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The microspore-derived embryo of *Brassica napus* L. as a tool for studying embryo-specific lipid biogenesis and regulation of oil quality

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Summary. A time-course study of lipid accumulation in microspore-derived embryos and developing zygotic embryos of rapeseed (*Brassica napus* L. ssp. *oleifera*) is presented. Rapid storage fat (triacylglycerol) biosynthesis was induced in microspore-derived embryos of oilseed rape (cv 'Topas') when the embryos were transferred from standing cultures (10 ml) to fresh medium (75 ml) and shake cultured. Triacylglycerols accumulated, after a lag period of 7 days, at a linear rate of approximately twice that of the developing zygotic embryo. The fatty acid composition of triacylglycerols in microspore-derived embryos closely parallelled that of the developing zygotic embryos. In the microspore-derived embryos, the amount of phosphatidylcholine, the major substrate for the production of polyunsaturated fatty acids in oilseeds, remained constant during the linear phase of triacylglycerol production, whereas it increased steadily in the zygotic embryos. The fatty acid composition of individual cotyledons from microspore embryos shake cultured for 15 days was compared with that of individual mature seeds. Relative amounts of the major fatty acids, i.e. palmitic, oleic and linoleic acids, were essentially the same, whereas the microspore-derived embryos had about 35% less stearic acid and 35% more linolenic acid than the mature seeds. Variation in the amounts of oleic, linoleic and linolenic acids between seeds was similar to that found between cotyledons of microspore-derived embryos, whereas variation in palmitic and stearic acid levels was significantly lower between microspore-derived cotyledons than between the seeds. The results indicate that microspore-derived embryos from shake cultures should be convenient for use in studying the regulation of oil biosynthesis and for rapidly screening for oil quality in genetically altered rapeseed.

Key words: *Brassica* – Embryo – Lipid biosynthesis – Microspore – Oil quality – Oilseed breeding – Screening method

Introduction

Embryos derived from microspores of oilseed rape (*Brassica napus*) accumulate storage products characteristic of the zygotic embryos. These products include proteins (Crouch 1982) and lipids (De La Roche and Keller 1977). De La Roche and Keller (1977) reported that relative amounts of the fatty acids differed greatly between microspore-derived embryos and zygotic embryos, although the seed-specific erucic acid accumulated in both types. Recently, however, Taylor and coworkers (1990) showed that at a certain stage of development microspore-derived embryos from both low and high erucic acid-containing varieties of rape cultivated in standing cultures in petri dishes can accumulate triacyglycerols with a fatty acid composition nearly identical to that of the oil in the seeds.

In light of these facts, we considered it worthwhile to evaluate the use of microspore-derived embryos when screening for oil quality in plant breeding programmes. In this article we present culture conditions under which microspore-derived embryos of rapeseed accumulate triacylglycerols at rates exceeding those of the zygotic embryos and with a quality very similar to that of the oil in the developing zygotic embryo.

Materials and methods

Plant material

Brassica napus L. ssp. *oleifera* cv 'Olga' (kindly provided by Weibulls, Landskrona, Sweden) was used for isolation of zygotic

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embryos. The plants were grown in climate chambers under a 16 h:8 h light/dark regime. The temperature was 24 °C during the light period and 16 °C during the dark period. Microspore cultures were obtained from *Brassica napus*, L. spp. *oleifera* cv 'Topas' (kindly provided by Svalöf AB, Svalöf, Sweden). The seed oil of the two rape varieties had virtually the same fatty acid composition with the following approximate molar % distribution: $C_{16:0}=4.5$, $C_{18:0}=1.5$, $C_{18:1}=63$, $C_{18:2}=19$, $C_{18:3}=10$, and with 2%–3% of other fatty acids.

