrophic and their growth depends on nutrients supplied by endosperm. Despite many years of experimentation, culture of proembryos is still a challenge since such explants require media containing not only standard components and an energy source but also an array of organic and hormonal substances that increases in complexity with decreasing embryo size (for review: Raghavan, 1976, 1980, 1986, 1997, 2000; Monnier, 1978, 1995; Raghavan and Srivastava, 1982; Collins and Grosser, 1984; Johri and Rao, 1984; Fisher-Iglesias and Neuhaus, 2001).

Carbohydrates are very important for the culture of zygotic embryos because besides being a carbon source they regulate medium osmolality, a critical factor in zygotic embryo development in vitro. The available data mainly concern sucrose, however. Rietsema et al. (1953) were the first to discover that immature embryos require higher osmotic pressure than mature embryos. Further experiments with various plant species confirmed these observations. On the basis of these data, Norstog (1963) and Raghavan and Torrey (1963) formulated media that allowed growth of early heart-shaped embryos into normal plants in vitro.

Methods for culturing proembryos have been developed which allow a gradual decrease of osmotic pressure during culture without the need to transfer embryos. Such systems have been successfully applied for culture of dicotyledons (Monnier, 1976; Liu et al., 1993; Góralski and Przywara, 1998).

The data on the effect of the carbon source on *Brassica napus* zygotic embryos are very scanty. Crouch and Sussex (1981) found that embryos ma-

* e-mail: l.przywara@iphils.uj.edu.pl

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tured if the sucrose concentration was lowered from 350 mM to 60 mM. In another experiment, morphologically normal seedlings were obtained from globular embryos only on medium with 1% sucrose (Góralski and Przywara, 1998). Similar results were reported for hybrid embryos *Brassica napus* × *Brassica oleracea* (Quazi, 1988) and for early globular stage embryos of *Brassica juncea* (Liu et al., 1993). Ilić-Grubor et al. (1998a,b) studied the effect of sucrose on microspore-derived embryos of *Brassica napus* cv. Topas. Bogunia and Przywara (2000) investigated the influence of different carbohydrates on callus induction and regeneration ability in *Brassica napus*.

The experiments described here compare the effects of different carbon sources and their concentrations on *Brassica napus* heart-shaped zygotic embryos cultured in vitro.

MATERIALS AND METHODS

PLANT MATERIAL

Brassica napus L. cv Topas plants were grown in a greenhouse under controlled conditions at 28˚C/22˚C (day/night) under a 16 h photoperiod. The pods were surface-sterilized by soaking in 70% ethanol for 30 sec and then for 10 min in commercial bleach diluted 1:1 with distilled water, followed by three rinses with sterile distilled water.

The ovules were isolated from the pods, and heart-shaped stage zygotic embryos (~150 µm long) were excised from them. The embryos were isolated with the use of needles in a drop of 9% glucose solution under a stereomicroscope (Nikon).

EMBRYO CULTURE

MS (Murashige and Skoog, 1962) was the basal medium. The medium was supplied with either 1%, 2%, 3%, 6%, 9% or 12% concentrations of fructose, glucose, maltose or sucrose. The dextro-rotatory form of carbohydrates was used. The sugars were added to the medium before autoclaving. In the case of fructose, filter-sterilized medium (Millex-GV Millipore; 0.22

TABLE 1. Results of in vitro culture of immature zygotic embryos with different sugars in media

Sugar*	Mean number of developing embryos per 100 embryos cultured**
Fructose	0.0 d
Glucose	11.0 c
Maltose	38.0 b
Sucrose	48.3 a

