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DEVELOPMENTAL BIOLOGY/MORPHOGENESIS

Optimization of methods for using polyethylene glycol as a non-permeating osmoticum for the induction of microspore embryogenesis in the *Brassicaceae*

A. M. R. Ferrie · W. A. Keller

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Abstract The objective of this work was to enhance the quality and quantity of microspore-derived embryos of cruciferous species by using polyethylene glycol (PEG) to replace sucrose in the culture medium. The main advantage in using PEG is that it produces embryos that are morphologically more similar to zygotic embryos and have enhanced germination capabilities. When microspores were cultured in full strength NLN medium supplemented with 25% (*w*/*v*) PEG, the addition of 3 ml of full strength NLN with 13% (*w*/*v*) sucrose at 14 d was beneficial for embryo quality and quantity. Experiments showed that this PEG system could be used for a number of *Brassica napus* cultivars, as well as a number of other cruciferous species. PEG enhanced microspore embryogenesis in *B. nigra*, *Crambe abyssinica*, and *Raphanus oleifera*. Microspore-derived embryos were obtained from all cruciferous species evaluated (*B. alboglabra*, *B. carinata*, *B. juncea*, *B. rapa*, *B. nigra*, *R. oleifera*, *Crambe abyssinica*, *Sinapis alba*) using either sucrose or PEG as the osmoticum. Microspore embryogenesis was induced in *B. napus* in PEG-based cultures without a 32°C heat shock (i.e., 4, 15, 18, and 24°C). These temperature conditions were non-inductive when sucrose was used as the osmoticum. Spontaneous chromosome doubling occurred in 64–92% of the regenerated plants when PEG was used in the NLN culture medium, whereas in culture medium containing sucrose, the spontaneous doubling rate was 2–18%.

A. M. R. Ferrie (✉) · W. A. Keller
Plant Biotechnology Institute,
110 Gymnasium Place,
Saskatoon, Saskatchewan S7N 0W9, Canada
e-mail: Alison.Ferrie@nrc-cnrc.gc.ca

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Introduction

Microspore culture is an important technique for both fundamental and practical research. A number of review articles have been written on the uses of microspore culture and the resulting doubled haploid plants (Forster and Thomas 2003; Szarejko 2003; Thomas et al. 2003). Isolated microspore culture in *Brassica napus* was first reported by Lichter in 1982. Since then, microspore culture protocols have been developed for many *Brassica* species (Lichter 1989; Cao et al. 1990; Duijs et al. 1992; Cao et al. 1994; Sato et al. 2002; Ferrie 2003). However, further optimization is required as some species and genotypes respond poorly in culture (Ferrie and Keller 2004). Among the known factors influencing embryogenesis are composition of the culture medium and culture conditions. More specifically, the carbon source and concentration have been shown to influence embryogenesis in a wide range of species (Sorvari and Schieder 1987; Finnie et al. 1989; Roulund et al. 1990). The most commonly used carbohydrate is sucrose, which is both a nutrient and an osmoticum. For *Brassica* species, sucrose concentrations are usually in the range of 8–17% (Keller et al. 1975; Dunwell and Thurling 1985; Baillie et al. 1992; Ferrie et al. 1995b). However, it has been shown (Yeung et al. 1996) that microspore-derived embryos take up excess sucrose when it is available in the medium. This results in greater starch accumulation in the embryos, and a subsequent reduction in conversion to plants. Sucrose starvation or reduced sucrose concentration has been used to induce embryogenesis in *Nicotiana* (Imamura et al. 1982;

Kyo and Harada 1985), rice (Ogawa et al. 1994), and wheat (Touraev et al. 1996a).

Polyethylene glycol (PEG) is a neutral compound, which is highly water-soluble. The high-molecular-weight PEGs are too large to penetrate the plant cell walls (Caripta et al. 1979; Rains et al. 1986). Ilic-Grubor et al. (1998) developed a novel culture system using the NLN culture medium with a very low level of sucrose, together with 25% (*w*/*v*) PEG 4000 as the osmoticum to induce embryogenesis in *B. napus* Topas 4079. Embryos were smaller than those induced in the NLN culture medium with sucrose, which was likely due to reduced carbon availability. PEG has also been used in the maturation of somatic embryos in conifers (Attree et al. 1991), horse chestnut (*Aesculus hippocastanum* L.; Capuana and Debergh 1997), and *Hevea brasiliensis* (rubber; Linossier et al. 1997).

