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INFLUENCE OF SUGARS ON ISOLATED MICROSPORE DEVELOPMENT IN MAIZE (*ZEA MAYS* L.)

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Carbon source and osmotic pressure are known to be of great importance in cultures in vitro. We compared the effectiveness of sucrose, maltose and mixtures of glucose with fructose in androgenic culture of isolated maize microspores. Also we examined changes in media during culture, focusing on osmotic pressure and sugar composition. The results suggest that osmotic pressure and kind of sugar have a great influence on androgenesis induction but much less influence on the number of macroscopic structures formed. In media containing sucrose the osmotic pressure rises significantly due to sucrose hydrolysis. In other media tested, changes in osmotic pressure are much smaller or not significant. These results suggest that the factors involved in androgenesis induction are different from those responsible for macroscopic structure formation. Changes in sugar composition and osmotic pressure increases in sucrose-based medium might decrease the effectiveness of androgenesis in maize microspore culture.

Key words: Androgenesis, maize, *Zea mays*, isolated microspores, pollen embryogenesis, sugars, osmotic pressure.

INTRODUCTION

Sugars are used for in vitro culture both as a carbon source and as an osmotic pressure regulator. Sucrose is the most commonly used sugar in plant cell and tissue cultures, including androgenic cultures of anthers or isolated microspores. However, in many cases, including cereal microspore cultures, other sugars have been found to be comparable or even more effective than sucrose. The beneficial effect of maltose has been demonstrated by many authors (Hunter, 1987; Finnie et al., 1989; Tanner et al., 1990; Olsen, 1991; Scott and Lyne, 1994a,b; Lentini et al., 1995). One possible explanation of the superiority of maltose in these cultures is that some products of sucrose degradation inhibit microspore development (Finnie et al., 1989). Several authors have reported that glucose and/or fructose produced in sucrose hydrolysis may decrease the efficiency of androgenesis in wheat (Navarro-Alvarez et al., 1994) and barley (Scott and Lyne, 1994a,b). By contrast, in maize anther culture these sugars are believed to be beneficial (Buter et al., 1993)

Besides the sugar products released into media as a result of enzymatic sucrose degradation, some toxic substances and pH changes have been observed in autoclaved media containing sucrose (Owen et al., 1991; Buter et al., 1993; Lemos and

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Baker, 1998). The presence of sucrose may also increase ethylene production in cultured tissues, which might be a cause of mortality in the explants (Adkins et al., 1990; Ghamemi et al., 1994; Lentini et al., 1995).

Finally, the effects of sugars may differ in various species and can be highly genotype-dependent. When the efficiency of maltose- and sucrose-based media is compared, one can expect different results, as has been demonstrated for wheat (Trottier et al., 1993).

The osmotic pressure in the medium, regulated mainly by sugars, is of critical importance in zygotic embryo development. In early stages, high osmotic pressure is required for embryo development, but at later stages the sugar concentration should be lower (Monnier, 1995). Osmotic pressure is also an important factor in the development of structures derived from microspores. Wei et al. (1986) compared the influence of different sucrose concentrations in the medium on callus formation in microspore culture of *Hordeum vulgare* and found a 6% concentration to be the best. Hoekstra et al. (1993) showed that high osmotic pressure in barley microspore culture resulted in a higher frequency of green plants produced. Van Bergen et al. (1998) improved the efficiency of both embryo-like structures (ELS) and plant production in barley microspore culture by increasing the amounts of mannitol and calcium and thereby the osmolality of the medium.

Changes in osmotic pressure during microspore culture and their influence on developing ELS have not been examined adequately.

This experiment investigates changes in osmotic pressure and sugar composition during androgenic culture of isolated maize microspores, with a view to increasing the still very low number of androgenic structures produced. Increased efficiency of culture would facilitate fundamental research on androgenic development.

MATERIALS AND METHODS

DONOR PLANTS

The experiments used androgenic maize hybrid DH5 × DH7 (Barloy et al., 1989) kindly donated by M. Beckert (INRA station, Clermont-Ferrand, France). The donor plants were grown in a growth chamber at 24/20 ± 20°C (day/night) with 16 h illumination provided by 400 W high-pressure sodium lamps (1800 W/m^2). Photosynthetic photon flux density was 880 μE m^{-2} s^{-1} measured at the top of the plants. The plants were watered daily with a nutritive solution. Tassels were harvested just before emergence from the leaf whorl. Prior to culture the tassels were wrapped in aluminum foil and pretreated for 7 to 21 days at 7°C before microspore isolation.

