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ORIGINAL PAPER

Microspore embryogenesis in Apiaceae

A. M. R. Ferrie · T. D. Bethune · M. Mykytyshyn

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Abstract Haploid technology is used to develop uniform, true-breeding lines, as well as to accelerate crop improvement programs. Among 20 Apiaceae species screened for response to doubled haploidy, 11 generated microspore-derived embryos, and all but one of the latter yielded doubled haploid plants. Donor plant conditions, basal media, and culture conditions were evaluated for their efficacy on inducing microspore-derived embryos. Growing donor plants at temperature conditions of 10/5 or 15/10°C promoted microspore embryogenesis in fennel (*Foeniculum vulgare* Mill.), whereas, growing donor plants at a temperature regime of 10/5°C, along with the use of a cold extraction method, enhanced embryogenesis in dill (*Anethum graveolens* L.) and anise (*Pimpinella anisum* L.). The culture of fennel and dill microspores in an NLN basal medium and caraway (*Carum carvi* L.) in AT-3 basal medium promoted the highest frequencies of embryo induction.

Keywords Caraway (*Carum carvi* L.) · Dill (*Anethum graveolens* L.) · Doubled haploid · Embryogenesis · Fennel (*Foeniculum vulgare* Mill.) · Microspore culture

Introduction

The Apiaceae (formally Umbelliferae) is a large family of plants with about 300 genera and more than 3,000 species. These species are distributed around the world, but are more common in the northern temperate regions. It is a diverse family that includes vegetables, herbs, and spices used for food, flavorings, and medicine. All plant parts have shown to be of some benefit, and those used include seeds and seed oils [e.g., dill (*Anethum graveolens* L.), caraway (*Carum carvi* L.), coriander (*Coriandrum sativum* L.)], and roots [e.g., carrot (*Daucus carota* L.) and parsley (*Pastinaca sativa* L.)], as well as stems, leaves, and petioles [celery (*Apium graveolens* L.), *Angelica* spp., lovage (*Levisticum officinale* Koch), and dill] (French 1971). Most of the Apiaceae are biennial or perennial, although many are grown and harvested as annuals. As the majority of the Apiaceae are not major economical crops, there have been very few breeding and improvement programs for this group of plants. Carrot is an exception, as there are vegetable breeding companies working on this economically important horticultural crop (Stein and Nothnagel 1995).

Very little work has been conducted on doubled haploid systems for the Apiaceae species. Limited response in terms of calli and/or embryoid production has been reported from anther cultures of celery, fennel (*Foeniculum vulgare* Mill.), and mitsuba (*Cryptotaenia japonica*) (Dohya et al. 1997), *Bupleurum falcatum* L. (Shon and Yoshida 1997), caraway (Smykalova et al. 2009), and carrot (Adamus and Michalik 2003; Matsubara et al. 1995; Górecka et al. 2005, 2009). Plantlets were regenerated from celery, carrot, caraway, and *B. falcatum* anther culture; however, the efficiency of production was low. Heart-shaped embryos were observed from isolated microspore cultures of celery, but no further development occurred (Dohya et al. 1997). In a previous study in the author's laboratory, over 80 different nutraceutical species, including 19 Apiaceae species, were screened for microspore culture response using a standard *Brassica napus* doubled haploid protocol (Ferrie et al. 2005). Several Apiaceae

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species were selected (dill, fennel, anise, and caraway) for further investigation of factors influencing microspore embryogenesis. In this paper, we give an overview of the experiments undertaken to develop doubled haploid protocols for several Apiaceae species.

Materials and methods

Donor plant seed of the different species, unless otherwise noted, was obtained from Richters Herbs (Goodwood, Ontario, Canada) or from the Department of Plant Sciences, University of Saskatchewan (Saskatoon, Saskatchewan, Canada). Donor plant seed of bulb fennel lines (VF1, VF2, VF3, VF4, VF8, and VF10) were obtained from Rijk Zwaan (Rijk Zwaan Zaadteelt en Zaadhandel B.V., P.O. Box 40, 2678 ZG De Lier, The Netherlands).

Four cultivars of dill ('Fernleaf', 'Vierling', 'Mammoth', and 'Hercules') and two dill DH lines developed during the project were tested in the microspore culture experiments. One cultivar of annual-type caraway ('Moran') and two caraway breeding lines (NN-1 and NN-2) were used, as well as one anise line obtained from Richters Herbs.

