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Induction of microspore-derived embryos of *Brassica napus* L. with polyethylene glycol (PEG) as osmoticum in a low sucrose medium

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Abstract Isolated microspores of *Brassica napus* were cultured on high concentrations of mannitol or polyethylene glycol (PEG 4000), with only a very limited amount of sucrose (0.08–0.1%) provided as carbohydrate source in the medium. While microspores cultured on high mannitol yielded no embryos and no embryogenic cell divisions were observed, microspores on high PEG developed into embryos within 2 weeks, and the embryo yield appeared comparable to that of the sucrose control. When placed under light, PEG embryos quickly changed color from yellow to dark green, while sucrose embryos first remained yellowish and then slowly changed color to pale green. Three-week-old PEG embryos were strikingly similar to immature zygotic embryos developed *in ovulo*, dissected at 14–15 days post-anthesis (DPA), while sucrose embryos differed from the latter in the size and shape, color and morphology of their cotyledons. These results demonstrate that in microspore embryogenesis of *Brassica napus*: (1) the level of metabolizable carbohydrate required for microspore embryo induction and formation appears to be substantially less than commonly used amounts, (2) sucrose as an osmoticum can be replaced with high-molecular-weight PEG. With further improvement the new method described here might be suitable for other *Brassica* species and would have a great potential application in breeding programs.

Key words Microspore-derived embryos · *Brassica napus* L. · Sucrose · Mannitol · Polyethylene glycol (PEG)

Abbreviations *PEG* Polyethylene glycol · *MD* Microspore-derived · *DPA* Days post-anthesis

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Introduction

Since haploid embryo formation from isolated microspores in *Brassica napus* was first reported by Lichter (1982), remarkable progress has been made in developing an efficient system for production of microspore-derived (MD) embryos in this rapeseed species (Chuong and Beversdorf 1985; Pechan and Keller 1988; Telmer et al. 1992). The high frequency of haploid embryos obtained using this system has made it a suitable tool for selection in oilseed rape breeding programs (Keller et al. 1987; Polsoni et al. 1988) and for various genetic manipulations (reviewed by Huang 1992).

Commonly used protocols for microspore and anther culture in *Brassica* include sucrose (usually at concentrations of 8–17%) as an optimal and essential osmotic and nutritional component required for haploid embryo induction (Keller et al. 1975; Lichter 1981, 1982; Dunwell and Thurling 1985; Baillie et al. 1992; Ferrie et al. 1995). In *Brassica campestris* anther culture, sucrose was superior to other compounds such as mannitol and sorbitol which failed to induce embryogenic division of pollen (Hamaoka et al. 1991). However, excess sucrose provided in the liquid medium could lead to substantial sugar uptake by MD embryos, resulting in abundant starch accumulation as observed by Rahman (1993) and Yeung et al. (1996). Moreover, zygotic embryos dissected from ovules at the torpedo stage and cultured on sucrose medium for 2 weeks accumulated starch grains in a similar manner (Rahman 1993).The only successful attempt to use sucrose-reduced or sucrose-free medium in pollen embryogenesis was reported in *Nicotiana*, when an initial few-day-long starvation period was followed by transfer to a carbohydrate-rich medium (Imamura et al. 1982; Kyo and Harada 1985).

In the study presented here we attempted to uncouple osmotic from nutritional requirements for embryo induction. Therefore, non-metabolizable osmotica such as mannitol and polyethylene glycol (PEG) were introduced as an alternative to sucrose, with only a very limited amount of sucrose provided as carbohydrate source in the medium.

Mannitol, which is commonly used as an external osmoticum, can easily penetrate cell walls, but the plasmalemma is considered to be relatively impermeable to it (Rains 1989), while high-molecular-weight PEG 4000 is too large to penetrate cell walls (Caripta et al. 1979; Rains 1989). Polyethylene glycol is a neutral polymer, highly soluble in water and nontoxic. In solution, PEG creates a more negative water potential, facilitating a water-restricted environment (Rains 1989). A high concentration of mannitol has been used to induce osmotic stress in MD embryos of *Brassica napus* (Huang et al. 1991) and in microspore (A. M. R. Ferrie, personal communication) and anther culture of *Brassica campestris* (Hamaoka et al. 1991). Also, it has been used in the pretreatment for barley anther (Roberts-Oehlschlager and Dunwell 1990) and pollen culture (Wei et al. 1986), tobacco anther (Imamura and Harada 1980) and pollen culture (Imamura et al. 1982) and in wheat microspore culture (Hu et al. 1995). The application of PEG has been attempted in combination with sucrose in barley anther culture (Thörn 1988) and in microspore culture in combination with Ficoll and glucose (Kao 1993). Also, it has been used as an osmotic agent of choice in the maturation of somatic embryos of conifers (Attree et al. 1991).

