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A microspore embryogenesis protocol for *Camelina sativa,* a multi-use crop

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Abstract

Camelina [*Camelina sativa* (L.) Crantz], a member of the Brassicaceae family, has a unique oil profile that has potential both for biofuels and as a food crop. It is essential to have a doubled haploidy protocol in order to enhance breeding of this crop for prairie conditions as well as improve the yield and quality characteristics, Microspore-derived embryos have been produced from *Camelina sativa*. Buds 1 – 3 mm in length were selected for culture. The microspores were isolated and purified in full-strength B5 extraction medium and cultured in NLN medium with 12.5% sucrose and 12.5% polyethylene glycol 4000 (PEG) without glutamine, at a density of 10,000 microspores per mL. Glutamine was added to the cultures 72 h after extraction to give a final concentration of 0.8 g/L. The microspore cultures were maintained at 24°C in the dark. After 28 days embryos were observed and these were regenerated to plants and selfed seed was produced. The highest embryogenic frequency achieved was 38 microspore-derived embryos from 100,000 microspores.

Keywords: *Camelina sativa*, doubled haploidy, haploids, microspore culture, microspore embryogenesis

Introduction

Camelina sativa (L.) Crantz is an underexploited member of the Brassicaeae family that has generated renewed interest because of its unique seed oil profile. The oil content of the seed is approximately 38 – 43% with the major fatty acids being linolenic (36.2 – 39.4%), oleic (12.8 – 14.7%), linoleic (16.3 – 17.2%), and eicosenoic (14.0 – 15.5%) (Gugel and Falk 2006). Erucic acid is low (3%; Zubr 1997), as are the glucosinolates, when compared to other species (Schuster and Friedt 1998). This species has potential for human and animal nutrition and as an industrial crop. The meal can be used for livestock feed (poultry, dairy, aquaculture), fertilizer, or as protein fractions. The oil has use as a biofuel, edible oil, cosmetic, pharmaceutical, or lubricant. *Camelina sativa* is more resistant to drought and more tolerant to flea beetles (Gugel and Falk 2006) when compared to *Brassica* species; it also has resistance to *Leptosphaeria maculans* (Salisbury 1987) and *Alternaria brassicae* (Conn et al. 1988). Camelina has great potential in western Canada because of these attributes: however, further improvements in germplasm, i.e. increased yield, larger seed, higher oil content, and better meal quality, would be advantageous. There is currently limited commercial production of camelina in North America and very few breeding programs. Mutation breeding (i.e. seed mutagenesis) has resulted in lines shorter or taller than the parental line or with higher linolenic levels (Vollmann et al. 1997; Buchsenschutz-Nothdurft et al. 1998).

There have been very few published *in vitro* studies on camelina (Tattersall and Millam 1999; Kuvshinov et al. 2002). Protoplasts of camelina have been used in somatic hybridization with *Brassica* species; however, in all cases, the focus of these studies was to improve the *Brassica* species (Narasimhula et al. 1994; Hansen 1998; Sigareva and Earle 1999; Jiang et al. 2009).

Doubled haploidy (DH) protocols have been used in breeding programs for many years to develop improved crop varieties (Thomas et al. 2003, Ferrie and Mollers 2011). In order for doubled haploidy to be effective in a breeding program, an efficient microspore culture protocol is required. To our knowledge, there are no published haploidy protocols for camelina. The conditions leading to the induction and development of microspore-derived embryos vary depending on the species, and therefore doubled haploidy methods have to be determined for each species. A number of factors influence microspore embryogenesis including genotype, stage of microspore development, donor

plant growing conditions, media composition, and culture conditions. The objective of this study was to evaluate factors influencing microspore embryogenesis, and to develop doubled haploidy protocol for camelina.

Materials and methods

Camelina sativa seed (genotype 933) was kindly provided by Dr Mark Smith and D. Puttick of the Plant Biotechnology Institute, Saskatoon, Saskatchewan, Canada.