Seeds were planted in 15-cm pots filled with a commercial soil-less mix containing approximately 1 g of slow-release fertilizer (14-14-14; Nutricote 100). Pots were placed in a growth cabinet (16-h photoperiod, 400 µmol m^{-2} s^{-1}) and watered three times a week with 0.35 g/l of 15-15-18 (N–P–K) fertilizer. For fennel, a temperature regime of 17/13°C was used and for the other species, the donor plants were grown at 20/15°C. Those species requiring vernalization (e.g., carrot, parsnip, and fennel) were grown at 10/5°C prior to use in microspore culture experiments.

The microspore culture protocols developed were based on a standard *Brassica napus* protocol (Ferrie and Keller 1995), with several modifications. Young buds less than 3 mm in length were collected, surface-sterilized and rinsed, and then gently crushed with a pestle in 1/2-strength B5 medium containing 13% sucrose (B5-13). The resulting suspension was filtered through a 90-µm nylon screen cloth into a 50-ml sterile centrifuge tube and then passed through a 41-µm screen for further purification. The filtrate was centrifuged at 145g for 3 min. The supernatant was discarded, fresh B5-13 was added, and the pelleted microspores were resuspended and centrifuged. This was repeated for a total of three washes. A hemocytometer was used to count the microspores. Culture medium was added to the final pellet to achieve a density of 5×10^4 microspores/ml for fennel and 1×10^5 microspores/ml for the other species (e.g., dill, caraway, and anise). The final plating density was determined based on preliminary experiments (data not shown). The microspore cultures were incubated in NLN media at 32°C for 72 h in the dark, and then maintained at 24°C in the dark. Embryos were counted after 4 weeks and then checked on a monthly basis. Experiments were repeated three to five times, with a minimum of 5–10 replicate plates per treatment for each experiment.

Parameters evaluated to improve microspore embryogenesis

Donor plant conditions

To evaluate the impact of donor plant conditions on microspore embryogenesis in fennel, donor plants were moved to growth cabinets with a 10/5, 15/10, or 17/13°C day/night temperature regime and a 16-h photoperiod following vernalization at 10/5°C for 8 weeks. Microspores were harvested as previously described and were cultured using NLN culture medium (Lichter 1982) with 25% sucrose (NLN-25) and were incubated at 32°C for 72 h to assess the effects of the donor plant temperature regimes.

Anise and dill donor plants were grown under conditions of 10/5 and 20/15°C. Buds were harvested as previously described and then isolated under room temperature conditions, wherein the equipment and media were kept at room temperature, or under cold conditions, wherein the equipment and media were kept on ice during experimentation. Anise microspores were cultured in NLN medium with 20% sucrose and dill was cultured in NLN medium with 15% sucrose. Isolated microspore cultures were incubated at 24 or 32°C for 3, 7, or 14 days and then transferred to 24°C.

Basal medium

Six established basal media were evaluated for the induction of microspore-derived embryos (MDE) in fennel and caraway. These included: B5 (Gamborg et al. 1968), NLN (Lichter 1982), N6 (Chu 1978), NN (Nitsch and Nitsch 1969), K&M (Kao and Michayluk 1975), and AT-3 (Touraev and Heberle-Bors 1999). AT-3 was evaluated in the caraway experiments, but not in the fennel experiments. For media compositions, the sucrose concentration was 13%, except for AT-3, which contained 9% maltose. The extraction/wash medium was B5-13, except when AT-3 was used as the culture medium, in which case Touraev B medium was the extraction/wash medium (Touraev and Heberle-Bors 1999). Donor plants were grown at 10/5°C and microspores were harvested as previously described. Cultures were incubated at 32°C for 72 h and then maintained at 24°C for a total experimental period of 28 days.