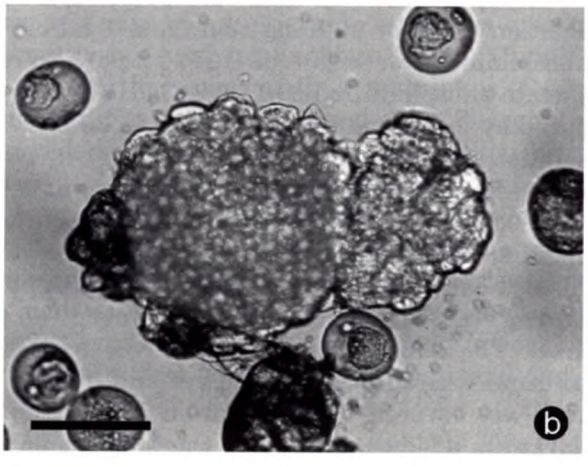
MICROSPORE ISOLATION AND CULTURE

Microspore isolation

Microspore isolation and culture techniques were based on the protocol given by Gaillard et al. (1991) with several changes. Before isolation, Alexander's (1969) staining method was applied to identify the parts of the tassels where microspores between the late uninuclear and early bicellular stages were present. These parts of the inflorescences were cut off and sterilized in 0.5% (w/v) sodium hypochlorite solution for 15 min and then rinsed three times in sterile distilled water. The sterile water and media were stored at 7°C. After sterilization the spikelets were removed from the tassels and collected in 50 ml tubes with ~25 ml isolation medium (ISO_S) for standard culture. Then the spikelets were pulverized at 8000 rpm for 3 sec with a precooled dispersing tool (Ultra Turrax T25 with an N18-G shaft). The slurry was passed first through a stainless steel sieve (pore size 100 μm) to remove large debris, and the microspore fraction was retained on a second filter (pore size 50 μm). The microspores were washed thoroughly with ISO_S and centrifuged (65 g, 3 min). The pellet containing microspores was resuspended in ISO_S (5 ml), loaded in a mixture of Percoll (1.5 ml) and ISO_S medium (3.5 ml), and centrifuged at 225 g for 3 min. The dense yellow layer that appeared between the deposit and the ISO_S medium was carefully removed, suspended in S150 medium, and centrifuged (100 g, 3 min). The live microspores floated and formed a ring or thin layer at the top of the tube. Floating microspores were removed, mixed with S30 medium, and centrifuged (100 g, 3 min). Finally the pellet containing isolated microspores was resuspended in culture medium, and viability and density tests were performed, and the microspores were suspended in the culture medium to adjusted concentrations of 50,000 or 70,000 microspores/ml.

Fig. 1. Structures observed in culture sucrose-based medium (S). (**a**) Multinuclear structures after 7 days (UV-DAPI staining), (**b,c**) Multicellular structures after two weeks of culture (UV-DAPI staining + visible light), (**d**) Macroscopic structures after six weeks of culture. Bars in Fig. 1a–c = 100 μm, in Fig. 1d = ~1 mm.


