MATURATION, DESICCATION, GERMINATION AND STORAGE LIPID ACCUMULATION IN MICROSPORE EMBRYOS OF BRASSICA NAPUS L.

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INTRODUCTION

Isolated microspore culture of Brassica napus has been well characterized (Lichter 1982; Chuong and Beversdorf 1985; Kott et al. 1990) and is now being used routinely in basic and applied research. This paper reports the results of studies on the developmental biology of embryogenesis in microspore embryos as affected by culture environment. Manipulation of the culture media and conditions produced microspore embryos of B. napus that will germinate readily (Mathias 1988), and somatic embryos of Medicago sativa to withstand dessication (Anandarajah and McKersie 1990). In vitro culture can produce zygotic embryos (Finklestein and Somerville 1989) and microspore embryos (Taylor et al. 1990) of B. napus that accumulate fatty acid compositions which are similar to seed fatty acid compositions. We describe a culture system that does all of these in B. napus microspore embryos.

MATERIALS AND METHODS

Donor plants:

The rapeseed (Brassica napus L.) genotypes used for the fatty acid accumulation study were the cultivars Profit, a canola type; BN0300, a proprietary high oleic mutant out of Regent; and a high erucic type, an F3 selection 89M003-5-66. The cultivars used for the maturation and germination study were the canola types Profit, Topas, and H51, a highly embryogenic line out of Altex. Donor plants for microspore culture were grown at 10°/5° C. day/night with a 16 hour day under cool white fluorescent light at 150 μE s^{-1} m^{-2}.

Microspore Culture:

The culture method used was similar to Chuong and Beversdorf (1985) with the following changes: the microspores were plated at a a density of 60,000 cells/ml in NLN medium (Nitsch and Nitsch 1967) as modified by Lichter(1981) but without potato extract or growth hormones. Cultures were incubated in the dark at 32° C. for three days followed by 18 days of culture at 25° C. After the initial 21 day culture period the cultures were transferred to various liquid media, shaken at 60 rpm, and cultured in no or low light (15 μE s^{-1} m^{-2}, 16 h day), to study aspects of embryo maturation. After maturation, the embryos were germinated on solid B5 medium (Gamborg et al. 1968) in bright fluorescent light (70-100 μE s^{-1} m^{-2} , 16 h day).

Embryo Maturation studies:

At 21 days after culture (DAC) the embryos were at various stages of development but most were at the late torpedo to cotyledonary stage, with the largest being about

5mm in length. The embryos were then moved to differing medium sequences, to study media effects on subsequent embryo development and germination. To transfer, the old medium was removed and fresh medium was added to the same plate. The maturation factors that were studied involved the presence or absence of three conditions: 1) an embryo enlargement phase; 2) an abcisic acid (ABA) phase; and 3) an embryo dessication phase. The enlargement phase involved transferring the embryos to NLN, B5W (B5 with 13% sucrose), or B5L (B5 with 2% sucrose) liquid media for 7-11 days. The ABA phase involved transferring the embryos to B5A (liquid B5 with 13% sucrose and 15 μM ABA,) or NLNA (liquid NLN with 13% sucrose and 15 μM ABA). After each of these phases the embryos were plated on B5 solid medium(0.8% agar) for germination. The media used are summerized in Table 1.

The cotyledonary stage embryos were dried by removing the remaining medium and putting the unwrapped Petri plates into a laminar flow hood for one week. The desiccated or non-desiccated embryos were scored for viability and germinability. Viability was defined as the ability of the embryos to turn green and grow roots on B5 medium. Germinability was defined as the ability of viable embryos to grow a shoot from the apex. In some cases embryo length was measured before germination and related to germinability.

Fatty Acid Accumulation Study:

Samples of the microspore cultures were taken after 21 days on NLN in the dark followed by 21 days on B5A in the light and analyzed using gas chromatography (GC). For the cultures 42 DAC five embryos of normal appearence 4-7mm in length were sampled and analyzed in bulk. Fatty acid composition was determined using GC analysis of methyl esterified total lipid extracts by the method of Christie (1982). Each extracted sample was analyzed twice. Seeds of each parental type were also analyzed.

RESULTS

Embryo Germination:

Table 2 shows the results of the various medium sequences on germination of embryos of the genotype H51. The highest rate of germination was 90.3% where the embryos, after 21 days in the induction medium NLN, were transferred to B5W for 10 days, then to B5A for 10 days before being germinated. The addition of an enlarging step alone was beneficial with B5W and NLN superior to B5L (26.7% and 25.0% germination vs. 11.1% germination, Table 2). The embryos in the high-sucrose media B5W and NLN grew slowly over the culture period with most of the embryos becoming 3-10mm long. The embryos in the low-sucrose B5L elongated up to 20-24mm in length. Transferring directly to the ABA-containing medium NLNA (43.3% germination) was an improvement over no maturation steps at all or the enlarging step alone.

The B5L medium in any part of the sequence was usually detrimental, and resulted in lower germination rates (Table 2). This illustrated the need for maintaining high sucrose levels throughout the maturation sequence. This negative effect could be partially overcome by using B5A as the next step in the sequence.

Embryo Desiccation, Germination, and Viability:

The data in Tables 2 and 3 show that desiccation tolerance is greatly enhanced when maturation medium sequences include an ABA-containing medium. The highest survival without ABA was 11% with only 2.3% germination, using B5W as an enlarging medium (Table 2). Table 3 shows no embryo viability at all for the cultivar Topas unless a B5A step is used before dessication. In a separate experiment (Table 4) cultivar Profit shows high rates of germination (85.2%) and viability (100%). Upon complete maturation a sample was plated to B5 for germination or desiccated and then germinated. Rapid drying of the embryos over just a few days resulted in low viability, so drying had to be done gradually over a period of about a week (unpublished data). Table 4 also shows that there is a distinct improvement in germination when the smaller size class is plated. This was consistant over several experiments (unpublished data) with the most responsive embryos in the 3-7mm range.