Addition of brassinosteroids

EBR (24-epibrassinolide; OlChemIm Ltd., Šlechtitelů 27, P.O. Box 22, 770 10 Olomouc, Czech Republic) was

dissolved in dimethyl sulfoxide (DMSO) and added at concentrations of 0.1 and 1 mM EBR to the culture medium (NLN-25), with the final DMSO concentration remaining constant at 0.1% v/v (Wilen et al. 1995). All experiments included untreated and 0.1% (v/v) DMSO-treated control cultures. Donor plants of the fennel line VF2 were grown as previously described, and the microspores were harvested and subsequently cultured at 32°C for 72 h prior to moving them to 24°C. After 72 h, the medium in some of the Petri plates was changed to NLN-10 (NLN medium with 10% sucrose) without EBR by pipetting medium with microspores into a 50-ml tube and centrifuging for 3 min at 130–150g. The supernatant was removed and NLN-10 without EBR was added.

Culture temperature

Caraway genotypes Moran, NN-1, and NN-2 were grown at 20/15°C. Based on the results of the basal medium experiment, caraway microspores were isolated in Touraev B medium, cultured in AT-3 medium, and incubated at temperatures of 24 or 32°C for 3, 7, or 14 days prior to incubation at 24°C. Embryos were counted after 28 days. The experiment consisted of 12 to 32 plates of 1×10^5 microspores/ml per temperature treatment for each genotype.

Regeneration

Once embryos from microspore cultures of fennel reached the cotyledonary stage of development, they were placed on a gyratory shaker in the light at 22°C for 1–2 weeks (16-h photoperiod with a light intensity of 100 µmol $m^{-2}s^{-1}$). For development into plantlets, these embryos were then transferred to solidified B5 germination medium (1% sucrose, 1% agar), with no growth regulators, in 100 × 25-mm sterile Petri dishes (Phoenix Biomedical, Canada). Once the plantlets developed vigorous shoots and roots, they were sub-cultured onto fresh, solidified B5 medium (2% sucrose, 0.8% agar) either in large Petri dishes (150 × 25 mm) or Magenta™ boxes and kept at 22°C with a 16-h photoperiod and a light intensity of 100 µmol $m^{-2}s^{-1}$. For the other species, microspore-derived embryos at the cotyledonary stage of development were plated directly onto solidified 1/2-strength B5 germination medium (1% sucrose, 0.8% agar), with no growth regulators, in 100 × 25-mm sterile Petri dishes to develop into plantlets. Plates were incubated at 22°C with a 16-h photoperiod and a light intensity of 100 µmol $m^{-2}s^{-1}$.

Chromosome doubling

Individual haploid plantlets were all initially treated with colchicine to develop doubled haploid plants. Once the root system was well developed, plantlets were removed from the culture vessel and the roots were immersed in a solution of 0.34% colchicine in water for 1.5 h, rinsed with distilled water, and then planted in a soil-less mix contained in 9-cm cell inserts (Kord Products, Bramlea, Ontario, Canada). Individual plantlets were covered with clear plastic drinking cups and trays were kept in a growth cabinet at 20/15°C with a 16-h photoperiod and a light intensity of 400 µmol $m^{-2}s^{-1}$. After a few weeks, surviving plantlets were hardened off and transplanted to larger pots. The pots were then placed in a 20/15°C growth cabinet or greenhouse. Plants were vernalized if required, then bagged to ensure self-pollination. Umbels were self-pollinated by hand when required and seed was harvested from each plant individually.

Ploidy level determination

A Partec Cell Counter Analyzer (CCA) was used to determine the ploidy of the donor plants, which served as a diploid control, and plants obtained from microspore culture experiments. Leaf tissue of size approximately 0.5 cm^2 was chopped several times with 400-µl nuclear extraction buffer (CyStain UV Precise P, Partec), incubated for up to 5 min, the solution filtered to remove plant material, 1.6 ml staining buffer (CyStain UV Precise P, Partec) added, and the samples were incubated for 1 min prior to analysis.

Results and discussion

Doubled haploid plants are routinely used as plant breeding tools with many crops (see Maluszynski et al. 2003). Although tissue culture regeneration protocols are available for some of the Apiaceae species, this family is considered to be recalcitrant to doubled haploid technologies. An efficient, reproducible protocol would be beneficial for Apiaceae breeding and genomics work. The main advantage of doubled haploid techniques in crop improvement is the reduced time required to achieve homozygosity. A number of factors have been identified as contributing to the induction and development of microspore-derived embryos, and these include the growth conditions of the donor plants, stage of microspore development, pre-treatment of the floral organs or microspores, genotype, culture medium, and culture conditions.