Four seeds were initially planted in 15 cm pots filled with a commercial greenhouse, soil-less mixture (Sunshine #4) supplemented with 14-14-14 slow release fertilizer (Nutricote). Once established, the plants were thinned to two per pot. Pots were placed in growth chambers with a 16 h photoperiod, a day/night temperature regime of 20/15°C, and a light intensity of 440 $\mu mols^{-1}m^{-2}$. Plants were watered three times a week with 0.35g/L of 20-20-20 (20% nitrogen, 20% phosphorus, 20% potassium) dissolved in tempered tap water.

Microspore culture

Inflorescences were removed from the plant, collected on damp paper towels, and kept on ice during bud selection. All chemicals and equipment were kept cold through-out the experiment. The buds (1 – 3 mm) were put into stainless steel baskets and rinsed in 70% EtOH for 10 - 12 s. The ethanol was decanted and replaced with sterile double-distilled water for 10 – 12 s to remove all traces of ethanol. After removing the water, the buds were sterilized for 15 min in 100% bleach (6% sodium hypochlorite) followed by 3 X 5 min rinses of cold, sterile water. Buds were then transferred to a Waring blender cup (37 – 110 mL capacity) containing 50 mL of half-strength B5 medium (Gamborg et al. 1968) supplemented with 13% sucrose (designated ½ B5-13) and macerated at low speed for 12 - 15 s followed by high speed for the same length of time. The crude microspore suspension was filtered through a sterile funnel lined with 41 µm nylon screen cloth into a 50 mL centrifuge tube. The funnel was rinsed several times to maximize microspore recovery. The crude microspore suspensions were then centrifuged at 130 – 150 x g for 3 min, the supernatant was decanted and 10 mL fresh ½ B5-13 was added to resuspend the pellet. This procedure was repeated twice more for a total of three washes. Prior to the last centrifuge spin, the number of microspores was determined using a haemocytometer and the pellet was then re-suspended in culture medium appropriate for each experiment and the microspores were plated at a density of 50,000 microspores per mL, unless

otherwise noted. Ten mL of the microspore\medium solution were dispensed per 100 x 15 mm petri plate and the plates were incubated in the dark at 24°C unless otherwise noted. Embryos were counted 28 days after initial culture. The pH of all media was adjusted to 5.8. B5 extraction and B5 solid media were autoclaved, whereas the NLN media was filter-sterilized. Experiments were repeated up to six times and each experiment consisted of 5 – 40 replicates with each petri plate being one replicate. All data from the replicated experiments were analyzed using analysis of variance procedures. Where significant ($P < 0.05$) F-test values were observed, means were compared using Duncan's multiple range test ($P < 0.05$).

Experiments evaluating the factors influencing microspore embryogenesis in *Camelina sativa* were as follows:

Culture medium

Donor plants were grown and microspores were extracted and purified as outlined above. Microspores were cultured in NLN (Lichter 1982) medium with 17% sucrose (632 mOsm) or NLN medium with 12.5% sucrose and 12.5% PEG 4000 (polyethylene glycol) (934 mOsm) and plated at a density of 50,000 microspores per mL. The PEG media was used within one week of preparation. Microspore cultures were incubated in the dark at 24°C continuously or 28°C for 24 h followed by 24°C. Embryos were counted after 28 days in culture.

Microspore density

Donor plants were grown and microspores were extracted as outlined above. Microspores were counted using a hemacytometer and based on this value, the required amount of NLN medium with 12.5% sucrose and 12.5% PEG 4000 was added to achieve densities of 10,000, 25,000, 50,000, 75,000, or 100,000 microspores per mL. The microspores were incubated at 24°C and embryos were counted after 28 days.

Influence of B5 extraction medium on induction of embryogenesis

Microspores were extracted and purified in B5 medium with quarter, half, or full-strength macro- and micro-nutrients and vitamins. The sucrose concentration of the B5 medium remained constant at 13%. The microspores were then cultured in NLN medium (12.5% sucrose and 12.5% PEG 4000) as outlined above at a density of 10,000 microspores per mL.