Fatty Acid Accmulation:

The use of NLN for the initial 21 days followed by B5A for 21 more days in cultures of Profit, BN0300, and 89M003-5-66 resulted in levels of oleic (18:1), eicosenoic (20:1), and erucic (22:1) acids very much like that of seed (Table 5). For Profit and BN0300 the fatty acid levels in the matured microspore embryos were almost identical to the fatty acid levels in the parental seed. The highest accumulation of erucic acid in microspore embryos was 21.1% with the total of eicosenoic and erucic acids being 37.6% in genotype 89M003-5-66 (Table 5).

DISCUSSION

Although producing embryos from rapeseed microspore cultures is now routine, germinating microspore embryos has been a problem in the past. Rapeseed microspore embryos that are not matured typically germinate at a rate of less than 5% (Mathias 1989). Our lab produces microspore embryos that germinate at 5-10% without maturation. Most of the embryos develop into structures with swollen cotyledons and elongated hypocotyls with secondary embryos growing on them.

The embryo maturation system described in this paper results in embryos that are easy to germinate and may be desiccated for future regeneration. The quality of the plants produced from the germinated microspore embryos is excellent, similar to seed-derived plants. In B. napus microspore embryos germination frequencies were improved up to 50% (Mathias 1989) by culturing torpedo stage embryos on high (1.5%) agar concentrations. Both of these systems involve a high osmotic or drying stage.

There is evidence that the application of high osmoticum or drying induces endogenous production of ABA in zygotic embryos grown in vitro (Finkelstein and Crouch 1987). Dessication tolerance has been induced with sucrose, heat shock and ABA in alfalfa (Anandarajah and McKersie 1990). It is not necessary to apply exogenous ABA to the culture medium for canola microspores to accumulate fatty acid profiles similar to parental fatty acid profiles (Taylor et al. 1990). Exogenous ABA is needed to produce matured embryos that germinate at a high frequency and are able to survive dessication. The accumulation of C22:1 fatty acids in

microspore embryos approaches but does not equal that of seed, even after maturing them on high sucrose and ABA for an extended time. Clearly there are other factors involved in the accumulation of C20:1 and C22:1 fatty acids besides ABA or high sucrose, although both are important in other aspects of embryo maturation. The fatty acid compositions in microspore embryos in all the genotypes studied were comparable to the parental seed.

Our results describe a simple efficient system that may be applied to *in vitro* studies of genetic and biochemical regulation in developing embryos. Researchers may also use the system to streamline regeneration for application in plant breeding.

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TABLES

Table 1. Media used in various maturation experiments.

	Maturation Phases			
Initiation	Enlargement	ABA	Desiccation	Germination
NLN	NLN	NLNA	1 week	B5
	B5L	B5A	none	
	B5W	none		
	none			

Table 2. The effects of various maturation medium sequences on embryo germination, genotype H51.

Enlargement medium	ABA medium	Dry stage	No. Germ.	No. Total	% Germ.	% Viable
B5L			5	45	11.1	100.0
B5W			12	45	26.7	100.0
NLN			10	40	25.0	100.0
	NLNA		87	201	43.3	100.0
B5L	B5A		14	20	70.0	100.0
B5W	B5A		28	31	90.3	100.0
B5L		DRY	1	30	3.3	3.3
NLN		DRY	1	60	1.7	8.0
B5W		DRY	1	44	2.3	11.0
B5W	B5A	DRY	13	42	31.0	45.0

Table 3. The effects of various maturation medium sequences on viability and germination of desiccated embryos, genotype Topas.

Enlargement medium	ABA medium	Dry stage	No. Germ.	No. Total	% Germ.	% Viable
B5L		DRY	0	30	0.0	0.0
B5W		DRY	0	30	0.0	0.0
NLN		DRY	0	70	0.0	0.0
B5L	B5A	DRY	0	32	0.0	0.0
B5W	B5A	DRY	9	52	17.3	33.0
NLN	B5A	DRY	50	111	45.0	65.0
B5L	NLNA	DRY	11	33	33.3	48.0
NLN	NLNA	DRY	13	80	16.3	46.3

Table 4. Embryo germination and viability in two size classes of pre- and post-desiccated embryos, genotype Profit.

Size (mm)	Pre-dry germinated No.	%	Post-dry germinated No.	%	Post-dry viability %
2-3	35/80	43.8	52/61	85.2	100.0
5-8	23/80	28.8	32/78	41.0	100.0

Table 5. Comparison of matured microspore embryo and seed oleic (18:1), eicosenoic (20:1), and erucic (22:1) acids. Standard deviation in parentheses.

Genotype	Tissue	18:1	20:1	22:1
Profit	embryos	70.6 (0.51)	0.6 (0.08)	0 (0.0)
	seeds	59.9 (1.18)	trace	trace
BN0300	embryos	80.8 (0.61)	0.7 (0.06)	0.0 (0.0)
	seeds	79.8 (0.84)	0.8 (0.12)	0.0 (0.0)
89M003 -5-66	embryos	33.8 (0.4)	16.5 (0.10)	21.1 (0.80)
	seeds	16.2 (2.23)	8.9 (0.83)	41.9 (2.14)