Our objective was to enhance the system developed by Ilic-Grubor et al. (1998) and to determine if PEG could be used in other *B. napus* genotypes and in other cruciferous species.

Materials and Methods

Donor plant material. Twenty-one *B. napus* cultivars were evaluated (Allons, Apollo, Cyclone, Defender, Dynamite, Elect, Excel, Garrison, Hero, Innovator, Legacy, Legend, LG 3235, N90-933, Quantum, Quest, Q2, Springfield, Sprint, Westar, and 46A65). These lines were obtained from their respective breeding organizations or the Plant Gene Resource Center of Agriculture and Agri-Food Canada (Saskatoon, SK, Canada). Embryogenic lines of *B. rapa* (Ferrie et al. 1995a), *B. nigra*, *B. carinata*, *B. juncea*, and *B. oleracea* developed at the Plant Biotechnology Institute were also evaluated (A. M. R. Ferrie, unpublished). The highly embryogenic *B. napus* line Topas 4079 was also used.

For all species, seeds were sown in 15-cm pots filled with a commercial greenhouse mixture (e.g., Redi-Earth®, W.R. Grace & Co. of Canada, Ajax, Ontario, Canada). Pots were placed in growth cabinets with a 16-h photoperiod, a light intensity of 400-µmol m^{-2} s^{-1}, and a day/night temperature of 20/15°C. Before bud formation, the temperature in the growth cabinet was lowered to 10/5°C. Plants were fertilized with slow release Nutricote® 100 (14% N, 14% P, 14% K, Westgro Sales Inc., Delta, British Columbia, Canada) once at seeding and then watered three times a week with 0.35 g l^{-1} of 15-15-18 (15% N, 15% P, 18% K) water soluble fertilizer.

Microspore culture conditions. For *B. napus*, 3- to 4-mm buds were selected and placed in stainless steel mesh baskets, with a maximum of 50 buds/basket. Buds were sterilized in approximately 150 ml of 6% sodium hypochlorite for 15 min with agitation, then rinsed with sterile, double-distilled water three times for 5 min. The buds were transferred to a 50-ml beaker containing 5 ml of half-strength B5 medium supplemented with 13% (*w*/*v*) sucrose (designated B5-13; Gamborg et al. 1968) and macerated with a glass rod, ensuring that all buds were crushed. The crude suspension was filtered through a funnel lined with 44-µm nylon screen into a 50-ml centrifuge tube. The filter and beaker were rinsed three times with 5 ml of B5-13 for a total of 20 ml in the tube. The microspore suspension was centrifuged at 130–150×g for 3 min, the supernatant was decanted, and 5 ml B5-13 was added to resuspend the pellet. This procedure was repeated twice for a total of three washes. Before the last centrifuge spin, the number of microspores was determined using a hemacytometer, and then the required amount of modified Lichter (1982) medium (NLN) was added to achieve a density of 10^5 microspores ml^{-1}. The media used were (1) full-strength NLN supplemented with 13% (*w*/*v*) sucrose and 0.83 mg l^{-1} potassium iodide, but without potato extract and growth regulators, pH 5.8 (designated as NLN-13% sucrose), or (2) full strength NLN supplemented with 25% (*w*/*v*) PEG 4000 (Fluka and Riedel-de Haën Biochemicals) and 0.83 mg l^{-1} potassium iodide, but without potato extract and growth regulators, pH 5.8 (designated as NLN-25% PEG). Osmolarity of the culture medium was determined using an osmometer (model 3300, Advanced Instruments, Inc., Norwood, MA). All media were filter-sterilized. Ten-milliliter aliquots of microspore suspension were dispensed into 100×15 mm sterile petri plates.

Plates were incubated in the dark at 32°C for 72 h to induce embryogenesis, and then at 24°C in the dark for 18 d. After 3 wk, the embryos were counted and the frequency of embryogenesis was determined as the number of embryos/100 buds. A minimum of three experiments was conducted with three to five replicates per treatment per experiment.