Influence of glutamine starvation on microspore embryogenesis

Microspores were extracted and purified in half-strength B5 medium and then cultured in NLN medium with 12.5% sucrose and 12.5% PEG 4000 with 0.8% glutamine (control), without glutamine, or initially without glutamine but with glutamine added at 72 h for a final concentration of 0.8% glutamine. For the glutamine addition, 1 ml of a 10X filter-sterilized stock was added to each petri plate. Microspores were cultured at a density of 10,000 microspores/ml at 24°C and embryos were counted after 28 days.

Combination of treatments

Microspores were extracted in full-strength B5 medium and then cultured at a density of 10,000 microspores/ml in NLN medium with 12.5% sucrose and 12.5% PEG 4000. A 72 h glutamine starvation was also applied and compared to cultures which had glutamine added from the beginning of the experiment.

Bud size

The floral buds were selected based on size, < 1mm, 1.1 – 2.0 mm, and 2.1 – 3.0 mm. Microspores were extracted in full-strength B5 medium and cultured at a density of 10,000 microspores/ml in NLN medium with 12.5% sucrose and 12.5% PEG 4000. A 72 h glutamine starvation was also applied.

Regeneration

Microspore-derived embryos were counted 28 days after microspore extraction. The embryos were then transferred to the light [on a shaker (New Brunswick G-10) at 50 rpm, 16 h photoperiod, 22°C, light intensity of 38 $\mu mol^{-1}m^{-2}$] for continued development and to allow the embryos to become green. After 5 - 10 days, the embryos were plated onto solid medium and kept at 22°C, with a light intensity of 110 $\mu mol^{-1}m^{-2}$, and a 16 h photoperiod. The standard *Brassica napus* regeneration media was used (½ strength B5, 1% sucrose, 0.8% agar, pH 5.8) (Gamborg et al. 1968). After approximately 6-8 weeks, plantlets with well developed roots and shoots were transferred to a commercial greenhouse, soil-less mixture (Sunshine #4). Plants were maintained at 20/15°C, 16 h photoperiod and a light intensity of 440 $\mu mols^{-1}m^{-2}$. Once flowering was initiated, plants were staked and bagged to ensure self pollination. Seeds were harvested upon maturation.

Results and Discussion

Camelina has potential as a crop for the Canadian prairies because of its drought tolerance, oil quality, disease resistance, and insect tolerance. Development of camelina germplasm with larger seed, higher seed yield, higher oil content, and better meal quality would be beneficial for the camelina industry. Doubled haploidy is a tool to assist the breeder in developing homozygous, true breeding lines. In order to develop an efficient doubled haploidy protocol, a number of factors must be evaluated. These factors include developmental stage of the pollen grain, medium constituents, and culture conditions. In the camelina experiments, variation in embryogenic frequencies was observed between the experiments of similar treatment (Table 1). This could be due to the plant to plant variation within the donor plants, as line 933 is a breeding line and not homozygous. Plant to plant variation for embryogenic response has been observed in *Brassica* and *Saponaria* species (Phippen and Ockendon 1990; Kernan and Ferrie 2006).

The composition of the medium in which microspores are cultured is an important factor influencing microspore embryogenesis. Preliminary experiments in camelina using basal media compositions commonly used in androgenesis indicated that there was no response from two formulations evaluated, i.e. N6 (Chu 1978) and KFWC (Kuhlmann and Foroughi-Wehr 1989). There was minimal response from NN (Nitsch and Nitsch 1969) (data not shown). Callus and limited embryo development was also observed in NLN. Microspore culture experiments utilizing NLN with 13% sucrose or 25% PEG gave little embryo development, despite being beneficial in *Brassica* (Ferrie and Keller 2007), a related species. Further evaluation with NLN medium compared two sucrose/osmoticum levels: NLN with 17% sucrose and NLN with 12.5% sucrose and 12.5% PEG 4000. Microspores were incubated at two different temperatures (24°C, 28°C for 24 h followed by 24°C). In all three experiments at both temperatures, more embryos were produced in the medium with sucrose and PEG 4000 compared to the medium with sucrose alone, however, this difference was only statistically significant in one experiment (Table 1). The frequency of microspore embryogenesis was low, therefore further evaluation was required. Medium with PEG 4000 has enhanced microspore embryogenesis in some *Brassica* species (Ferrie and Keller 2007). Embryos derived from the PEG-containing medium were smaller but germinated better than embryos from sucrose-containing medium (Ferrie and Keller 2007).