The objective of the experiments described here was to determine whether the initiation and formation of MD embryos of *Brassica napus* are affected by extremely reduced sucrose level in a mannitol- or PEG-mediated low water potential environment.

Material and methods

Growth conditions of donor plants

Plants of *Brassica napus* L. cv 'Topas' line 4079 were grown in a growth chamber at a 20°/15°C day/night temperature regime and 16-h day illumination provided by VHO (very high output) Sylvania cool-white fluorescent lamps. Prior to bolting, the temperature was lowered to 10°/5°C (day/night), and plants were maintained at this regime with water and nutrients provided twice per week as 0.35 g l^{-1} of 15-15-18 (N-P-K) nutrient solution (Ferrie and Keller 1995). Another set of plants was grown at a 22°/15°C day/night temperature regime and 16/8-h (day/night) photoperiod, and hand-pollinated flowers were tagged to indicate days after anthesis (DPA). Immature zygotic embryos were dissected from developing ovules (14 and 15 DPA) and used for comparison with MD embryos.

Isolation of microspores

A mixed population consisting of microspores from mid- and late-uninucleate stages to young bicellular pollen was isolated from the flower buds using a protocol for *Brassica napus* modified from that described by Ferrie and Keller (1995). For each treatment, 12 surface-sterilized buds (3.6–4.2 mm in length) were macerated in 5 ml half-strength B5 washing medium (Gamborg et al. 1968) containing 13% sucrose (w/v) in 50-ml beakers. The crude suspension was filtered through 44-μm Nytex nylon mesh, both beakers and meshes were rinsed, and a total of 20 ml was collected into 50-ml centrifuge tubes and centrifuged at 150 g for 3 min. The pellets were resuspended in 5 ml of washing medium, the washing procedure was repeated twice, then the final supernatant was carefully pipetted out, leaving the pellet in approximately 0.3–0.4 ml of washing medium. Final resuspension was done by adding filter-sterilized half-strength NLN medium (Lichter 1982) containing 13% (w/v) sucrose as control treatment; in the other two treatments, half-strength NLN sucrose-free medium with 8% (w/v) mannitol or 25% (w/v) PEG 4000 (Fluka Chemika) was added, respectively. Therefore, both half-strength NLN-mannitol and -PEG medium contained only about 0.08%–0.1% sucrose that remained from the washing medium. Aliquots of 10 ml of microspore suspension were plated into 100×15-mm sterile petri dishes, with an approximate plating density of 40,000 microspores/ml. Plates were first incubated at 35°C for 1 h, followed by 33°C for the next 14–18 h and then placed on a shaker (50 rpm) in the dark at 24°C for 14 days. During the first 8 days in culture, PEG and mannitol media were gradually diluted by adding an adequate volume of a simple half-strength NLN medium containing no sucrose, mannitol or PEG to the plates, to give a final concentration of approximately 22% PEG and 7% mannitol. The concentration of sucrose in the control treatment was lowered the same way to a final concentration of 11%. After 2 weeks, plates with MD embryos were placed under light [16/8-hour (day/night) photoperiod] at 24°C for an additional week. Experiments dealing with mannitol as osmoticum were repeated three times with five replicas, while experiments dealing with PEG and sucrose as a control were repeated at least ten times with a minimum of three replicas.