The first experiment evaluated the Ilic-Grubor et al. (1998) protocol. The isolated microspores were cultured in half-strength NLN culture medium with 25% (*w*/*v*) PEG 4000, incubated at 35°C for 1 h, then 32°C for 18 h followed by 24°C. Ten days after culture, 500 µl of basal medium (NLN medium but no PEG or sucrose) was added to each petri plate containing 10 ml of microspore suspension for a final PEG concentration of 22%. The cultures were then incubated for an additional 11 d at 24°C. Embryos were counted after 3 wk.

Concentration of PEG and time of sucrose addition. A second experiment evaluated seven concentrations of PEG 4000 (5, 10, 15, 17, 20, 25, 30% *w*/*v*) in full strength NLN culture medium. The time of NLN-13% sucrose (3 ml)

addition (7, 14, 21 d) to make a final concentration of 3% sucrose was also evaluated. NLN-13% sucrose was added to each petri plate containing 10 ml of microspore suspension. Microspores were cultured at 32°C for 3 d before incubation at 24°C.

Culture Temperature

A third experiment evaluated the culture temperature. Microspores in NLN-13% sucrose or NLN-25% PEG were incubated at five different temperatures (4, 15, 18, 24, 32°C) for 3 d before incubation at 24°C. Three milliliters of NLN-13% sucrose was added to the PEG cultures at 14 d to improve embryo quality. NLN-13 was added to each petri plate containing 10 ml of microspore suspension for a final sucrose concentration of 3%.

Regeneration. Microspore-derived embryos from the experiments utilizing either PEG or sucrose as the osmoticum were placed on a gyratory shaker (70 rpm) at 22°C with a 14-h photoperiod and a light intensity of 100-μmol m^{-2} s^{-1}. The embryos turned green after approximately 1 wk. At that time, morphologically normal embryos from each of the treatments (PEG or sucrose) were transferred to solid B5 medium (Gamborg et al. 1968) free of growth regulators and maintained at 22°C with a 14-h photoperiod and a light intensity of 150- μmol m^{-2} s^{-1}. Root and shoot development was recorded after 3–4 wk.

Genotype and species evaluation. The *B. napus* protocol, as outlined previously, was followed for all 21 *B. napus* genotypes as well as for *Sinapis alba*, *Crambe abyssinica*, and *R. oleifera*. For the other *Brassica* species (i.e., *B. alboglabra*, *B. carinata*, *B. juncea*, *B. rapa*, and *B. nigra*), protocols were followed as outlined by Ferrie (2003). For these *Brassica* species, modifications to the *B. napus* protocol were primarily in bud size, sucrose concentration of the NLN culture medium, and heat shock duration. For *B. rapa*, 2- to 3-mm buds were used; the initial culture medium consisted of full-strength NLN with 17% (*w*/*v*) sucrose and 0.44-μM benzyladenine (BA) but no glutamine, followed by a medium change after 48 h to full strength NLN with 10% (*w*/*v*) sucrose (designated as NLN-10% sucrose) with glutamine but without BA (Baillie et al. 1992). For *B. juncea*, bud size and initial induction medium were similar to *B. rapa*; however, cultures were initially incubated at 35°C for 48 h. The medium was then changed to full-strength NLN-13% sucrose and glutamine but no BA (Ferrie 2003). The standard *B. napus* protocol was used for induction of *B. carinata* microspore-derived embryos with the following exceptions: 2- to 4-mm-sized buds, half-strength NLN with 10% (*w*/*v*) sucrose, and an incubation treatment of 32°C for 48 h. For *B. nigra* and *B. alboglabra*, donor plants were grown at 20/15°C, 2- to 3-mm buds were selected for culture, and microspores were cultured in NLN-10% sucrose. For each species, NLN medium with sucrose was compared to NLN-25% PEG using the specific protocols described above.

Ploidy analysis. Flow cytometry was used to determine plant ploidy levels. Plant material was prepared using the Partec CyStain UV precise P kit (Partec GmbH, Otto-Hahn-Str. 32, D-48161 Münster, Germany). Leaf tissue or whole embryos were placed in microfuge tubes with 4–5 drops of Partec nuclear extraction buffer. The tissue was cut five times with a sharp blade, and 2 ml of Partec staining buffer was added. The solution was mixed well and stored at 4°C in the dark until the samples were read (within 24 h). The samples were filtered to remove plant tissue debris before reading on a Partec cell counter analyzer (Partec GmbH). As a control, nuclei were similarly extracted from the diploid tissue of the microspore donor plants and used to set the diploid peak. For the experiments using microspore-derived embryos, 220 embryos from each culture condition were analyzed. Using leaf tissue, 150 plant samples were analyzed from each culture system (i.e., sucrose or PEG).