The density at which the microspores are plated can influence the embryogenic response in terms of both the quantity and quality of the resulting embryos. From the experiments with camelina, the microspores plated at the lower densities (10,000 or 25,000 microspores per mL) produced more embryos when compared to the microspores plated at the higher densities of 50,000, 75,000 or 100,000 (Table 2). A low microspore density was reported beneficial in *Saponaria vaccaria* L. (Kernan and Ferrie 2006), but higher densities of 50,000 microspores/mL were advantageous for tobacco (*Nicotiana tabacum* L.; Touraev and Heberle-Bors 2003) and *B. napus* L. (Fan et al. 1988).

Microspores were extracted and purified in quarter, half, or full-strength B5-13 medium prior to culture in NLN medium. The microspore embryogenic response varied with the strength of the extraction media. Full-strength B5 gave a higher embryogenic response compared to the lower strengths, although there was extreme variability between the experiments in terms of overall embryo yields (Figure 1). Very little research has been conducted on the extraction medium, making this a unique finding.

Microspores were cultured in NLN medium with or without glutamine. For some of the cultures initially without glutamine, glutamine was added after 72 h. Results show that glutamine is essential for microspore embryogenesis as no embryos resulted from the treatments without glutamine (Figure 2). In all experiments, there was a significant difference between the cultures which had glutamine from the start of culture and those cultures which had glutamine added after 72 h (Figure 2). More embryos resulted when the microspores were temporarily starved of glutamine, although embryos were still produced from treatments with glutamine in the media from initial culture. This is similar to *B. rapa* L. (Ferrie 2003), but in contrast to *Nicotiana tabacum* L. and *N. rustica* L. (Kyo and Harada 1985, 1986) where embryos were only produced from cultures starved of glutamine.

Further experiments involved a combination of the "best" treatments (i.e. density of 10,000 microspores/mL, full-strength B5, NLN with 12.5% sucrose and 12.5% PEG with glutamine added at 72 h, 24°C culture temperature). Although the combination of all beneficial treatments did show a significant difference in the embryogenic

frequency when compared to the control, this was not a large increase and therefore further experimentation is required (data not shown).

It has been shown in some species that bud size can be correlated to the developmental stage of the microspore (Kernan and Ferrie 2006). This allows for a quick and easy method of selecting the microspores that would be most embryogenic. Although embryos were produced at all three bud sizes, the larger bud size (2.1 mm – 3.0 mm) is not beneficial for microspore embryogenesis (Table 3). The smaller bud sizes correlated to the early – late uninucleate stage, whereas the larger bud size (2.1 – 3.0 mm) correlated to the binucleate to trinucleate stage of development (Figure 3). Further experiments will focus on the smaller bud sizes.

Like most tissue culture systems, genotype plays a major role in embryogenic response. There have been numerous studies in *Brassica* species evaluating microspore embryogenic response of different genotypes (Ferrie et al. 1995; Kuginuki et al. 1999; Barro and Marti 1999; Lionneton et al. 2001). Preliminary microspore studies evaluated six camelina genotypes (data not shown). Genotype 933 was the most consistent and responsive line in these studies and therefore was selected for studies on methodology development. Further studies will evaluate different camelina genotypes to the developed protocol.