a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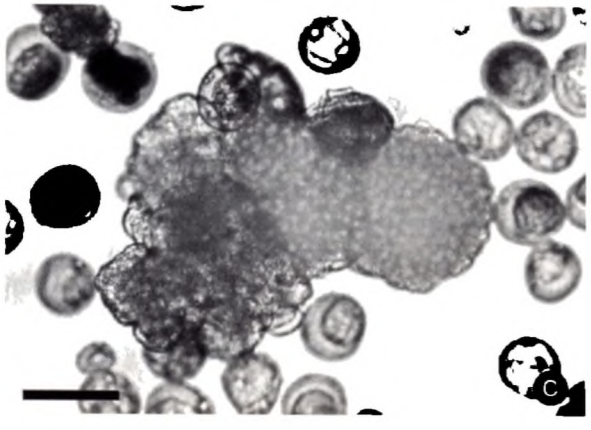
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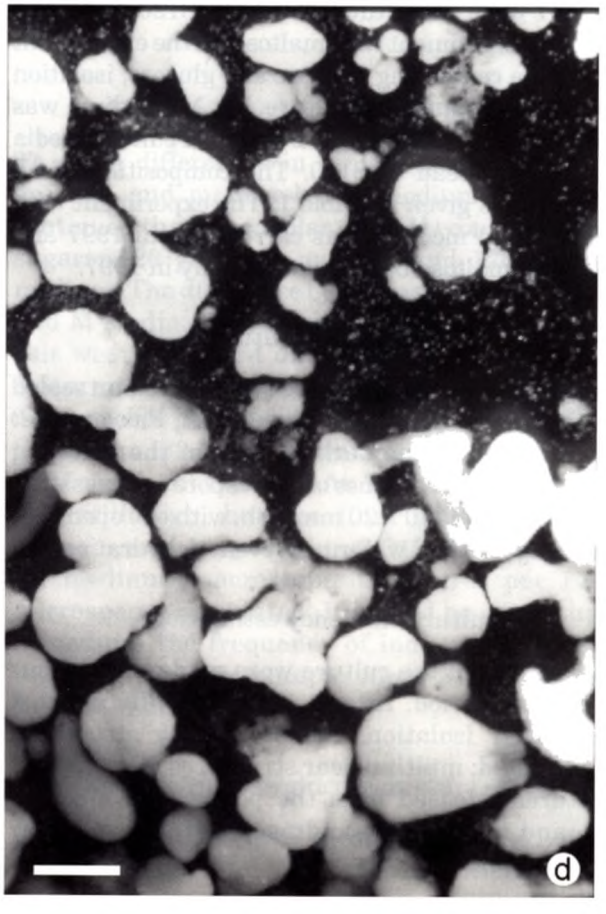
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TABLE. 1. Media used for microspore culture

Ingredients	Media										
	ISO_S	S120	S150	S30	ISO_M	M126	M157	M31	Mt	FG1	FG2
Macroelements* [ml]	50	100	100	100	50	100	100	100	100	100	100
MS microelements [ml]	0.5	1	1	1	0.5	1	1	1	1	1	1
MS FeEDTA [ml]	5	5	5	5	5	5	5	5	5	5	5
Sucrose [g]	50	120	150	30	–	–	–	–	–	–	–
Maltose [g]	–	–	–	–	52	126	157	31	–	–	–
Fructose [g]	–	–	–	–	–	–	–	–	–	31.8	63.2
Glucose [g]	–	–	–	–	–	–	–	–	–	31.8	63.2
Mannitol [g]	–	–	–	–	–	–	–	–	16	–	–
Myo-Inositol [g]	1	0.1	0.1	0.1	1	0.1	0.1	0.1	0.1	0.1	0.1
Glutamine [g]	1	1	1	1	1	1	1	1	1	1	1
Serine [g]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
MOPS [g]	2.3	–	–	–	2.3	–	–	–	–	–	–
H_2O adjust to:	1000 ml	1000 ml	1000 ml	1000 ml	1000 ml	1000 ml	1000 ml	1000 ml	1000 ml	1000 ml	1000 ml
pH	7.0	5.8	5.8	5.8	7.0	5.8	5.8	5.8	5.8	5.8	5.8
Vitamins** [ml]	10	10	10	10	10	10	10	10	10	10	10
Sterilization	A	F	A	A	A	F	A	A	A	F	F

*YP macroelements (Ku et al., 1978).
**Strauss (1960); A – autoclaved; F – filtration 22 μm.

Different carbon sources were tested in microspore culture: sucrose, maltose, fructose and glucose. For isolation and culture, ISO_M, M126, M157 and M31 were substituted for ISO_S, S120, S150 and S30 in the experiment with maltose. In the experiment with media containing fructose and glucose, isolation was done as in standard culture, but Mt medium was used instead of S30; also, FG2 and FG1 culture media were used instead of S120. The composition of all media used is given in Table 1. The experiment with sucrose-based medium was carried out in 1997 and 2000; with maltose-based medium only in 1997.

Microspore culture

The microspores were cultured in 60 × 15 mm sealed Petri dishes (4 ml suspension per dish, Falcon 3002) and incubated in the dark at 28°C in the following arrangement: three dishes of microspore cultures were placed in a large 140 × 20 mm dish, with one open dish containing distilled water to prevent dehydration.

Culture efficiency estimation

Observations of the culture were made throughout the culture period. After 1, 2, and finally about 6 weeks after isolation, the developing structures were counted: multinuclear structures after 7 days, structures released from the pollen wall after 14 days, and macroscopic androgenic structures after ~40 days (Fig. 1).