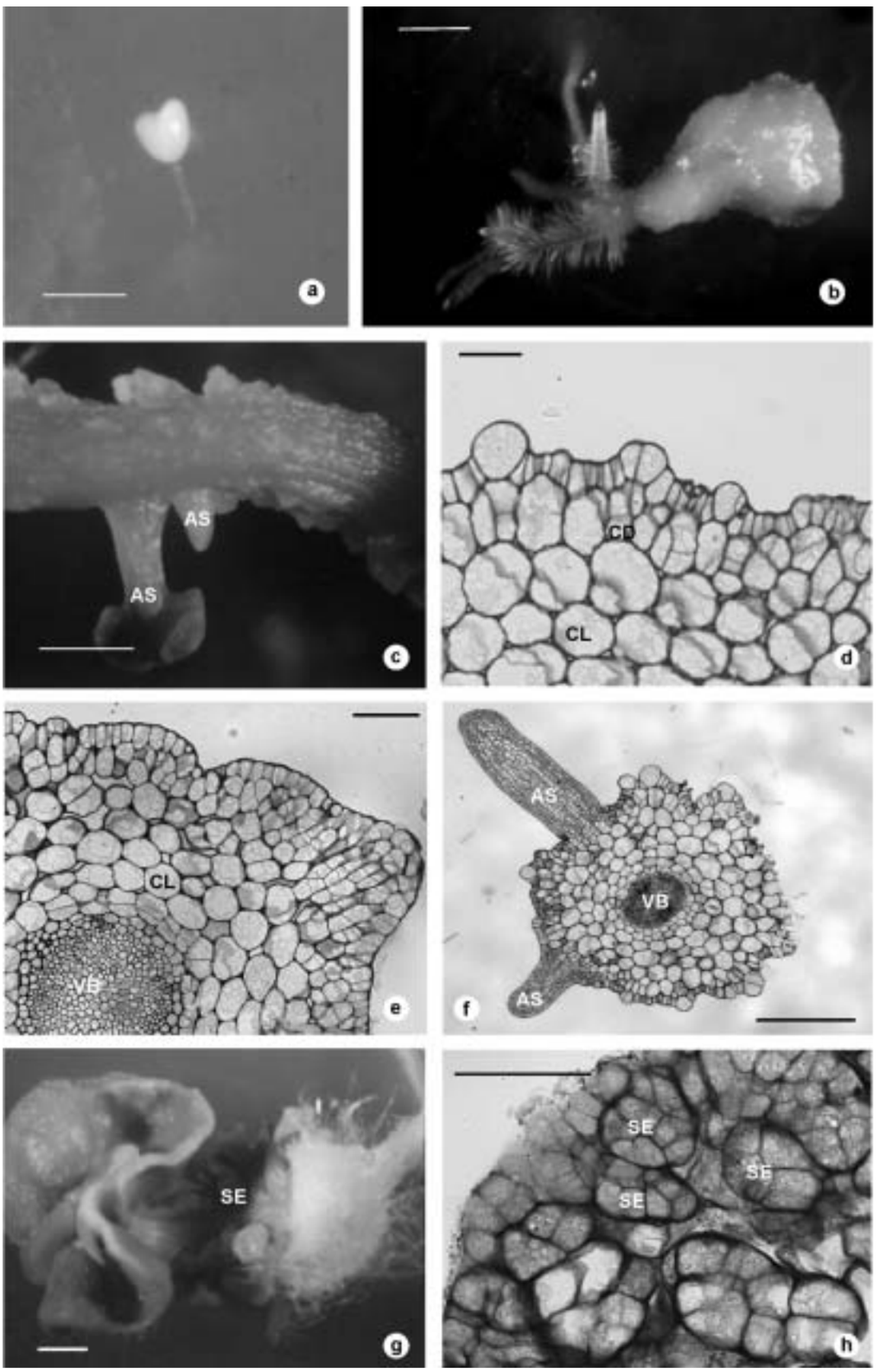
*For each sugar 6 concentrations were used.
**Means with different letters indicate significant differences by Duncan's multiple-range test ($P < 0.05$).

TABLE 2. Results of in vitro culture of immature zygotic embryos with different sugars and sugar concentrations in media

Sugar	Concentration (%)	Mean number of developing embryos*
Fructose	1	0.00 d
	2	0.00 d
	3	0.00 d
	6	0.00 d
	9	0.00 d
	12	0.00 d
Glucose	1	0.00 d
	2	0.20 cd
	3	0.80 cd
	6	1.60 cd
	9	2.00 c
	12	2.00 c
Maltose	1	0.60 cd
	2	0.40 cd
	3	0.40 cd
	6	5.60 b
	9	7.40 a
	12	8.40 a
Sucrose	1	0.20 cd
	2	0.80 cd
	3	4.00 b
	6	7.60 a
	9	8.20 a
	12	8.20 a

*Means of five replicates (Petri dishes) with 10 explants each. Means with same letters indicate no significant difference by Duncan's multiple-range test ($P < 0.05$).