Embryos (Figure 4 a) and calli developed after 28 days, but only the embryos were counted and plated on to solid media for further development (Figure 4 b). Calli were predominant in the initial cultures with the high sucrose concentrations, but embryos were predominant in the cultures with sucrose and PEG combined. Utilizing the standard *Brassica* regeneration protocol (full-strength B5, 2% sucrose and 0.8% agar) there was poor response, with approximately 1% of the embryos converted to plantlets (Figure 4 c; data not shown). Despite the low frequency of embryo to plantlet conversion, there was a high survival rate of plantlets transferred from *in vitro* to *in vivo*. Up to 70% of plantlets appeared to spontaneously double, as evidenced by the production of fertile flowers, such that the use of colchicine or other doubling agents was not required (data not shown). A total of 123 DH camelina plants with seed have been produced to date (Figure 4 d).

Although a highly efficient doubled haploidy protocol was not obtained for *Camelina sativa*, we have shown that it is feasible to produce microspore-derived embryos and regenerate these to doubled haploid plants. Further research is continuing and will focus on evaluating factors influencing microspore embryogenesis and plant regeneration. These factors will be genotype, donor plant growing conditions, media composition, and culture conditions. A doubled haploid protocol will enhance basic research and breeding of *Camelina sativa*.

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Tables and Figures:

Table 1: Effect of 17% sucrose or 12.5% sucrose and 12.5% PEG 4000 on microspore embryogenesis of *Camelina sativa* line 933 at two culture temperatures (24°C, 28°C).

	Exp 1		Exp 2		Exp 3	
	24°C	28°C	24°C	28°C	24°C	28°C
NLN basal media composition	Embryos per 5 x 10^5 microspores (plate)					
17% Sucrose	0.8 a	1.0 a	0.7 a	0.4 a	2.8 a	1.0 b
12.5% Sucrose + 12.5% PEG 4000	2.0 a	1.5 a	2.6 a	0.6 a	7.5 a	8.0 a

Means followed by different letters in a column are significantly different at $p = 0.05$ level as determined by Duncan's multiple range test.

Table 2: Effect of microspore density on microspore embryogenesis of *Camelina sativa* line 933.

Density Microspores/mL	Exp 1	Exp 2	Exp 3	Exp 4
	Embryos per 1 x 10^5 microspores			
10,000	1.60 a	4.40 a	4.40 a	3.20 a
25,000	0.72 b	4.72 a	3.12 a	2.40 b
50,000	0.80 b	0.72 b	0.28 b	0.56 c
75,000	0.13 c	0.26 b	0.08 b	0.05 c
100,000	0.06 c	0.06 b	0 b	0.04 c

Means followed by different letters in a column are significantly different at $p = 0.05$ level as determined by Duncan's multiple range test.

Table 3: Influence of bud size on microspore embryogenesis of *Camelina sativa* line 933.

Bud size	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5
	Embryos per 1 x 10^5				
< 1mm	4.5 a	2.5 a	1.6 b	0.1 b	0.1 b
1.1 – 2.0 mm	0.8 b	2.2 a	2.9 a	2.8 a	4.3 a
2.1 – 3.0 mm	0.2 b	0.1 b	0.4 b	0.4 b	0.9 b

Means followed by different letters in a column are significantly different at $p = 0.05$ level as determined by Duncan's multiple range test.

Figure 1: Effect of quarter-strength, half-strength, and full-strength B5 was medium on microspore embryogenesis of *Camelina sativa* line 933.