Results and Discussion

Microspore culture.

A. Ilic-Grubor et al. (1998) *protocol* Isolated Topas 4079 microspores were cultured in half-strength NLN culture medium with 25% PEG 4000, incubated at 35°C for 1 h, then 32°C for 18 h, followed by 24°C. Ten days after induction, 500 μl of basal medium (half-strength NLN medium but no PEG) was added to each petri plate of 10 ml of microspore suspension that was then incubated for an additional 11 d at 24°C. There was no significant difference in the number of embryos produced using the PEG system or the sucrose system (data not shown). However, embryos produced in the half-strength NLN with PEG medium were quite small, making it very difficult to manipulate the embryos in culture. To facilitate embryo handling, further optimization of the PEG protocol was required.

B. *Concentration of PEG and time of sucrose addition* Seven concentrations of PEG 4000 (5, 10, 15, 17, 20, 25, 30% *w*/*v*), as well as the addition of NLN-13% sucrose at three times (7, 14, 21 d), were evaluated to determine if embryo quality (i.e., size, morphology) and quantity could be enhanced. Embryo induction was consistently higher with 25% PEG 4000 as the osmoticum (Table 1). Medium with 20% PEG 4000 also gave good quality embryos,

Table 1. Effect of PEG 4000 concentration on *B. napus* microspore embryogenesis with the addition of 3 ml of 13% sucrose (3% sucrose final concentration) at 7, 14, or 21 d after culture

% Osmoticum (osmolarity mOsm)								
Time of sucrose addition	5 PEG (48)	10 PEG (136)	15 PEG (276)	17 PEG (364)	20 PEG (564)	25 PEG (872)	30 PEG (1,560)	13 S (458)
Embryos/100 buds								
0 (control)	0	0	0	258a	988a	4227a	725ab	12,711
7 days	0	0	0	126b	3235bc	17,037c	3851ab	NA
14 days	0	0	0	131b	3929c	10,160b	4457b	NA
21 days	0	0	0	0c	2521b	9103b	376a	NA

Means followed by different *letters* in a *column* were significantly different at $p=0.05$ level as determined by least significant differences (LSD) test.
NA Not applicable

although the frequency of embryogenesis was less than the cultures with 25% PEG 4000. Embryos induced in NLN culture medium with 17 or 30% (*w*/*v*) PEG were morphologically abnormal. In culture media containing the lower concentrations of PEG 4000 (5, 10, 15%), there was no embryo development (Table 1).

The addition of 3 ml of NLN-13% sucrose to PEG-containing media (3% sucrose in the final concentration of culture medium) to each 10 ml petri plate at 7, 14, or 21 d was beneficial for embryo production except when 17% PEG was used in the initial NLN culture media or 30% PEG with NLN-13% sucrose at 21 d (Table 1). When sucrose was added at 7 d to the plates with 25% PEG 4000, embryo production was 17,037 embryos/100 buds, which was higher than with the standard sucrose protocol (12,711 embryos/100 buds). Although there were fewer embryos when the sucrose addition was delayed until 14 d, the embryos produced from these cultures were larger. NLN-13% sucrose added at day 7 most likely provides energy for more microspores to develop into embryos, hence, the higher embryo numbers. As development proceeds past day 7, deposition of storage products begins, for which energy reserves are required. This could account for the larger embryos when sucrose is added at day 14.

C. *Culture temperature* Pollen development follows a precise sequence of events. Stress is usually required to trigger the microspores to switch from gametophytic development to sporophytic development. This can be a thermal stress, nutrient starvation, colchicine treatment, or the addition of caffeine (Touraev et al. 1996a; Touraev et al. 1996b; Zhao et al. 1996; Bueno et al. 1997; Chen et al. 1998; Zaki 1999). In the case of *Brassica*, a thermal stress (32°C) is required (Keller and Armstrong 1979; Baillie et al. 1992). In these experiments, we evaluated five incubation temperature conditions (4, 15, 18, 24, 32°C) to determine if PEG could alleviate the need for thermal stress. Three milliliters of NLN-13% sucrose was added to each petri plate for a final concentration of 3% sucrose at 14 d to improve embryo quality.