DAPI STAINING

The structures occurring in the culture at days 7 and 14 of culture were stained with DAPI. The DAPI solution used for staining contained 1 ml citrate/phosphate buffer with 5 μl DAPI stock (5 $\mu g \cdot ml^{-1}$). Citrate/phosphate buffer (0.1/0·2M, pH 4.0) was prepared by mixing citric acid (0.63 g) in 30 ml water solution with $Na_2HPO_4 \cdot 12H_2O$ (1.43 g) in 20 ml water solution. The culture suspension was mixed and ~0.5 ml was drawn into a pipette with a wide opening and transferred to an eppendorf tube. Then 1 ml distilled water was added and, after mixing, the suspension was centrifuged (65 g, 3 min). The supernatant was removed and DAPI solution was added to the pellet and mixed. The tube was covered with aluminum foil and kept in darkness at least 1 h. Then the suspension was mixed and observed under UV with a Nikon Optiphot-2 microscope.

OSMOTIC PRESSURE MEASUREMENTS

The Petri dishes with the examined cultures were tilted for ~10 min. When the microspores and developing structures settled on the bottom of the dish, a 400 μl sample of the medium from the top of the culture was drawn into an eppendorf tube. Osmotic pressure was measured for three probes per examined culture with an OSMOMAT 030 osmometer. At least three replicates were measured.

TABLE 2. Efficiency of maize microspore culture on media with various carbon sources. S – sucrose; F – fructose; G – glucose; M – maltose

Sugar	Mean number of developing androgenic structures/10,000 microspores		
	7 days	14 days	final yield
	Experiment 1997		
S	82.3 ± 40.1	27.3 ± 10.6	2.0 ± 1.0
M	4.8 ± 2.5	6.8 ± 2.8	2.9 ± 1.9
	Experiment 2000		
S	45.3 ± 22.1	15.0 ± 7.1	1.4 ± 0.5
FG1	29.5 ± 18.4	16.4 ± 6.5	0.8 ± 0.4
FG2	2.2 ± 3.8	< 0.8	0.5 ± 0.2

SUGAR ANALYSES

To determine the quantity and type of carbohydrates in the culture media, the samples were analyzed with a Dionex HPLC system, Series 4500i (Sunnyvale, CA), equipped with an elutant degas module, a BioLC gradient pump (GPM-II), liquid chromatography module (LCM-2) and pulse amperometric detector (PAD-II). The samples were separated by anion exchange chromatography at a flow rate of 1ml/min on an analytical column (Carbo PacPA1, 4 × 250 mm) equipped with a precolumn (Carbo PacPA1:10-32) and a 50 µl sample loop. Reagents of high grade and ultrapure water were used throughout. Elution was done at room temperature with ultrapure water for 17 min. NaOH (300 mM) was added to the postcolumn effluent at a flow rate of 1.6 ml/min using a Dionex Autiion reagent pump to optimize detection and minimize baseline drift. Carbohydrates were detected using a PAD with a gold working electrode (Dionex). The pulse potentials and durations were E1 = +0.05V (t1 = 120 ms), E2 = +0.6V (t2 = 300 ms), and E3 = -0.8V (t3 = 300 ms). The PAD response was in millivolts and the response time was set to 1 sec. Chromatograms were recorded and integrated using Dionex AI 450 software. The monosaccharides were identified according to their retention times, and their amounts were determined relative to commercial standards (Sigma).

RESULTS

CULTURE EFFICIENCY

To estimate culture efficiency, developing androgenic structures were counted after 1, 2 and 6 weeks of culture in all media tested. The criteria for identifying developing structures were as follows: at day 7 of culture, structures containing at least 9 nuclei ("induced microspores"); at day 14, structures with ruptured pollen walls; at 6 weeks, macroscopic androgenic structures (MAS) at least ~1 mm in length (Fig. 1). The results are presented in Table 2.

Sucrose-based medium (S medium)

Microspore culture in medium containing sucrose at 120 g/l concentration was regarded as the standard culture. In all cultures there were noticeable differences in the number of developing androgenic structures at each stage of culture, between different cultures (i.e., Petri dishes) and between experiments. Generally the number of growing structures was very low compared to the number of isolated microspores, and gradually decreased during culture (Tab. 2).

After 7 days of in vitro culture the mean number of "induced" microspores per 10,000 microspores between two experiments run in two different years was ~63, whereas five weeks later it was dramatically lower; only ~2 macroscopic ELS developed per 10,000 microspores inoculated (~12 structures per dish).