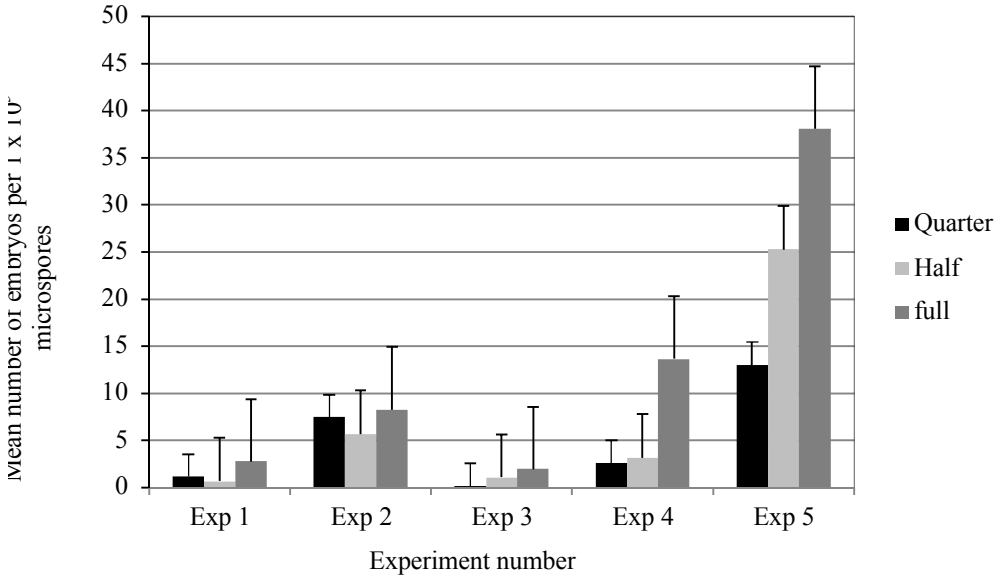
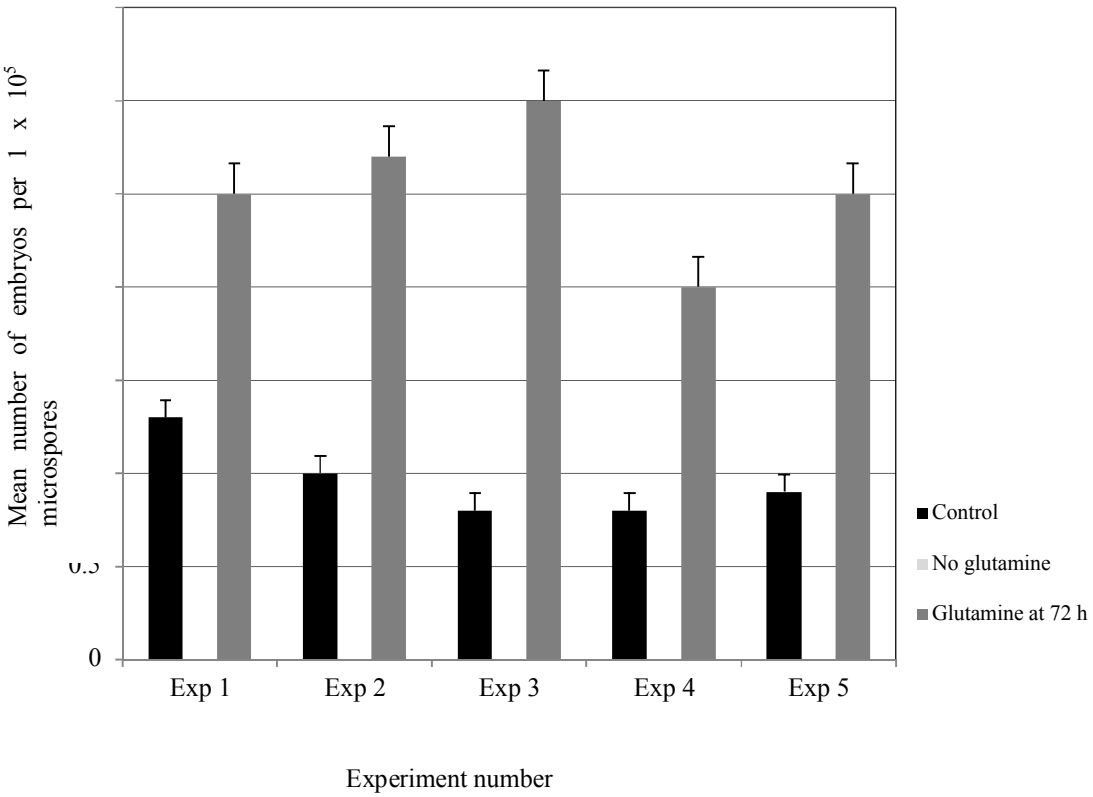


Figure 2: Effect of glutamine in the NLN culture medium on microspore embryogenesis of *Camelina sativa* line 933.