For microspores induced in NLN-13% sucrose, embryos were produced only after a thermal stress of 32°C for 3 d (Fig. 1). No induction was observed at the lower temper-

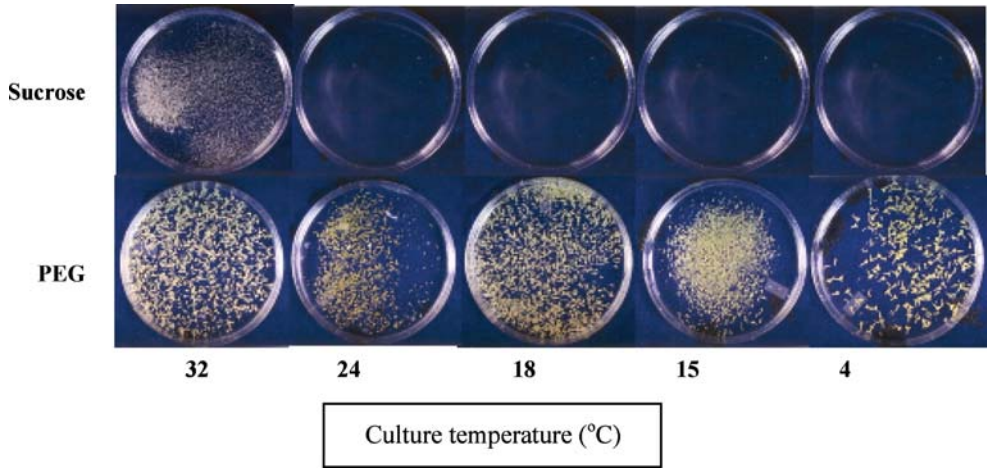

Figure 1. Microspore embryogenesis of *B. napus* induced in NLN culture medium with 13% sucrose or 25% PEG 4000 at different temperature regimes.

atures (4, 15, 18, 24°C for 3 d and then maintained at 24°C). In contrast, embryos were induced using NLN-25% PEG at all incubation temperatures, although the frequency of embryogenesis was slightly decreased at the lower temperatures. The manner in which PEG enables microspore embryogenesis at these low temperatures is unknown. Other studies in *Brassica* have shown that heat shock proteins (HSPs) are involved in embryogenesis (Seguín-Simarro et al. 2003). However, it has been difficult to discriminate between those HSPs that are triggered because of an elevated temperature and those HSPs involved in embryogenesis.

Further experiments evaluated the duration of the different temperatures when using either 25% PEG 4000 or 13% sucrose as the osmoticum in the NLN culture medium. Microspores were first incubated at 4, 15, or 18°C, and then some plates were transferred to 24°C everyday for 21 d. Embryos were produced at all temperature regimes and at all times with PEG in the culture media (data not shown). However, under cold conditions (4°C), the embryos developed slower than those at the warmer temperatures. Microspores incubated at 4°C for 21 d had developed to the globular stage only. Further development occurred when the plates were moved to a warmer temperature (24°C). Embryos were produced in sucrose media when incubated at 32°C for 1–21 d, but no embryo induction/development was observed at any of the other temperatures.