Cultivation of microspore embryos

Microspores were isolated and cultivated according to a modification of the protocol of Choung and Beversdorf (1985). Plants (cv 'Topas') were cultured under greenhouse conditions until young flower buds had developed. The plants were thereafter transferred to a growth chamber and cultured for 7 days under a 16 h/8 h light/dark regime (10 °C and 4 °C during the light and dark periods, respectively and with a light intensity of 280μmol m^{-2} s^{-1} obtained from halogen lamps). To isolate microspores the young flower buds (2–4 mm) from the main raceme and lateral branches were surface sterilized for 3–5 min in 70% ethanol. The buds were immediately macerated in B5 medium (Gamborg et al. 1968) supplemented with 13% (w/v) sucrose, filtered through a 50-μm nylon mesh, pelleted by centrifugation at 100 *g* for 5 min and washed in the same medium. The microspores were thereafter resuspended in culture medium containing the macro- and micronutrients and vitamins (without folic acid) essentially according to Nitsch and Nitsch (1967) but with 13% sucrose and no hormones, and adjusted to pH 5.8. Ten milliliter aliquots of the suspension ($20–40\times10^3$ microspores/ml) were dispensed in 9-cm petri dishes. The microspores were incubated for an initial period of 72 h at 33 °C in the dark followed by 23 days of dark culture at 25 °C. On average, about 0.5% of the isolated microspores developed into embryos. Twenty-six days after isolation of the microspores, the microspore-derived embryos were transferred to 100-ml Erlenmeyer flasks (ca. 100 embryos/flask) containing 75 ml fresh culture medium and incubated while undergoing continuous circular shaking (200 rpm) in darkness at 25 °C. After defined periods of time the embryos were removed for lipid analysis.

Lipid extraction and analysis

Twenty to fifty embryos were dissected out from developing seeds at defined times after anthesis and homogenized with an Ultra-Turrax in a mixture containing 1 ml 0.15 *M* acetic acid, 1.25 ml chloroform and 2.5 ml of methanol. By adding 1 ml of water and 1.25 ml of chloroform a two-phase system, according to Bligh and Dyer (1959), was obtained. The lipid-containing chloroform layer was removed, evaporated to dryness under nitrogen and resuspended in a small volume of chloroform. Lipid extraction of microspore-derived embryos from shake cultures (at least duplicate samples with 50 randomly chosen embryos from each of two incubation flasks at each developmental stage) was performed in a similar way. Triacylglycerols and phosphatidylcholine were separated from the chloroform extracts on silica-gel TLC plates (Merck silica-gel 60) in *n*-hexane:diethylether:acetic acid (70:30:1 by volume) and chloroform:methanol:acetic acid:water (85:15:10:3.5 by volume), respectively. Lipids in areas corresponding to those to which reference substances had migrated were removed from the gel and methylated *in situ* with 2 ml 4% HCl in methanol at 85 °C for 45 min. The methyl esters were extracted in hexane and separated by gas chromatography (GC) on a 2.5 m × 3 mm i.d. glass column containing 3% SP-2300 on a Supelcoport 100/120 mesh (Supelco, Bellefonte, P., USA), and their amounts quantified relative to added methyl-heptacanoate (17:0-Me).

Fatty acid composition was determined in half cotyledons that had been removed from either microspore embryos after 15 days in shake culture (fresh weight ca. 35 mg/embryo and mean cotyledon diameter of 4 mm) or from 41-day-old embryos (fresh weight ca. 5 mg) retained in standing culture, and from whole, crushed seeds. The cotyledons and seeds were directly methylated in 1 ml of a mixture of 4% HCl in methanol at 85 °C for 45 min. The methyl esters were extracted by addition of 400 μl *n*-hexane and 1 ml water. An aliquot (100 μl) of the hexane phase was transferred to micro-vials and methyl ester composition determined by GC as described above except that the aliquots were injected by a Shimadzu AOC-9 automatic sample injector.

Results

Lipid accumulation in microspore-derived embryos and in developing zygotic seeds

After a certain lag period, microspore-derived embryos in shake culture began to accumulate triacylglycerol in a rapid and linear fashion. There was a great variation in the size of the microspore-derived embryos in standing cultures, but when the embryos were transferred to shake-cultures embryo growth became synchronized during the lag period prior to the start of the oil accumulation phase. As a result, embryos in the same flask were similar in size after 15 days in the shake culture. Increasing the number of embryos per unit volume medium substantially decreased the rates of oil deposition and embryo growth (data not shown). Microspore-derived embryos retained in the standing culture in the petri dishes accumulated triacylglycerol slowly (data not shown) and produced an oil with a much lower content of polyunsaturated fatty acids (see Table 1).