Maltose-based medium (M medium)

To avoid differences in osmotic pressure between sucrose- and maltose-based medium, both media contained the same molar (0.35 M) concentration of sugars; 120 g/l for S medium and 126 g/l for M medium. The difference between standard-culture S and M media in their ability to induce androgenesis was significant during the first two weeks of culture. After 7 days the mean number of induced microspores per 10,000 was many times higher in S medium (82.3 in S medium and 4.8 microspores in M medium) (Tab. 2). At 14 days the difference was still high, but the final yields in the two cultures were very similar, 2.0 (S medium) and 2.9 (M medium) macroscopic structures per 10,000 microspores inoculated. It should be noted that in S medium the frequency of induced microspores reaching the macroscopic stage was much lower than in M medium.

Media with fructose/glucose mixture (FG media)

Osmotic pressure in S medium increased significantly during culture. We suspected that this phe-

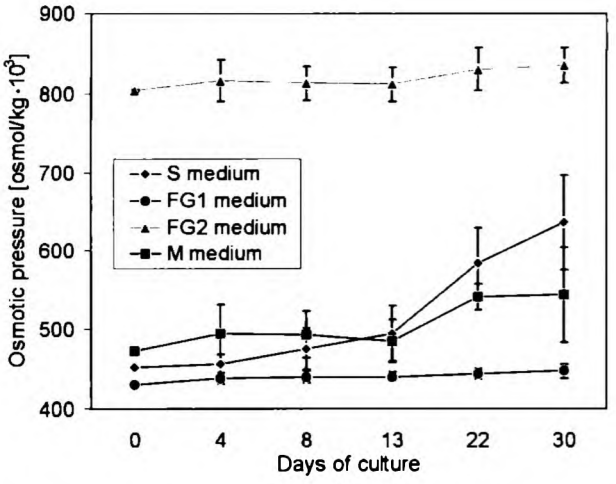

Fig. 2. Osmotic pressure changes in four media based on sucrose (S), maltose (M), or two concentrations of fructose and glucose (FG1, FG2).

nomenon was caused mainly by hydrolysis of sucrose to glucose and fructose. Because osmotic pressure is a critical factor for embryogenesis, in our experiments we applied media containing glucose and fructose in a 1:1 proportion (as in sucrose). We believed that the osmotic pressure in medium containing glucose and fructose instead of sucrose would be osmotically more stable since these sugars are monosaccharides; qualitatively the sugar composition would be the same.

The FG1 medium contained the sugars in a molar concentration similar to the molar sucrose concentration in S medium, while in the FG2 the concentration of glucose and fructose was twice the sucrose molar concentration in S medium. Thus the FG1 medium simulated the osmotic pressure expected at the beginning of culture in S medium. The FG2 medium simulated the osmotic pressure expected under total hydrolysis of all sucrose content in S medium.

The most significant feature of FG2 medium was its very low efficiency in inducing androgenesis. Only ~2 microspores per 10,000 started development. The mean number of macroscopic structures was also the lowest of all media tested (~0.5 structures developed per 10,000 microspores). However, the fraction of induced microspores that continued development and finally reached macroscopic size was higher in FG2 medium than in S and FG1 media.

The final efficiency of FG1 medium was between that of S and FG2 media (0.8 ELS/10,000 microspores), although the mean number of induced microspores was lower than in standard medium (Tab. 2).