D. *Genotype screening of B. napus cultivars* As with many tissue culture systems, genotypic differences are reflected in androgenic response. Genotype screening studies have shown that there are differences among genotypes and even individual plants within the same genotype for both embryo induction and plantlet regeneration (Phippen and Ockendon 1990; Hiramatsu et al. 1995). The line Topas 4079 is a selection from the *B. napus* cv. Topas, which is used in many studies because of its high embryogenic capability. However, this cultivar is no longer under cultivation. In this experiment, we evaluated 21 *B. napus* cultivars for their response to microspore culture using PEG in the culture medium. Comparisons were made between NLN-13% sucrose and NLN-25% PEG with the addition of 3 ml of NLN-13 sucrose at 14 d of culture. Embryos were counted at 21 d. Differences in embryogenic response were observed among the *B. napus* genotypes when sucrose or PEG was used in the culture medium (Table 2). From these experiments, we concluded that using PEG in the microspore culture-induction medium not only enhances microspore embryogenesis in the highly embryogenic line, Topas 4079, but also may improve embryogenesis for a number of poorly embryogenic *B. napus* genotypes. In these experiments, several cultivars, i.e., Cyclone, Elect, Legend, Q2, Springfield, Sprint, and 46A65, produced more embryos when PEG was used as the osmosticum compared to sucrose, whereas the cultivars, Defender, Excel, Hero Innovator, LG 3235, N90-933, Quantum, and Westar were more embryogenic when sucrose was incorporated into the NLN medium rather than PEG. For six of the 21 cultivars, there was no significant difference between the NLN sucrose medium or the NLN PEG medium in terms of embryogenic frequency.

Table 2. Effect of culture osmoticum, 13% sucrose or 25% PEG 4000, on microspore embryogenesis of *B. napus* cultivars

Cultivar	NLN with 13% sucrose	NLN+25% PEG 4000+3 ml of NLN-13 at 14 days
	Embryos/100 buds	
Allons	2a	0a
Apollo	3,976a	2,331a
Cyclone	1,122a	7,853b
Defender	24,174a	4,307b
Dynamite	20,187a	19,553a
Elect	977a	1,771b
Excel	27,475a	19,110b
Garrison	1,168a	2,937a
Hero	28,401a	10,539b
Innovator	24,462a	7,592b
Legacy	19,271a	18,333a
Legend	16,872a	26,666b
LG3235	17,220a	11,172b
N90-933	4,619a	174b
Quantum	1,444a	0b
Quest	19,681a	18,078a
Q2	18a	19,615b
Springfield	1,699a	16,282b
Sprint	299a	1,972b
Westar	8,252a	2,716b
46A65	561a	4,727b

Means followed by different *letters* in a *row* were significantly different at $p=0.05$ level as determined by LSD test.

E. *Microspore culture of recalcitrant cruciferous species* The use of PEG or sucrose in the NLN culture medium was compared in a number of cruciferous species (i.e., *B. alboglabra*, *B. carinata*, *B. juncea*, *B. nigra*, *B. rapa*, *C. abyssinica*, *R. oleifera*, and *S. alba*). There have been very few or no published protocols for some of these species (e.g., *B. nigra*, *C. abyssinica*, *R. oleifera*, and *S. alba*; Klimaszewska and Keller 1983; Govil et al. 1986; Hetz and Shieder 1989; Takahata et al. 1996). Embryos and doubled haploid plants were produced in all eight species evaluated (Table 3). For some species, i.e., *B. alboglabra*, *B. carinata*, *B. juncea*, and *B. rapa*, embryo induction was higher when sucrose was used in the culture medium, although embryos still developed with the PEG medium. For *C. abyssinica*, a recalcitrant species, PEG was beneficial (16 embryos/100

Table 3. Effect of culture osmoticum, sucrose or 25% PEG 4000, on microspore embryogenesis of eight cruciferous species

Species	NLN + Sucrose	NLN+25% PEG 4000+3 ml of NLN-13 at 14 days
	Embryos/100 buds	
Brassica alboglabra	4,200a	158b
Brassica carinata	2,286a	70b
Brassica juncea	1,633a	240b
Brassica rapa	2,000a	500b
Brassica nigra	12a	2,229b
Raphanus oleifera	25a	1,000b
Crambe abyssinica	0a	16b
Sinapis alba	42a	0b

Means followed by different *letters* in a *row* were significantly different at $p=0.05$ level as determined by LSD test.

buds) as no embryos were produced using sucrose. A total of 40 embryos developed from all *Crambe* microspore culture experiments. PEG was also beneficial for *R. oleifera* and *B. nigra*. Embryogenic frequency of *B. nigra* induced in PEG was 2,229 embryos/100 buds compared to 12 embryos/100 buds in sucrose; as for *R. oleifera*, embryogenic frequency in PEG was 1,000 embryos/100 buds, whereas it was only 25 embryos/100 buds with sucrose. For *S. alba*, another recalcitrant species, embryos were only produced from the sucrose-containing medium. Doubled haploid plants were produced, and seeds were collected from all plants. Based on this experiment, PEG could be used for some of the cruciferous species to enhance microspore embryogenesis.