Twenty species were evaluated for microspore culture response. Over the years, numerous experiments were carried out evaluating donor plant conditions, media composition, and culture environment. Microspore-derived embryos were observed from 11 species (Table 1). Plants were regenerated from 10 of the 11 responding species and

Table 1 List of Apiaceae species evaluated for androgenic response and the response in terms of microspore-derived embryos, haploid/doubled haploid plants, and number of lines with seed and field evaluation (updated from Ferrie et al. 2005)

Latin name	Common name	Androgenic response				
		No response	Microspore-derived embryos	Haploid/doubled haploid plants	No. of lines with seed	Field evaluation
Ammi majus L.	Laceflower		x	x	4	
Ammi visnaga L.			x	x	0	
Anethum graveolens L.	Dill		x	x	134	x
Angelica archangelica L.	Angelica		x	x	0	
Angelica sinensis Oliv.	Dong quai	x				
Anthriscus cerefolium L.	Chervil		x			
Apium graveolens L.	Celery	x				
Bupleurum falcatum L.	Bupleurum	x				
Carum carvi L.	Caraway		x	x	60	x
Carum copticum L.	Ajowan	x				
Centella asiatica L.	Gota kola	x				
Coriandrum sativum L.	Coriander	x				
Cryptotaenia japonica L.	Mitsuba	x				
Cuminum cyminum L.	Cumin	x				
Daucus carota L.	Carrot		x	x	17	x
Foeniculum vulgare Mill.	Fennel		x	x	183	x
Levisticum officinale Koch.	Lovage		x	x	21	
Pastinaca sativa L.	Parsnip		x	x	2	
Petroselinum crispum Miller	Parsley	x				
Pimpinella anisum L.	Anise		x	x	61	x

field evaluations were conducted with five of them (dill, fennel, caraway, anise, and carrot) (Ferrie et al. submitted). There were some similarities between the species in terms of requirements, although in-depth studies were not carried out on all species. Generally for the species which responded, culture in NLN medium resulted in embryo development, with caraway being an exception. Sucrose concentrations of 10–25% in the NLN medium resulted in embryogenesis, with the higher concentrations (20, 25%) being beneficial for some species (e.g., anise, laceflower, parsnip, lovage, and carrot). Embryo development was more frequently observed after a heat shock (32°C), although embryos were also obtained at a continuous 24°C.

In our research, many factors were evaluated with the Apiaceae, but response remained very low and inconsistent in some species. This has made it very difficult to determine specific conditions required to develop a routine, efficient system. For example in dill, embryos were observed in NLN media but not in any of the other basal media formulations used (B5, N6, K&M, AT-3, NN). However, embryos were noted in sucrose concentrations ranging from 10 to 25% with culture conditions of 24 or 32°C for 3, 7, or 14 days followed by 24°C. Microspore-derived embryos of dill were observed from the four cultivars evaluated (Fernleaf, Vierling, Mammoth, and Hercules) and the two DH lines tested. The results varied greatly from experiment to experiment and were inconsistent within each experiment. Although embryos developed and regenerated into plants, it was very difficult to determine if there were significant differences between the microspore culture treatments. Similar results were observed for other Apiaceae species evaluated. When compared to responsive species (e.g., *Brassica*), the frequency of embryogenesis was low, but compared to previous reports for the Apiaceae, the results presented here are encouraging.

Donor plant conditions

Donor plant conditions play a major role in pre-disposing plant material for microspore embryogenesis (Dunwell 1976; Lee et al. 1988; Jones and Petolino 1987; Keller et al. 1987). In our experiments, donor plants were grown in growth chambers where the temperature and photoperiod can be controlled. Fennel plants were vernalized prior to transfer to one of three temperature regimes (10/5, 15/10, and 17/13°C). Differences in embryogenic response were observed among the donor plant temperature regimes (Table 2). For five genotypes (VF1, VF3, VF4, VF10, and LF1), plants grown at 15/10°C were the most responsive.