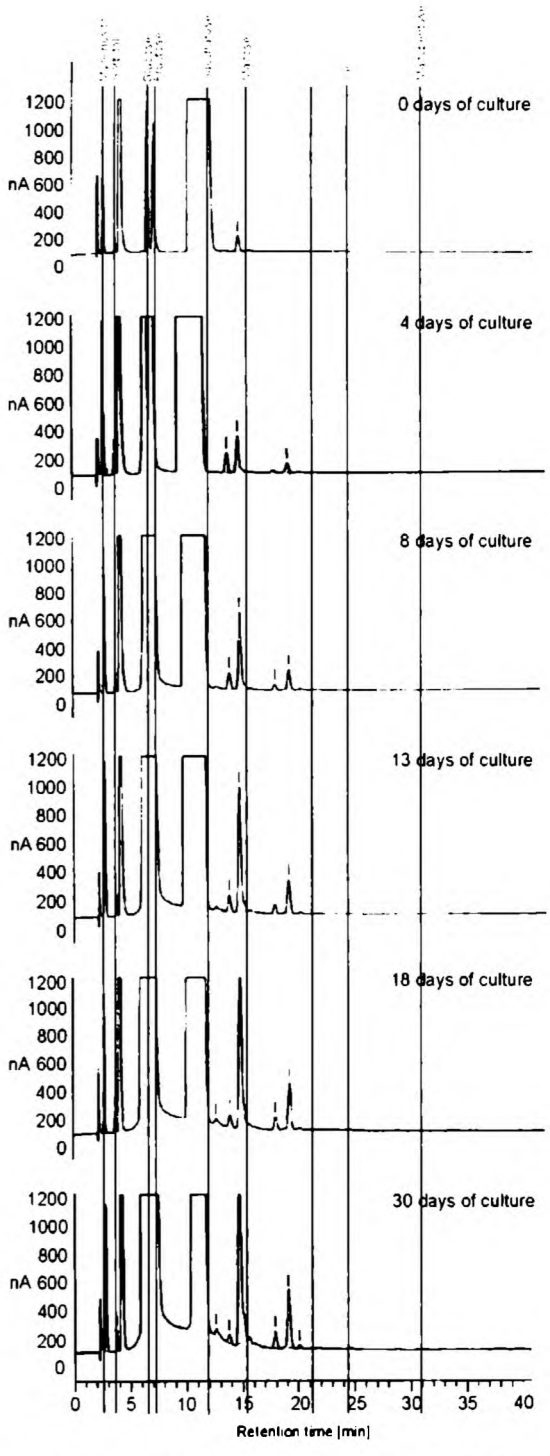

Fig. 3. Changes in sugar composition in media during culture (high-performance anion-exchange chromatography-pulsed amperometric detection). Sucrose-based medium (S).