Results and discussion

In order to meet specific osmotic requirements for microspore embryogenesis and to avoid potential abundant sugar uptake by embryos, we applied mannitol and PEG as alternative osmotic agents in concentrations that provided an osmotic potential similar to that created by sucrose. Therefore, sucrose as a mere carbohydrate source was present in the medium only in a residual amount (approximately 0.08–0.1%). Induction of MD embryos depended on the type of osmoticum used: microspores cultured on sucrose and PEG medium developed into embryos within 2 weeks, while mannitol-cultured microspores yielded no embryos. After 2 weeks in culture, some of the PEG embryos were about 1–3 mm in length, reaching the cotyledonary stage; however, most of them were smaller, still in the globular, heart-shape or torpedo stages. When placed under light, these embryos quickly became green (Fig. 1A, C) and further increased in size due to elongation of the embryo axis and further differentiation of the cotyledons. An assessment of frequency of embryo formation was not attempted due to the technical difficulty of counting PEG embryos of such a small size. Nevertheless, the number of microspores induced to undergo embryogenic development appeared comparable to that of the sucrose control (Fig. 1A) based on visual observations of microspores and developing embryos with the inverted light microscope. Since the size of MD embryos rather than their total number was affected on PEG medium, this indicated that a high concentration of PEG indeed provided a proper osmotic environment for the induction of microspore embryogenesis in *Brassica napus*. The smaller size of PEG embryos compared to sucrose embryos is likely due to a restricted carbon supply, as expected given the minute amount of carbohydrate provided in the medium. Interestingly, the high viscosity of the PEG solution and restricted water supply did not affect early microspore development upon inductive heat shock