Table 2 Effect of donor plant growth conditions on the microspore embryogenesis of bulb fennel. The results represent the total number of phenotypically normal embryos plated from five experiments

	The effect of donor plant growing temperature °C on the total no. of embryos plated from five experiments		
Genotype	10/5°C	15/10°C	17/13°C
VF1	2	117	0
VF2	491	350	0
VF3	0	120	0
VF4	47	331	85
VF8	149	125	3
VF10	20	215	0
LF1	101	449	3

For two genotypes (VF2 and VF8), the 10/5°C growing conditions for the donor plants were more favorable, although a high frequency of embryos was also observed at 15/10°C. A temperature regime of 17/13°C did not enhance embryo induction in fennel. For our experiments with anise and dill, we evaluated two donor plant temperature regimes (10/5, 20/15°C), along with two extraction, purification, and resuspension temperatures (cold and room temperature). The results indicated that the colder donor plant growing conditions (10/5°C) coupled with cold extraction, where all of the media and equipment were kept on ice, was beneficial for both anise and dill (Table 3). These conditions were the best regardless of the subsequent culture conditions (24 and 32°C for 3, 7, or 14 days). Cold stress (10/5°C) slows the growth of the plant, thereby, allowing a longer period for the production and harvest of buds. It may also slow the natural degradation processes in the anther, and, therefore, protect microspores from any detrimental compounds exuded from the anther (Duncan and Heberle 1976). The transfer of the newly cultured microspores, which were extracted under cold conditions to 24 or 32°C, also provides a significant initial temperature shock. There have been few reports on donor plant growing conditions and their effects on embryogenesis in the Apiaceae. Donor plants for anther culture experiments have been grown under field (Shon and Yoshida 1997; Dohya et al. 1997; Matsubara et al. 1995; Hu et al. 1993) or greenhouse conditions (Górecka et al. 2005). In carrot, Adamus and Michalik (2003) evaluated anther culture response from greenhouse, growth chamber (14°C), or field-grown plants. Differences were observed between the growth conditions in terms of callus production, with greenhouse plants producing the most callus and field-grown plants producing the least; however, there was no difference in embryo production. Smykalova et al. (2009) grew caraway donor plants for anther culture in the greenhouse, but provided different temperature regimes, including a cold treatment. The best response was from plants grown at 22°C for 9 days, followed by 19 days at 6°C. A cold treatment also appeared to influence coriander pollen grains by increasing the number of androgenic microspores (Bugara et al. 1985). Donor plants were initially grown in the greenhouse, and the stems were cut and kept for 15 days at 7°C; however, no embryo development was obtained.

Media composition

Media composition is another key factor influencing microspore embryogenesis. There are numerous studies evaluating basal media, carbohydrate type, and concentration, as well as the addition of biochemical supplement (Armstrong and Keller 1981; Roulund et al. 1990; Baillie et al. 1992; Powell and Uhrig 1987). In our experiments, several established basal media were compared in order to develop a DH protocol for fennel and caraway. We evaluated B5 medium, which is commonly used in many tissue culture protocols and has been used in Apiaceae anther culture and isolated microspore culture (Górecka et al. 2005; Smykalova et al. 2009; Dohya et al. 1997). NLN is used for microspore culture in *Brassica* (Lichter 1982), N6

Table 3 Response of anise and dill to donor plant conditions, temperature during microspore isolation, and incubation temperature

Donor plant growing temperature °C	Temperature during isolation	Incubation temperature °C average embryos/plate			
		24°C	32°C 3 days	32°C 7 days	32°C 14 days
Anise					
10/5°C	Room	1.06 b*	1.64 b	2.14 b	0.46 b
10/5°C	Cold	4.36 a	4.86 a	4.76 a	2.94 a
20/15°C	Room	0 b	0.02 b	0 c	0.02 b
20/15°C	Cold	0.02 b	0.04 b	0.02 c	0 b
Dill DH-12					
10/5°C	Room	0.08 ab	0.04 b	0 a	0.04 b
10/5°C	Cold	0.40 a	3.20 a	2.54 a	0.78 a
20/15°C	Room	0 b	0 b	0 a	0 b
20/15°C	Cold	0 b	0.08 b	0.02 a	0 b

*Within each species, values within one column followed by the same letter are not significantly different ($P = 0.05$)

has been used in anther culture of cereals (Chu 1978), NN has been used in anther culture of dicots (Nitsch and Nitsch 1969), and K&M has been used with *Vicia* protoplasts (Kao and Michayluk 1975). For fennel, there were no significant differences among B5, N6, and NN for embryo yield (95–107 embryos), while no embryos developed in K&M medium. The best basal medium for fennel was NLN, which yielded double (210 embryos) the number of normal embryos produced with B5, N6, or NN media (data not shown).