Oil deposition was monitored in microspore-derived embryos for up to 22 days in shake culture (about 100 embryos/flask), and their rates of triacylglycerol accumulation was compared with those of zygotic embryos obtained from plants grown under similar temperature regimes. During the linear phase of triacylglycerol deposition (i.e. beginning on the 7th day in shake culture for the microspore-derived embryos and on the 18th day after anthesis for the zygotic embryos) the rates of accumulation in microspore-derived embryos were about twice those of the zygotic embryos (91 and 56 nmol triacylglycerols accumulated per embryo and day, respectively [Fig. 1 A, B]).

Phosphatidylcholine, the major phospholipid in both the microspore-derived embryos and the developing zygotic embryo (data not shown), approximately tripled in content in the microspore-derived embryos during the first 7 days in shake culture, but remained more or less constant after this (Fig. 1 C). By contrast, in zygotic embryos there was a linear increase in the content of this lipid between days 17 and 38 after anthesis (Fig. 1 D).

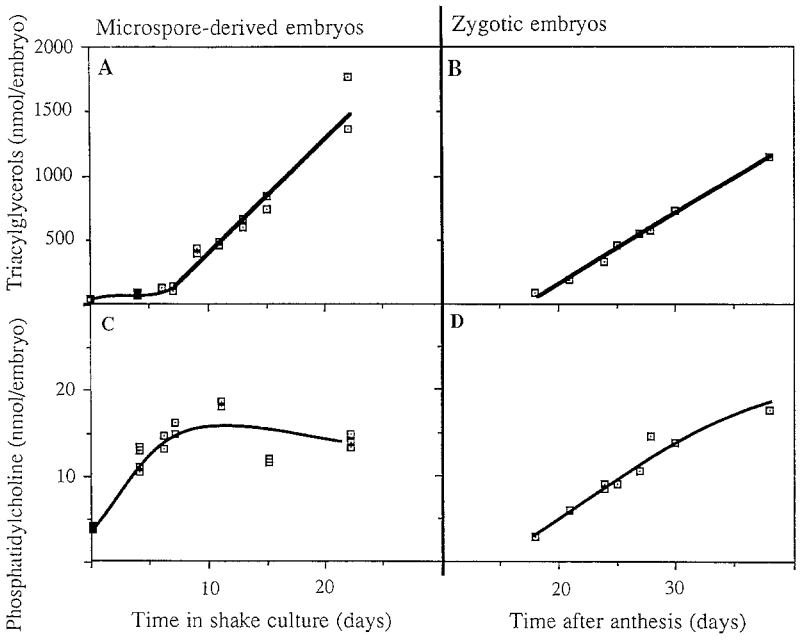

Fig. 1 A–D. Accumulation of triacylglycerol (**A** and **B**) and phosphatidylcholine (**C** and **D**) in microspore-derived embryos in shake cultures (**A** and **C**) and in developing zygotic embryos (**B** and **D**) of rape

Table 1. Relative amounts of the five major fatty acids in cotyledons from 41-day-old individual microspore-derived embryos and in individual mature seeds of rape (cv 'Topas'). Cotyledons from the microspore-derived embryos were analysed after 41 days in standing cultures or after subculturing 26-day old embryos from standing culture for 15 days in shake culture. The individual values for the microspore-derived embryos in shake culture and the seeds are presented in graphic form in Fig. 4

Fatty acid	Tissue	*n*	mol%		
			Mean	Median	Standard deviation
Palmitic	Microspore embryo in standing culture	87	5.24	5.18	0.50
	Microspore embryo in shake culture	93	4.14	4.08	0.27
	Seed	87	4.68	4.63	0.49
Stearic	Microspore embryo in standing culture	87	2.20	2.07	0.61
	Microspore embryo in shake culture	93	1.00	0.97	0.13
	Seed	87	1.56	1.54	0.20
Oleic	Microspore embryo in standing culture	87	73.3	73.5	3.12
	Microspore embryo in shake culture	93	63.3	63.5	2.66
	Seed	87	63.2	63.2	2.87
Linoleic	Microspore embryo in standing culture	87	11.2	11.2	1.71
	Microspore embryo in shake culture	93	18.5	18.0	2.07
	Seed	87	19.3	18.9	2.01
Linolenic	Microspore embryo in standing culture	87	6.54	6.36	2.00
	Microspore embryo in shake culture	93	13.1	13.0	1.20
	Seed	87	9.77	10.0	1.23

Relative amounts of the major fatty acids (palmitic, stearic, oleic, linoleic and linolenic) in the triacylglycerols showed similar changes during the linear phase of oil deposition in microspore-derived embryos and zygotic embryos (Fig. 2). Palmitic, stearic and linoleic acids showed a relative decrease, whereas there was a corresponding increase in oleic acid and the level of linolenic acid remained relatively constant. However, during the first 7 days in shake culture there was a relative increase in linoleic acid at the expense of oleic acid in the microspore embryos (Fig. 2).