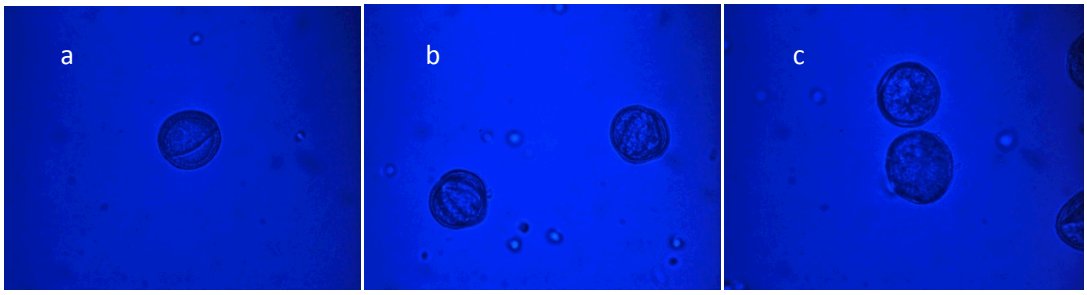


Figure 3a: Microspore from 1 mm *Camelina sativa* line 933 bud, b: microspore from 2 mm *Camelina sativa* line 933 bud, c: microspore from *Camelina sativa* line 933 3 mm bud.

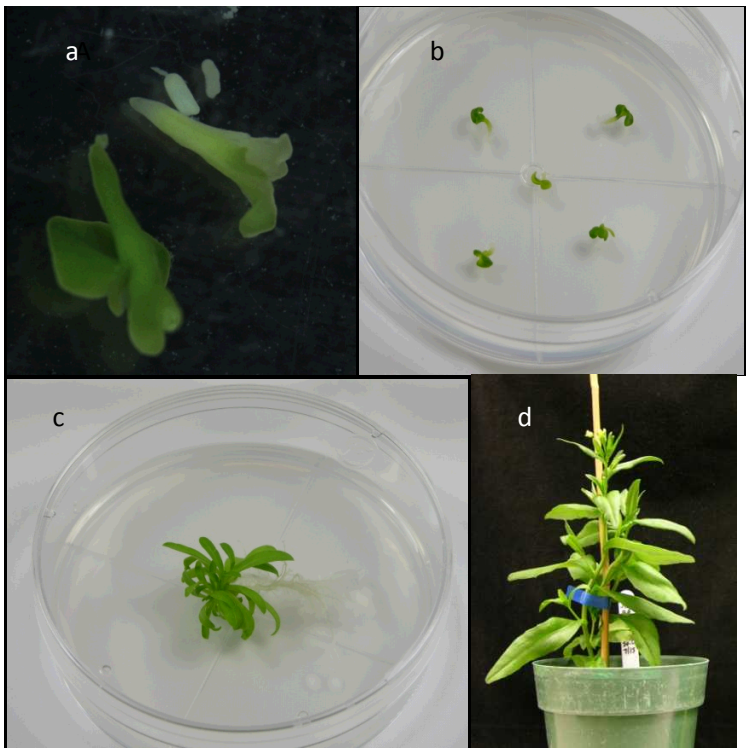


Figure 4a: Microspore-derived embryos of *Camelina sativa* line 933, b: Microspore-derived embryos of *Camelina sativa* line 933 on regeneration medium, c: microspore-derived plantlet of *Camelina sativa* line 933, d: Doubled haploid *Camelina sativa* plant.

Figure legends:

Figure 1: Effect of quarter-strength, half-strength, and full-strength B5 was medium on microspore embryogenesis of *Camelina sativa* line 933.

Figure 2: Effect of glutamine in the NLN culture medium on microspore embryogenesis of *Camelina sativa* line 933.

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