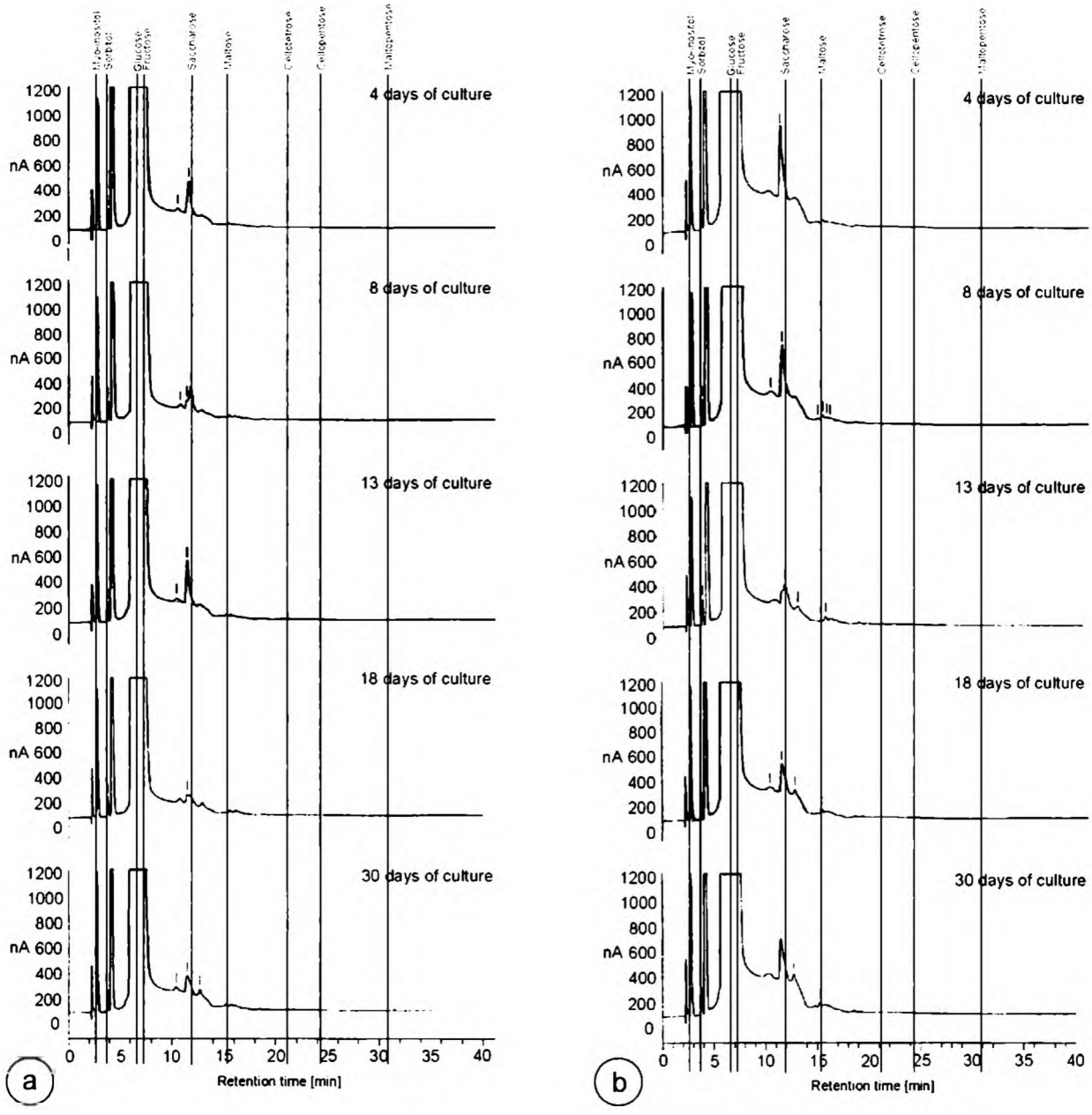

Fig. 4. Changes in sugar composition in media during culture (high-performance anion-exchange chromatography-pulsed amperometric detection). (**a**) Fructose- and glucose-based medium (FG1); molar concentration similar to sucrose-based medium, (**b**) Fructose- and glucose-based medium; molar concentration twice that of sucrose-based medium (FG2).

MEDIUM CHANGES DURING THE CULTURE

Osmotic pressure

Osmotic pressure was measured during 30 days of microspore culture in four media containing different carbon sources. The results are presented in Figure 2.

The measurements revealed distinct differences in osmotic pressure changes depending on the sugar used. The biggest changes were found in sucrose-based medium, in which the osmotic pressure increased more than 40% within one month. Growth was slower during the first two weeks than in the remaining period of culture.

In the medium containing maltose (M medium) the total increase of osmotic pressure was less than in standard culture (~14%). In media containing a mixture of fructose and glucose (FG media), the osmotic pressure was very stable during the whole period of culture, despite great differences in the initial pressure related to the amount of sugar (804 and 430 osmol/kg·10^3 for FG2 and FG1, respectively).

SUGAR COMPONENTS IN THE CULTURE

Changes in medium composition were examined in S and FG media. The results revealed dramatic changes in S medium during culture, whereas changes observed in FG media were far less marked (Figs. 3, 4).

Sucrose-based medium

Although neither glucose nor fructose were added to the solution when the standard medium was prepared, both sugars were found in the medium, even at the beginning of culture: 0.01 mg/ml for both sugars. At that time the sucrose concentration was much higher and the peak for sucrose was significantly wider. The exact concentration of sucrose is not known because it was too high for the method used, so the changes in sucrose concentration were estimated from the visible field of the peak. During the following days of culture the concentration of glucose and fructose increased, and after eight days the fructose and glucose peaks were represented as one peak only (Fig. 3).

The diagrams presented indicate that the increase in fructose and glucose concentration was accompanied by a decrease in the amounts of su-crose. Finally the sucrose peak was much sharper than at the start of culture, but the peak representing fructose and glucose was distinctly wider than previously, and wider than the sucrose peak at that time.

Besides the changes in sucrose, fructose and glucose concentration, changes in the content of other sugars were also observed. From the beginning of culture a small peak with retention time 14.8 min was visible. At the fourth day of culture, peaks with 13.8 and 19.1 min retention times appeared, and a peak with retention time 18 min at the eighth day. Later these peaks rose but the substances indicated by them were not identified.

Fructose and glucose-based media

The qualitative changes observed in FG media were very similar and were much less distinct than in S medium (Fig. 4a,b). In both media, the peak containing sucrose was always present from the fourth day of culture. During evolution of the culture this peak decreased, indicating diminution of sucrose amount. The main difference between FG1 and FG2 media regarded the amounts of glucose and fructose. The FG2 medium was also enriched in sucrose. Near the sucrose peak, two small peaks of unidentified substances and a small peak probably indicating the presence of maltose were also seen.

DISCUSSION

The environmental conditions of culture may have a great influence on the development of the embryo. Osmotic pressure is a very important factor in embryo formation, and uncontrolled changes in it may disturb this process. In cultures in vitro, androgenic structures and zygotes are maintained in a nutrient medium whose chemical and physical conditions differ from those within the ovule. For example, in standard procedures the medium is not changed for weeks, and osmotic pressure may change significantly. In natural conditions the embryo is surrounded by endosperm which, besides playing nutritive and protective roles, also regulates osmotic conditions (Brink and Cooper, 1947; Smith, 1973; Vijayaraghavan and Prabhakar, 1984; Becraft et al., 2001; Olsen, 2001). In culture, rapid degradation of sucrose to glucose and fructose during culture may significantly increase osmotic pressure in the medium.