For caraway, five basal media (NLN, N6, NN, K&M, and AT-3) were evaluated for microspore culture response. There was no response of the microspores in any of the media except for AT-3, where fewer than ten microspore-derived embryos per plate were observed (data not shown). AT-3 medium has been used previously for tobacco microspore culture experiments (Touraev and Heberle-Bors 1999), and it differs from other media in using maltose, rather than sucrose, as a carbohydrate source. The culture medium carbohydrate source has been shown to be very important, with sucrose being the most commonly used. Sucrose has been shown to be superior to other carbohydrates for *Brassica* microspore embryogenesis (Hamaoka et al. 1991), while for many cereals, maltose is the more effective carbohydrate (Last and Brettell 1990; Orshinsky et al. 1990).

Touraev B isolation and wash medium was used in conjunction with the AT-3 culture medium. Touraev B medium contains mannitol and is buffered to pH 7.0 with phosphate buffers. Substituting mannitol for sucrose in the 1/2-strength B5 isolation/wash medium and substituting maltose for sucrose in the other basal media formulations evaluated did not produce any microspore-derived embryos. A similar experiment substituting maltose and mannitol with sucrose in the AT-3 and Touraev media did not yield any embryos. It appears that the benefits of the AT-3 culture medium and Touraev B isolation/wash media extend beyond the presence of maltose and mannitol, respectively. Further studies with caraway used the AT-3 medium.

There are very few reports in the literature of studies comparing culture media for isolated microspores of Apiaceae species. Dohya et al. (1997) evaluated three media compositions used with celery microspores. Heart-shaped embryos were observed when microspores were cultured in B5 or 1/2-strength MS medium (Murashige and Skoog 1962), but there was no response with NLN medium. For carrot-isolated microspore culture, 1/2-strength MS medium supplemented with 2,4-D (2,4-dichlorophenoxyacetic acid) and BA (N^6-benzyladenine) resulted in calli production after 5 months, but no further development was noted (Matsubara et al. 1995).

Brassinosteroids are naturally occurring, growth-promoting substances active at low concentrations and known to influence many aspects of growth and development in plants. They have also been shown to protect plants from abiotic and biotic stresses. Brassinosteroids have been used to promote somatic embryogenesis (Pullman et al. 2003) and cell division (Nakajima et al. 1996), and to increase microspore embryogenesis in several *Brassica* species (Ferrie et al. 2005). The addition of 0.1 or 1.0 mM EBR to NLN-25 resulted in a decrease in embryogenesis of fennel line VF2, as compared to the NLN-25 control media (Fig. 1c, d vs. a), while a media change to NLN-10 from NLN-25 after 3 days also had a severe negative effect (Fig. 1e, f). However, a media change after 3 days induction at 32°C from NLN-25 brassinosteroid-supplemented media to NLN-10 without EBR was stimulatory to embryo formation (Fig. 1c, d vs. g, h). The data suggest that a 72-h pulse of EBR at the beginning of the culture period is beneficial. While a change to NLN-10 from NLN-25 is quite detrimental after the 72-h 32°C heat stress treatment (Fig. 1), when the NLN-25 contains EBR, it is very beneficial to embryogenic response to change to NLN-10 in order to remove the EBR (Fig. 1). NLN-25 with DMSO also appeared to have a stimulatory effect on embryogenesis; however, after a media change to NLN-10, embryogenesis was similar to the control.

Culture conditions

The culture conditions that microspores are subjected to just after isolation are extremely important in determining the developmental pathway that the microspores will follow. After determining that AT-3 fostered embryogenesis