Fig. 1. (**a**) Heart-stage zygotic embryo just after isolation (Bar = 0.2 mm), **(b)** Embryo after 3 weeks of culture on MS + 2% glucose, callogenesis (Bar = 0.5 mm), **(c)** Embryo after 3 weeks of culture on MS + 6% maltose (Bar = 1 mm), (**d–f)** Cross sections of the hypocotyl region of embryos cultured for 5 weeks on MS + 3% maltose, different stages of adventitious shoot formation (Bars in d,e = 0.1 mm, in f = 0.5 mm), **(g)** Embryo after 2 weeks of culture on MS + 6% sucrose, somatic embryo formation (Bar = 1 mm), **(h)** Cross section of the hypocotyl region of the embryo after 2 weeks of culture on MS + 6% sucrose, somatic embryo formation (Bar = 0.1 mm). AS – adventitious shoot; CD – cell divisions; VB – vascular bundle; CL – cortex layer; SE – somatic embryo.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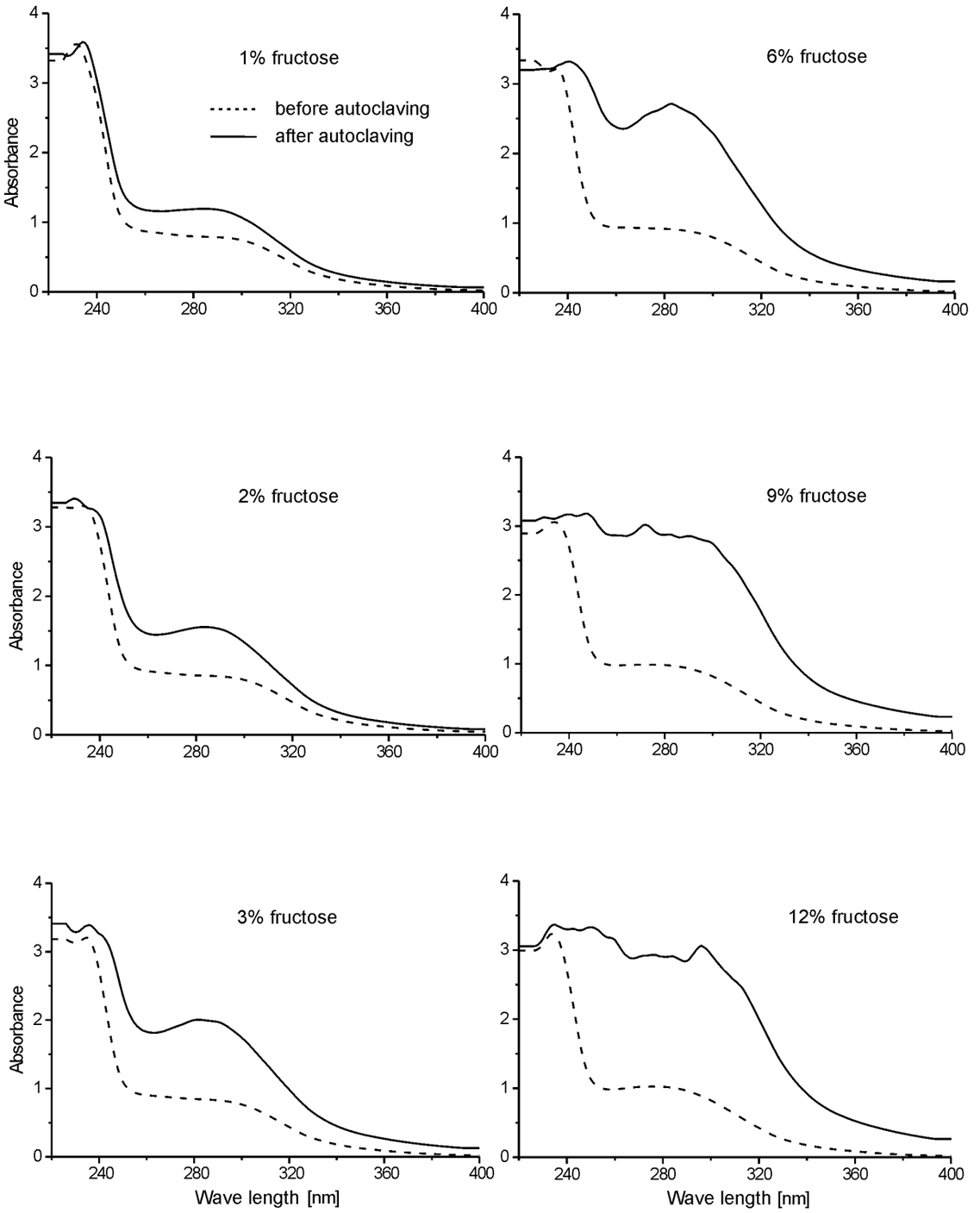