F. *Regeneration experiments* A major problem in doubled haploid plant production is embryo germination and conversion to plants. Embryos derived from full strength NLN medium containing PEG 4000 or sucrose were plated onto solid medium (B5) to evaluate germination and regeneration. Although embryos derived from PEG 4000 medium were too small in the early experiments to individually transfer with forceps, they were pipetted directly onto the solid embryo culture media and germinated. Regeneration frequency was determined from our later experiments, where the PEG-derived embryos were larger and could be transferred easily. The frequency of normal plantlets (i.e., root and shoot development similar to zygotic development) depended on the experiment, age, and quality of the embryos, and genotype. For *B. napus* line Topas 4079, 97% of the plantlets derived from PEG-containing medium were normal compared to 76% for sucrose-derived plantlets. This would be beneficial as less sub-culturing would be required.

G. *Chromosome doubling* Doubling the chromosome number of microspore-derived plants is necessary for the production of doubled haploid plants. For some species, a chemical treatment is required because spontaneous doubling occurs infrequently. This is the case for the *Brassica* species. Traditionally, colchicine has been used to double the chromosome number, but antimicrotubule agents such as oryzalin, trifluralin, amiprophos-methyl, and pronamide can also be used for this purpose (Zhao and Simmonds 1995; Hansen and Andersen 1996). Stress treatments, like cryopreservation, have also been observed to double the chromosome number (Chen and Beversdorf 1992). Our objective was to determine the effect of PEG on spontaneous chromosome doubling.

B. napus line Topas 4079 embryos developed in NLN-13 sucrose, NLN-20% PEG, or NLN-25% PEG and cultured at either 32 or 15°C were regenerated into plantlets. The plants were not treated with colchicine or any other doubling agent, but were grown in the greenhouse to

Table 4. The frequency of haploid and doubled haploid plants or embryos of *B. napus* Topas 4079 developed from microspores cultured in NLN-13% sucrose, NLN-20% PEG or NLN-25% PEG

Culture medium	Temperature (°C)	Number of plants/ embryos evaluated	% Haploid plants or embryos	% Doubled haploid plants or embryos
Leaf tissue samples				
Sucrose 13%	32	150	82	18
PEG 20%	32	150	23	77
PEG 20%	15	150	8	92
PEG 25%	32	150	32	68
PEG 25%	15	150	22	78
Embryo samples				
Sucrose 13%	32	220	98	2
PEG 25%	32	220	36	64

When NLN-20% PEG or NLN-25% PEG were used as microspore culture medium, 3 ml of NLN-13% sucrose was added per plate at 14 d.

determine how many had spontaneously doubled. Leaf tissue analysis showed that when sucrose was used in the culture medium, 82% of the plants were haploid, whereas 18% had spontaneously doubled (Table 4). For both PEG concentrations (20, 25%) and culture temperatures of 32 or 15°C, the frequency of spontaneous doubling was higher (68–92%) than the sucrose control (18%; Table 4). Similar results were observed when the microspore-derived embryos rather than the resulting plantlets were used to determine ploidy levels, indicating that doubling occurs during the early embryo stages (Table 4). PEG has a beneficial effect on chromosome doubling; however, the mechanism of chromosome doubling is not known.

In conclusion, experiments showed that improvements to the Ilic-Grubor et al. (1998) protocol resulted in enhanced quality and quantity of microspore-derived embryos of cruciferous species by using PEG to replace sucrose in the NLN culture medium. When microspores were cultured in NLN-25% PEG, the addition of 3 ml of 13% sucrose at 14 d was beneficial for embryo quality and quantity. The advantages of using PEG are that embryos are morphologically more similar to zygotic embryos, germination capabilities are enhanced, and there is an increased frequency of spontaneous chromosome doubling in the regenerated plants. Experiments also showed that the PEG system could be used for a number of *B. napus* cultivars, as well as a number of other cruciferous species (*B. nigra*, *C. abyssinica*, and *R. oleifera*). Microspore embryogenesis was induced in *B. napus* in PEG-based cultures grown at 4, 15, 18, and 24°C. These temperature conditions were non-inductive when sucrose was used as the osmoticum.

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