The changes in the relative amounts of the fatty acids during development were more pronounced in phosphatidylcholine than in the triacylglycerols for both the microspore and the zygotic embryos, but the trends were similar (Fig. 3).

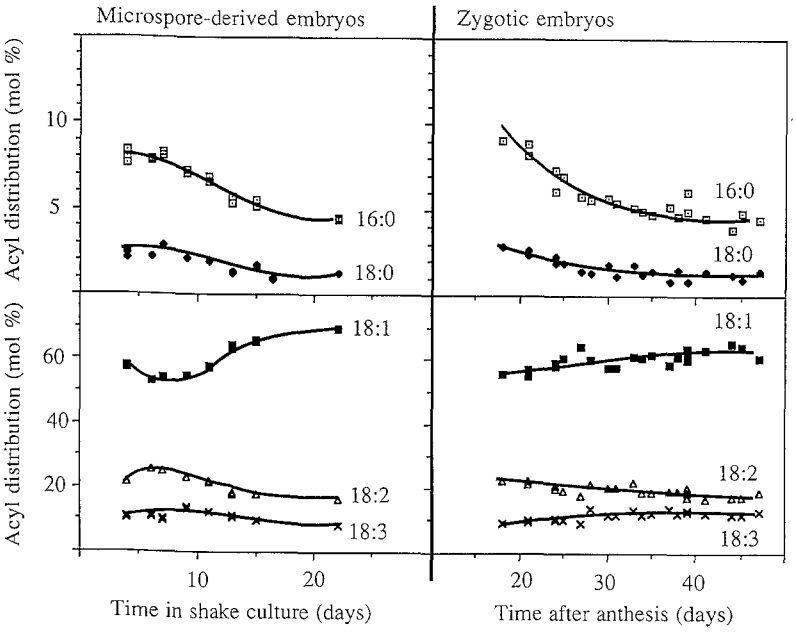

Fig. 2. Relative contents of fatty acids in the triacylglycerols from rape microspore-derived embryos in shake culture and in developing zygotic embryos. □ Palmitic acid (16:0), ◆ stearic acid (18:0) ■ oleic acid (18:1), △ linoleic acid (18:2), × linolenic acid (18:3)

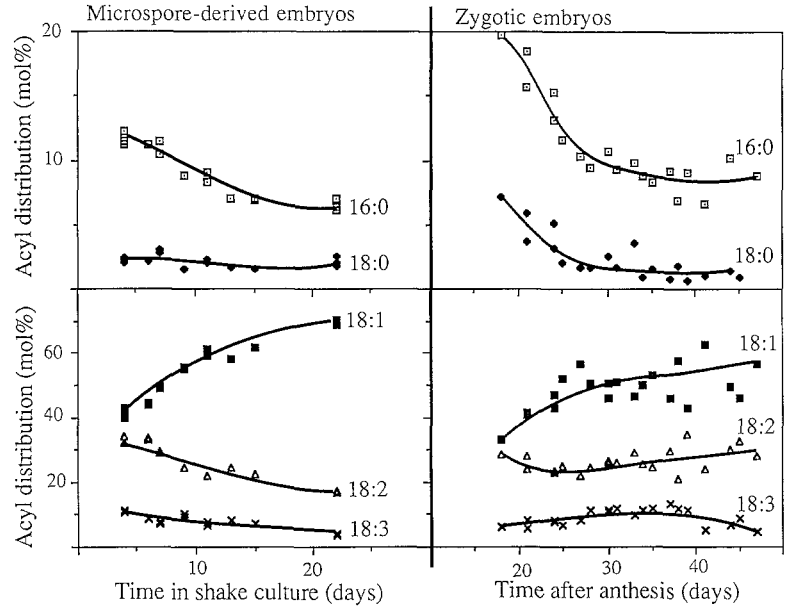

Fig. 3. Relative contents of fatty acids in the phosphatidylcholine from rape microspore-derived embryos in shake culture and in developing zygotic embryos of rape. □ Palmitic acid (16:0), ◆ stearic acid (18:0) ■ oleic acid (18:1), △ linoleic acid (18:2), × linolenic acid (18:3)