Fig. 2. Absorbance of fructose medium as a function of autoclaving.

µm) was additionally tested. To check the influence of autoclaved fructose a test culture with *Capsella bursa-pastoris* embryos was additionally made.

The media were solidified with 0.7% Difco Bacto-Agar and adjusted to pH 5.7–5.8 with 0.1N HCl or NaOH after the agar was dissolved and prior to autoclaving at 121˚C and 108 hPa for 20 min. No growth regulators were added to the media.

The embryos were cultured under a 16 h photoperiod provided by cool-white fluorescent tubes (60–90 µm photons $m^{-2}s^{-1}$) at 25 ± 3˚C. Ten embryos per Petri dish were inoculated; five replicates (Petri dishes) were used for each sugar concentration.

HISTOLOGICAL ANALYSIS

The material for sectioning was prepared by embedding tissues in Technovit 7100 (2-hydroxyethyl-metacrylate) (Heraeus Kulzer). The cultured embryos were sampled at one-week intervals from the 2nd to 5th weeks after the beginning of culture and fixed in glutaraldehyde for 24 h. Then the samples were rinsed four times in phosphate buffer (PBS) and dehydrated in a graded ethanol series (10%, 30%, 50%, 70%, 96%) for 15 min in each concentration, and kept overnight in absolute ethanol. The next day the samples were infiltrated in a mixture of absolute ethanol and Technovit in proportions 3:1, 1:1 and 1:3 (v/v) (1 h in each mixture) and stored for 12 h in pure Technovit. The resin was polymerized with the addition of hardener. The material was sectioned 5 µm thick using a rotary microtome (Microm, Adamas Instrumenten), stained with toluidine blue and mounted in Entellan (Merck).

Photographs of in vitro cultures were taken under a Zeiss Stemi SV 11 stereomicroscope equipped with an MC80 microphotographic attachment on Kodak film. Microscope sections were photographed with a Zeiss Axio Cam MRe digital camera (Zeiss Axio Vision 3.0).

SPECTROPHOTOMETRIC ANALYSIS

MS medium supplied with 1%, 2%, 3%, 6%, 9% or 12% fructose (without agar) was spectrophotometrically analyzed before and after autoclaving using a Beckman DU-640 spectrophotometer (USA). The samples were scanned at 220–400 nm.

STATISTICS

The statistical analyses were carried out using ANOVA followed by Duncan's multiple range test using the Statgraphics Plus package (Statistical Graphics Corp. U.S.A.).

RESULTS

MORPHOLOGICAL OBSERVATIONS

A total of 1200 heart-shaped stage zygotic embryos (~150 µm long) were cultured (Fig. 1a) on MS medium supplemented with sucrose, maltose, glucose or fructose at 1%, 2%, 3%, 6%, 9% or 12% sugar concentrations.