It is well known that a high sugar concentration in the medium may cause various structural deformations in cultured embryos. This phenomenon should be considered when the structural aspects of androgenesis are discussed and culture procedures are elaborated. After they reach the transitional stage, androgenic embryoids probably should be transferred to a double-layer system, which has proved very suitable for embryo and embryoid culture in different species (Liu et al., 1993; Iglesias et al., 1994; Monnier, 1995; Góralski and Przywara, 1998).

An important feature of our maize microspore culture was its low efficiency; only a tiny proportion of the microspores induced to divide continued development to the macroscopic stage. One way to improve culture efficiency is to change the media components. In our experiments we focused on the influence of carbon source and changes in osmotic pressure, two factors found to be important to the development of zygotic and androgenic embryos or embryoids (Wei, 1986; Hoekstra et al., 1993; Liu et

al., 1993; Monnier, 1995; Góralski and Przywara, 1998; Matthys-Rochon et al., 1998; Van Bergen et al., 1998).

It is striking that despite the great differences in the frequency of microspore induction observed after seven days of culture, the final yield was similar in all media tested; only a very small number of induced microspores reached macroscopic size. It raises the question of whether the factors responsible for microspore induction efficiency and macroscopic structure formation are independent of each other. From the data it appears that they are indeed independent.

In our study, osmotic pressure changes in the culture were strictly connected with the sugar used. The most noticeable changes were observed in sucrose-based medium. The medium containing maltose displayed much smaller changes in osmotic pressure, and in the media containing glucose and fructose the changes were very small.

The differences in osmotic pressure values were connected with chemical variations observed in the media during culture. In FG media the changes in sugar composition were much less marked than in S medium. The osmolality increase in the sucrose-based medium was due mainly to hydrolysis of sucrose to glucose and fructose, which altered the physical as well as the chemical conditions of the culture.

The S and FG1 media proved most efficient in microspore induction, but in both cases only a small fraction of the induced microspores could develop into ELS. The similar effects of these two media may have resulted from early hydrolysis of the disaccharide sucrose to the monosaccharides fructose and glucose. In the FG media some amount of sucrose was detected, so all three of these sugars were available to the developing structures, though in different proportions. It is possible that the presence of glucose and fructose inhibits the development of androgenic structures, especially in early stages of culture development. Sucrose-based medium, containing low amounts of fructose and glucose, seems more efficient than FG1 medium.

The osmotic pressure values were similar at the beginning of culture in S and FG1 media, but later the osmotic pressure increased significantly in sucrose-based medium, while it did not change in FG1 medium. As mentioned earlier, the final efficiency was similar in S and FG1 media, so the osmotic pressure apparently was not critical to the number of macroscopic structures formed. On the other hand, the efficiency of microspore induction differed very substantially between FG1 and FG2 media. Because these media contained the same sugars and differed only in sugar concentration, probably microspore induction was strongly influenced by osmotic pressure in this case.

Our results with maltose-based medium showed that this sugar also induced androgenesis and supported the development of the structures produced. The induction efficiency of maltose was much lower than that of S and FG1 media, but the final yield was very similar in all media.

Besides sugar type and osmotic pressure, ELS production might have been affected by other factors connected with culture conditions. For example, the high mortality of induced microspores might have been caused by toxic substances released into the medium by the growing structures, or a lack of compounds essential for development. If so, the highest mortality should occur in cultures containing a high number of growing structures at early stages of culture. Although our results seem to support this suggestion, it needs to be explained why the final yield was similar in all media. The factors present in the medium that participate in ELS growth stimulation should be identified.

We have demonstrated that the mean numbers of macroscopic ELS produced were very similar in all media tested. Apparently the final yield in androgenic maize microspore cultures did not depend on the culture conditions studied. It seems that only a small fraction of the microspores are potentially able to complete androgenesis. This may be due to the gene composition (the genotype used is a hybrid of two different genotypes) or gene expression pattern. An examination of this hypothesis requires more experiments, including microspore segregation, examination of ELS structure, and studies of gene expression in different types of microspores and androgenic structures.

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