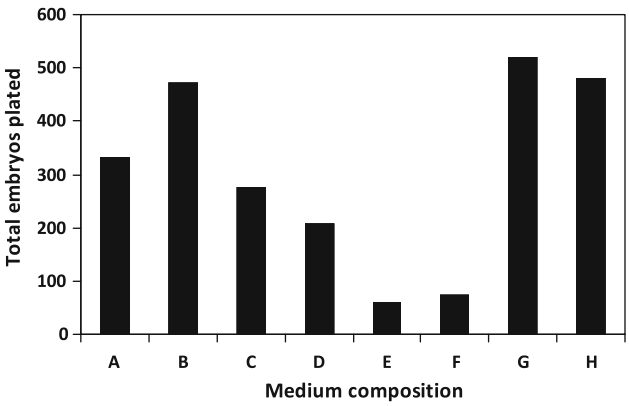

Fig. 1 Effect of 24-epibrassinolide on microspore embryogenesis of bulb fennel (genotype VF2) cultured at 32°C for 72 h, followed by transfer to 24°C. Media compositions include the following: *A* NLN-25; *B* NLN-25 with DMSO only; *C* NLN-25 + 0.1 mM EBR; *D* NLN-25 + 1.0 mM EBR; *E* NLN-25 changed to NLN-10 at transfer to 24°C; *F* NLN-25 plus DMSO changed to NLN-10 at transfer to 24°C; *G* NLN-25 + 0.1 mM EBR changed to NLN-10 at transfer to 24°C; *H* NLN-25 + 1.0 mM EBR changed to NLN-10 at transfer to 24°C. The results represent the total number of normal embryos available for plating from five experiments

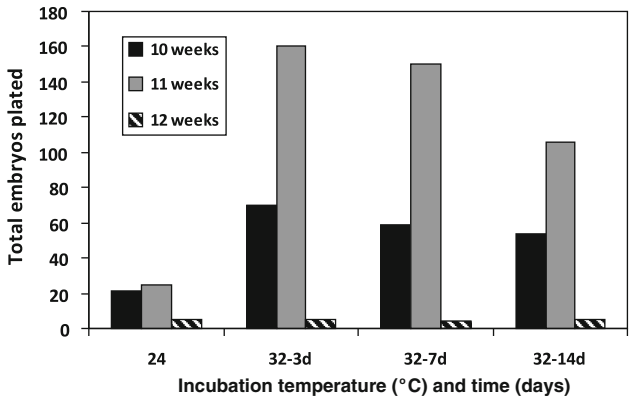

Fig. 2 Frequency of caraway (breeding line NN-1) microspore embryogenesis from three donor plant ages (10, 11, and 12 weeks) and from cultures incubated under four temperature regimes. The results represent the total number of normal-appearing embryos available for plating on germination medium

in caraway, further experiments were conducted to evaluate other culture conditions to improve embryo induction. A heat shock of 32°C had a positive influence, although embryos were also observed in cultures maintained at 24°C (Fig. 2). No significant differences in embryogenesis were observed when the microspores were cultured for 3, 7, or 14 days at 32°C. An interesting observation was noted regarding the age of the caraway donor plants and the embryogenic response of the microspores. With two of the three lines, it was obvious that microspores from 11-week-old donor plants were more embryogenic than those from either 10- or 12-week-old donor plants. For the other caraway line, NN-2, the highest frequency of embryogenesis was observed with 10-week-old plants (data not shown). The age of the donor plant at the time of microspore isolation has been shown to play a role in the induction of embryogenesis in other species (Dunwell 1976; Takahata et al. 1991); however, there have been no reports of this in the Apiaceae.

Regeneration and chromosome doubling

Plants were regenerated from 10 of the 11 Apiaceae species from which microspore-derived embryos were obtained. Colchicine treatment for chromosome doubling contributed to poor survival of the anise, dill, and caraway plantlets upon transfer to soil. When the colchicine treatment was eliminated from the regeneration protocol, plantlet survival was enhanced and spontaneous chromosome doubling occurred, as determined by flow cytometry and the presence of fertile flowers in approximately 50% of the established plantlets. For this study, genetic markers were not utilized but further investigations could use such an approach to confirm homozygosity of the diploid plants.

Conclusion

Although routine, highly efficient, doubled haploid protocols were not obtained, embryo development was reported in 11 out of 20 Apiaceae species evaluated, and doubled haploid plants and seed were produced from 10 of the 11 responsive species. The numbers of embryos produced were low, but we have shown that it is feasible to produce microspore-derived embryos and regenerate them into plants that can be incorporated into breeding programs (Ferrie et al. submitted). Further research focused on evaluating the different factors influencing embryogenesis is needed to produce an improved doubled haploid protocol. Such a protocol could be used to enhance basic research on and the breeding of the Apiaceae species.

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