Fatty acid composition of cotyledons of individual microspore-derived embryos and zygotic seeds

To evaluate the use of microspore-derived embryos when screening for oil quality in plant breeding programmes, we compared the total fatty acid compositions of cotyledons dissected from individual embryos after 15 days in shake culture with those of embryos of the same age that had been retained in standing culture. At this age, the embryos in standing culture had small cotyledons (average diameter: 1 mm) and an average fresh weight of 5 mg, whereas the embryos from shake cultures had well-developed cotyledons (average diameter: 4 mm) and a fresh weight of approximately 35 mg. The fatty acid composition of part of a cotyledon was determined after dissecting it from an embryo, the rest of the embryo was cultured to a plant. For comparative purposes the fatty acid compositions of individual mature seeds were also determined.

The relative amounts of plamitic, oleic and linoleic acids in the microspore-derived embryos from the shake culture were about the same as those in the mature seeds.

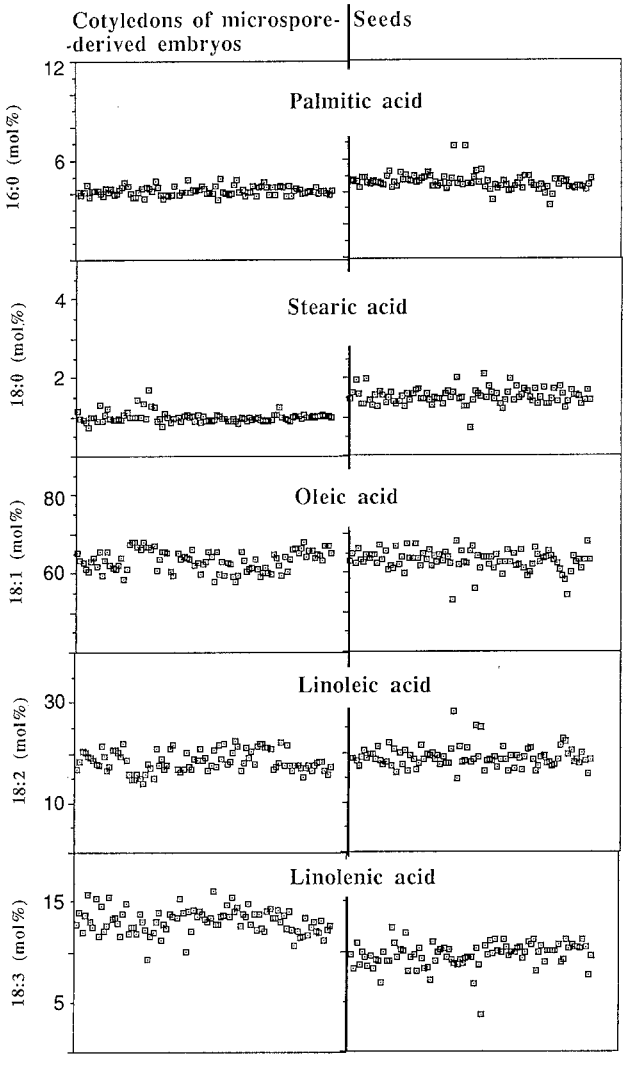

Fig. 4. Relative contents of fatty acids in individual cotyledons from microspore-derived embryos (cv 'Topas') shake cultured for 15 days and in individual mature seeds of rape (cv 'Topas'). Statistical analysis of the data is presented in Table 1

On the other hand, the microspore-derived embryos had about 35% less stearic acid and 35% more linolenic (Table 1, Fig. 4). Variation (standard deviation) in the relative amounts of oleic, linoleic and linolenic acids between seeds was similar to that found between cotyledons of microspore embryos in shake culture, whereas for palmitic and stearic acids there was significantly less variation between microspore-derived cotyledons (Table 1). The percentage of oleic acid was considerably higher in cotyledons from microspore-derived embryos in standing cultures (14%) than in seeds, whereas the relative amounts of polyunsaturated fatty acids were only 60% as high as those in the seeds (Table 1). Individual variation in the levels of stearic and linolenic acids between cotyledons from the embryos in standing cultures was considerably higher than that found between seeds.