Stimulation of immature embryos for development was strictly dependent on the type and concentration of sugar in the medium (Tabs. 1, 2). Sucrose, maltose and glucose stimulated embryo development, while fructose did not at any concentration used. The frequency of developing embryos increased with the sugar concentration.

Sucrose and maltose were shown to be the best sugars for embryo development: 48.3% of the inoculated embryos showed development on sucrose, and 38.0% on maltose. On glucose-based medium only 11% were induced to develop. The differences in frequencies of developing embryos between sugars were statistically significant ($P < 0.05$).

The earliest induction of embryo growth occurred on medium with maltose (on the second day of culture). On MS containing glucose and sucrose the embryos cultured started to grow 5 to 7 days after inoculation.

Normal development and embryo germination occurred only on MS with 1% sucrose and maltose. On media with higher amounts of sugar, prolonged culture caused callogenesis (Fig. 1b) and/or organ formation (Fig. 1c–h). On MS supplied with glucose the growth of cultured embryos was completely inhibited due to abundant callus formation. On MS with maltose, callus was produced on most embryos but did not stop their growth. On sucrose-based medium, callus tissue was produced sporadically.

After two weeks of culture, organ regeneration from the hypocotyls of the embryos cultured was observed on MS with 2, 3, 6, 9 and 12% sucrose and maltose.

HISTOLOGICAL STUDY

Histological analysis showed that adventitious shoot formation was the dominant morphogenetic process in embryos cultured on media with 2% and 3% sucrose and maltose. Shoots were produced from the groups of

meristematic cells located in the subepidermal layer of the cortex (Fig. 1d–f). On media with 6% sucrose and 12% maltose, somatic embryos were formed in addition to adventitious shoots (Fig. 1g,h).

EMBRYO CULTURE ON FILTER-STERILIZED FRUCTOSE

Because embryos cultured on MS supplemented with autoclaved fructose did not show any morphogenetic response, filter-sterilized fructose at the same concentrations as in the previous experiment was used. In this case, 38% of the cultured embryos started to grow 2–3 days after inoculation. The highest frequency of developing embryos (56%) was observed on 6% and 9% fructose. On medium with 12% fructose, callogenesis occurred. As with the other sugars tested, lower concentrations of fructose (2% and 3%) induced adventitious shoot formation on the embryos.

TEST CULTURE

A test culture of heart-shaped stage zygotic embryos of *Capsella bursa-pastoris* was made to see whether the lack of morphogenetic reaction of *Brassica napus* embryos cultured on MS with autoclaved fructose is species-specific. The procedures of sterilization and embryo isolation, the culture conditions and sugar concentrations were the same as for oilseed rape embryos.

A total of 60 embryos were inoculated and none of them responded.

SPECTROPHOTOMETRIC ANALYSIS

Samples of liquid MS medium supplied with 1%, 2%, 3%, 6%, 9% or 12% fructose were measured before and after autoclaving. Spectrum analysis confirmed an absorbance rise of autoclaved fructose-based media of 260–320 nm (Fig. 2), which could correspond to the formation of furfural derivatives.

DISCUSSION

The success of embryo culture depends on the availability of an in vitro system that allows normal development of an embryo maintained in chemical and physical conditions different from inside the ovule. Generally the embryos require a higher amount of sugar than other plant tissue cultures. An 8–12% sucrose concentration has approximately the same osmotic potential as the intracellular environment of the young embryo sac. Smith's (1973) experiments on *Phaseolus vulgaris* revealed that during embryo development the osmolality of ovular sap decreases from 0.7 M at heart-shaped stage to 0.5 M at cotyledon stage. It is suggested that high medium osmolality during proembryo culture enables effective flow of different metabolites (Raghavan and Srivastava, 1982). Monnier (1995) found that the optimal sucrose concentration required for the fastest growth of embryos depended on the size of cultured embryos; for heart-shaped stage *Capsella bursa-pastoris* embryos, 12% sugar concentration was found to be optimal.