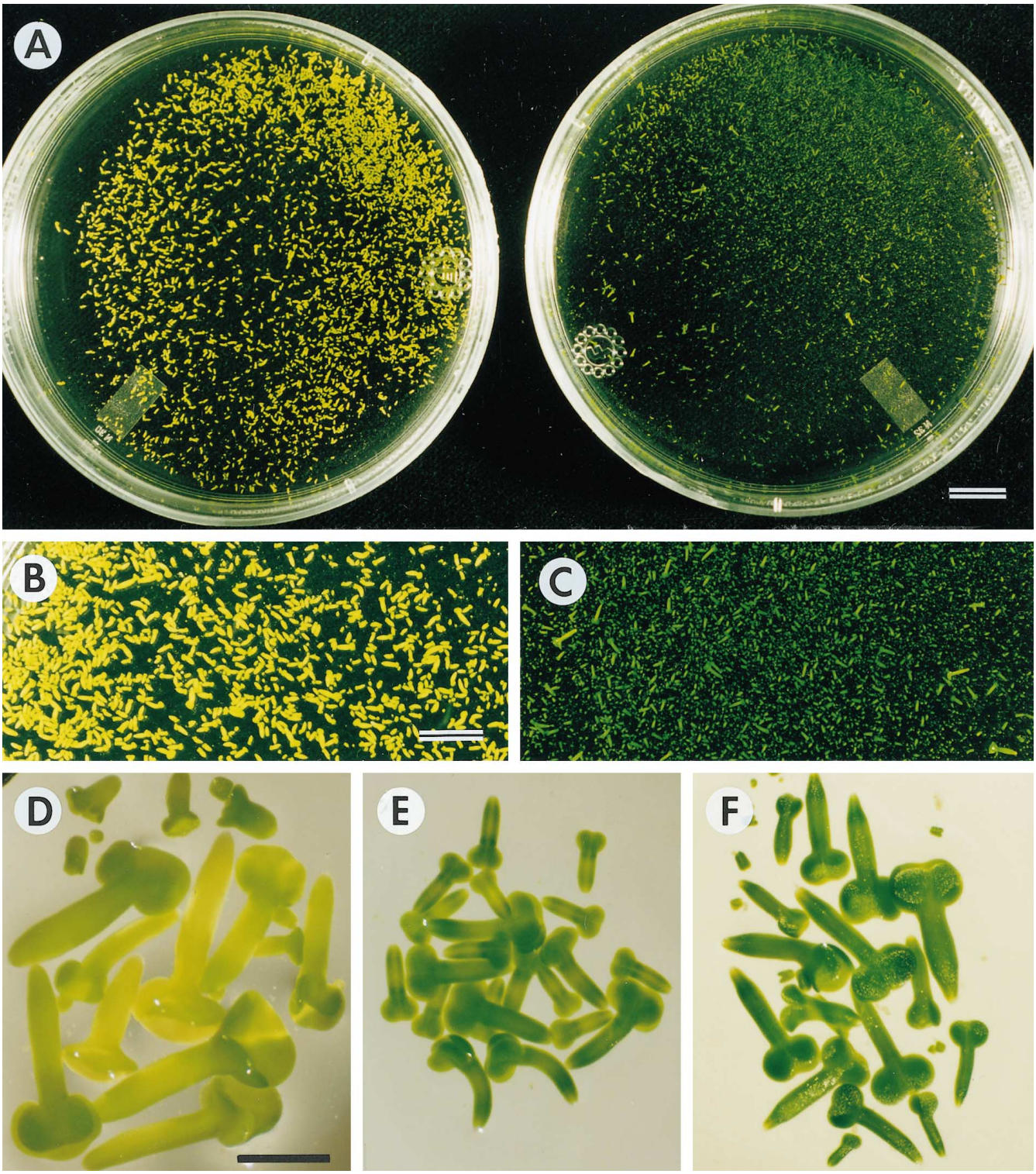

Fig. 1A–F **A** Sixteen-day-old microspore-derived embryos of *Brassica napus* on different types of osmoticum: sucrose (*left*) and polyethylene glycol 4000 (*right*). Plates were kept under the light for 2 days. *Bar*: 10 mm. **B** Enlargement of sucrose embryos (from **1A**), **C** enlargement of PEG embryos (from **1A**). *Bar* (**B–C**): 5 mm. **D** Three-week-old sucrose embryos: **E** Zygotic immature embryos developed in ovulo, dissected at 14 and 15 DPA. **F** Three-week-old PEG embryos. *Bar* (**D–F**): 1 mm

treatment, which is considered to be a sensitive stage. In contrast, mannitol, a solute generally considered to penetrate cells passively and very slowly (Cram 1984), had a negative effect on the induction of microspore embryogenesis. Microspores in the mannitol solution became swollen after heat shock treatment, but no divisions were observed and the microspores were presumably dead within a week in culture. A similar effect of mannitol was observed in *Brassica campestris* microspore (A. M. R. Ferrie, personal communication) and anther culture (Hamaoka et al. 1991). It remains to be investigated why mannitol had such a detrimental effect. Possible uptake of this solute by microspores and its accumulation in the cytoplasm cannot be ruled out; uptake and metabolism of mannitol by barley and maize root cells was reported by Cram (1984). Also, uptake of mannitol and its transport to the shoots was reported in *Brassica napus* and *Triticum aestivum* seedlings grown in vitro (Lipavská and Vreugdenhil 1996).

Under restricted sugar and water supply, the size of the PEG embryos was reduced when compared to sucrose embryos; however, they were morphologically very similar to immature zygotic embryos dissected from ovules at 14 and 15 DPA (Fig. 1E, F).Three-week-old PEG embryos had well-developed cotyledons, often widely opened and dark green, suggesting possible photosynthetic activity (Fig. 1F). MD embryos on sucrose medium, when compared to both zygotic and PEG embryos, were pale green, had elongated embryo axes and relatively small underdeveloped cotyledons. These features have already been reported in *Brassica napus* MD embryos (Rahman 1993; Yeung et al. 1996). Sucrose has been widely used as an osmoticum as well as a carbon and energy source in anther and microspore culture of a number of species (Ferrie et al. 1995). As a metabolite, sucrose can be absorbed into cell symplasts, readily utilized and/or stored in vacuoles or converted into starch and stored in plastids. Based on the results of our experiments, we conclude that a sucrose-abundant environment affects not only the internal cell and tissue structure of MD embryos, as reported by Rahman (1993), but also their external morphology, i.e. size and shape of cotyledons as well as size of the whole embryos (Fig. 1D).

Our preliminary results showed that both sucrose and PEG embryos were capable of forming plantlets upon transfer to solid medium, and further work is being undertaken to improve plantlet conversion. Methods to facilitate maturation and subsequent desiccation of PEG embryos are being developed.

In conclusion, the results of our experiments demonstrate that sucrose as an osmoticum can be replaced with high-molecular-weight PEG in microspore embryogenesis of *Brassica napus*. The amount of metabolizable carbohydrate required for MD embryo induction and formation appears to be substantially less than that reported previously. Morphologically, PEG embryos are strikingly similar to immature zygotic embryos dissected from ovules. This is the first report of microspore embryogenesis being induced using PEG as an osmoticum with only a minute quantity of carbohydrate in the medium. Further improvement of this method, perhaps by supplying small amounts of other carbohydrates (e.g. glucose or maltose) would likely result in a more synchronous population of MD embryos. With such improvements and a defined protocol for maturation and desiccation of PEG embryos, this method might be suitable for other *Brassica* species, such as self-incompatible *Brassica rapa,* and would have wide potential applications in breeding programs. Moreover, low sucrose culture systems involving non-permeating osmotica might be suitable for studies of in vitro embryogenesis with a broad range of plant species.

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