Discussion

Lipid accumulation

Taylor et al. (1990) found that in microspore-derived embryos of rapeseed in standing cultures the relative proportions of polyunsaturated fatty acids progressively decreased and, at a certain stage in their development, they had triacylglycerols with an acyl composition similar to that of mature seed. In our work we have shown that the subculture of microspore-derived embryos to large volumes of medium and subsequent shake culture results in the synthesis of triacylglycerols with acyl compositions at rates that closely resemble those occurring in the developing zygotic seed. The enhancement of embryo growth and oil accumulation obtained by shaking was probably due to a dilution of inhibitory substances excreted by the embryo, as indicated by two observations: the rate of accumulation was inversely related to the number of embryos per unit volume of medium in the shake culture; the transfer of young embryos to petri dishes with semi-solid medium containing activated charcoal affected embryo growth in a way similar to that of the shake culturing (Lars Johansson, personal communication). Changes in the acyl composition of the triacylglycerols during the development of the microspore-derived embryos in shake culture showed trends similar to those observed in the seed embryos. This indicates that the genes determining the composition of the triacylglycerols are expressed similarly in both the microspore-derived embryo and the zygotic embryo.

The production of polyunsaturated fatty acids in Sycamore cell suspensions has been shown to depend on oxygen concentration (Bligny et al. 1980). The fact that the relative amounts of polyunsaturated fatty acids in embryos retained in standing cultures were lower than those in shake cultures might have been due to low oxygen levels in the non-aerated petri dishes, which could have retarded the desaturation process. However, this possibility was not further investigated.

Whereas the developing microspore-derived embryos in shake culture and the developing zygotic seed were similar in terms of their triacylglycerol deposition and composition, phosphatidylcholine accumulation and content differed between the two types of embroys, i.e. the phosphatidylcholine content of microspore-derived embryos remained more or less constant throughout the linear phase of triacylglycerol accumulation, but the zygotic embryos showed a steady increase in the content of this lipid during the corresponding period. The fatty acid compositions of phosphatidylcholine and triacylglycerols were similar in both types of embryos. Since phosphatidylcholine is the major substrate for linoleic and linolenic acid production in oilseeds (Stymne and Stobart 1987) these similarities suggest that the levels of phosphatidylcholine in the embryos do not play an im-

portant role in determining the levels of polyunsaturated fatty acids in the triacylglycerols. In both types of embryos the acyl groups in phosphatidylcholine seem to be in close equilibrium with the substrates of the glycerol 3-phosphate pathway [(i.e. acyl-CoA and diacylglycerols, see Stymne and Stobart (1987)] that leads to the formation of triacylglycerols. It is therefore probable that the main factor controlling the polyunsaturation level in rapeseed oil is the relation between desaturase activities and the rates of triacylglycerol production (Mazliak 1988).

In view of the striking similarities between shake-cultured microspore-derived embryos and the zygotic embryos in terms of their triacylglycerol deposition rate and composition, we suggest that microspore-derived embryos would be highly suitable for use in studying the control of oil biosynthesis in rapeseed. Such an experimental system would allow us to study the effects of parameters that are difficult to manipulate in the developing zygotic embryo, for example, the influence of external factors (sucrose concentration, light, hormones, etc.) on lipid biogenesis.

Oil quality

Microspore embryos from shake cultures could also be used in screening for oil quality in plant breeding programmes, especially since the amount of variation in the major fatty acids between microspore embryos was equal to or less than that found in the seeds and because there were strong similarities between embryo types with respect to relative amounts of the major fatty acids. The use of microspore-derived embryos offers several advantages over the conventional method of screening seeds. Because microspore-derived embryos are haploid, characteristics determined by recessive genes will also be expressed. Except for plant cultivation for microspore production and crossings of selected individuals all of the work can be done in the laboratory thereby avoiding labour-intensive and expensive field work. Furthermore, if mutagenesis or gene transfer aimed at modifying oil quality were to be done at the microspore level, screening could be carried out after only 1.5 months. Consequently, mucht time could be saved, since experiments performed on zygotic material take much longer. Since individual microspore-derived embryos can be regenerated to fertile double-haploid plants this approach is a promising reality for the future.

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