In some species, embryo development can be regulated by changing the sugar content of the medium. Lowering the sucrose concentration to 2% (58 mM) induced early germination of immature pea embryos, while higher sugar content stopped embryo development (Cook et al., 1988). In contrast, low sucrose concentration inhibited the maturation of globular and heart-shaped *Citrus aurantium* embryos (Carimi et al., 1998).

Our study demonstrated that the type and concentration of sugar have important effects on the growth of *Brassica napus* L. cv Topas heart-shaped zygotic embryos in vitro. The best carbon source for embryo culture was sucrose, followed by maltose and glucose (~50%, 38% and 11% of embryos stimulated to develop, respectively). Fructose did not stimulate embryo growth at all. The highest percentage of stimulated embryos occurred on media with 12% sucrose and 12% maltose (more than 80%). By contrast, on media with low sugar concentrations (1–3%) only ~17% embryos showed growth on sucrose and ~5% on maltose. Statistical analysis demonstrated that with sucrose and maltose the mean numbers of developing embryos were very similar on 1%, 2%, 9% and 12% sugar concentrations, but differed significantly on media with 3% and 6% sugars; the number of growing embryos was much higher on sucrose. Glucose-based medium stimulated the growth of only 20% of the embryos at the most effective sugar concentrations (9% and 12%).

High amounts of carbohydrates in the medium caused various abnormalities in embryo growth. Cultured embryos callused, and frequently organogenesis was induced from hypocotyls. To obtain normal seedlings it is necessary to transfer embryos after 2–3 days of culture onto MS with 1% sucrose or maltose.

The reaction to the carbon source may differ in different species and can be highly genotype-dependent. When the efficiency of maltose- and suc-

rose-based media is compared, one may expect different results even within species. The use of maltose instead of sucrose reduced the frequency of regeneration from immature zygotic embryos of *Pennisetum glaucum* (Oldach et al., 2001). Matthys-Rochon et al. (1998) reported different results in *Zea mays*: maize embryos developed only when the culture medium was supplied with maltose. The authors suggested that a possible explanation for the superiority of maltose in nutrition of maize embryos might be that this homodisaccharide is hydrolyzed into two molecules of glucose, while sucrose is hydrolyzed into glucose and fructose. Since fructose is thought to be less effective than glucose, it is likely that the embryo cells at first absorbed glucose. Another explanation for the inefficiency of sucrose might be that some products of sucrose degradation were toxic, as was demonstrated for barley microspores (Finnie et al., 1989).

In the present experiment we did not obtain embryo growth on fructose-based medium. Apparently fructose had an inhibitory effect on the cultured embryos. However, when medium was supplied with filter-sterilized fructose, 38% of the inoculated embryos developed. As with the other carbon sources tested, the highest number of embryos developed on 12% sugar concentration (56% developing embryos).

Why the inhibitory effect of autoclaved fructose? De Lange (1989) observed that pollen germination was stopped when fructose-based media were autoclaved. Druart and De Wulf (1993) found that furfural derivatives were products of fructose degradation after autoclaving. Our study seems to confirm this. Spectrophotometric analysis of fructose-based media showed an absorbance rise of 260–320 nm after autoclaving, which could correspond to the formation of furfural derivatives. Thus it is likely that products of fructose degradation inhibited the growth of cultured embryos.

However, data on the influence of autoclaved fructose on tissue culture are contradictory. Perhaps the reaction to fructose degradation products is species-specific.

Histological analysis of hypocotyl sections revealed that adventitious shoot formation was the dominant morphogenetic process at low sugar concentrations (2% and 3%). On media supplied with 6% sucrose and 12% maltose, somatic embryos were formed in addition to adventitious shoots.

Interesting results on the influence of sugars on immature zygotic embryos have been reported for sunflower. In this species, the sugar concentration in the medium controls regeneration. Shoots were induced at 3% sucrose concentration, and somatic embryos at 12% (Bronner et al., 1994; Jeannin et al., 1995, 1998; Jach and Przywara, 2000). The same morphogenetic reaction of sunflower embryos was observed with other carbon sources, but sucrose was the most efficient among the carbohydrates tested, including glucose, maltose and maltotriose (Charriere et al., 1999).

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