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Full Length Article

Cytological Characterization of Nucleus Development in Microspore Embryogenesis and Improvement in Healthy Embryo Production using Polyethylene Glycol in *Brassica napus*

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Abstract

This study characterized microspore embryogenesis at each stage of cell development in oilseed *Brassica napus* (cvs. Zheshuang 758 and Zheshuang 72). Isolated microspores of *B. napus* were cultured with various concentrations of polyethylene glycol (PEG 6000) as an osmoticum and sucrose (0.1%) as carbohydrate source in liquid NLN culture medium. The most embryos were produced from NLN culture medium with 6.25% PEG 6000. Approximately 9% of the microspores swelled after one day heat treatment at 30°C in darkness. After 2 days culture, some swollen microspores began to divide into 2-cell and 3-cell proembryos with "T" shaped and gradually into multi-cellular proembryos, which further developed into globular, hearted, and torpedo embryos. Two kinds of division were observed under electron microscope: symmetrical division and asymmetrical division. It showed that 3 weeks culture was suitable for microspore embryogenesis. The optimal concentration of 6-benzyl-aminopurineand naphthalene acetic acid for shoot regeneration was 2.5 $mg \cdot L^{-1}$ and 0.4 $mg \cdot L^{-1}$, respectively. The highest doubling frequency was observed in Zheshuang 758 (78%) and Zheshuang 72 (73%), when 200 $mg \cdot L^{-1}$ colchicine was employed in culture medium. The ploidy level of microspore-derived plants could be checked by flow-cytometer or staining the lower epidermis of leaves with fluorescein diacetate by observing fluorescence of guard cell chloroplasts with a microscope under ultraviolet light. From these findings, it can be concluded that the swelling of microspore cell and symmetric division are key factors to start microspore embryogenesis and healthy embryos could be produced using PEG 6000 in microspore culture. © 2019 Friends Science Publishers

Keywords: *Brassica napus*; Microspore embryogenesis; PEG 6000; Symmetric division; Shoot regeneration; Doubling frequency

Introduction

Brassica napus L. is the third important vegetable oil crop in the world (Zhou *et al.,* 2002a). *Brassica* has six critical species including three tetraploid species (*B. napus, B. carinata* and *B. juncea*) and three basic diploid species (*B. oleracea, B. nigra* and *B. rapa*). *B. napus* (AACC) is an amphidiploid species derived from the hybridization of *B. rapa* (AA) and *B. oleracea* (CC). Since, Lichter (1982) firstly established the successful microspore culture techniques on *B. napus*, microspore-derived (MD) culture has been developed as a vital tool for plant breeding, molecular biology, gene expression and genetic mapping (Zhang *et al.,* 2008). Subsequently, microspore culture has been used in other *Brassica* species, such as cauliflower, broccoli and cabbage (Xu *et al.,* 2007). Microspore-derived culture technique has been widely applied in different areas because of its gene purity and high efficiency of haploid production (Palmer *et al.,* 1996). With the rapid development of scientific and technological innovations, more and more expansion of its applications has brought about a series of improvements in doubled haploid (DH) breeding and MD culture techniques in higher plants (Shi *et al.,* 2008).

Doubled haploid has been extensively used in recent years. Compared with traditional procedure of breeding for producing genetically completely stable homozygous lines, DH is more effective to accelerate and shorten the process of breeding, and to select the useful traits (Zhou *et al.,* 2002b; He *et al.,* 2006; Brian *et al.,* 2007). In *B. napus*, MD culture has high production rate of embryogenesis, which can produce more than thousands of embryos during only one isolation (Gu *et al.,* 2004; Cousin *et al.,* 2009). Now days, several relevant studies have been carried out to improve the quality and quantity of MD embryos and enhance the efficiency of embryo production (Zhang *et al.*,2006a, b). This study investigated the microspore cell development and embryogenesis of two *B. napus* cultivars

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(Zhengshuang 758 and Zheshuang 72) under fluorescence microscope and transmission electron microscope.

The NLN-13 medium (liquid NLN culture medium with 13 % sucrose) has been used popularly for microspore culture in *Brassica* species (Lichter, 1982). In NLN-13 medium, mostly a small amount of sucrose (0.08–0.1%) is used for carbohydrate source and the left as an osmoticum. However, high molecular weight of polyethylene glycol (PEG 6000) cannot cross the cell wall and penetrate into plant cell, which make it as an important osmoticumin scientific research and practical application. Sucrose as an osmoticum in microspore culture medium could be replaced with high-molecular-weight PEG 6000 (Ilic-Grubor *et al.,* 1998).

Colchicine is the most common agent for chromosome doubling to produce DH plants, which can be both used in culture medium with isolated microspores and roots for doubling purpose (Takahira *et al.,* 2010). Nowadays, colchicine is the most commonly used reagent for chromosome doubling in *B. napus* (Mohammadi *et al.,* 2012). The ploidy level of plants could be measured by chromosome counting, guard cell chloroplast (GCC) observation, flow cytometry (FCM) and other ways (Li *et al.,* 1995; Compton *et al.,* 1999; Cousin *et al.,* 2009). During embryogenesis and plant regeneration, different concentrations of plant growth regulators (6-benzyl-aminopurine (6-BA), naphthalene acetic acid (NAA) and ect.) in solid culture medium are also important for embryogenesis and root growth (Wan *et al.,* 2011).

In this experiment, two leading oilseed *B. napus* cultivars (Zheshuang 758 and Zheshuang 72) were selected and applied with different treatments to optimize an efficient protocol for microspore embryogenesis, shoot and root regeneration, chromosome doubling and ploidy analysis, which could be beneficial for other *Brassica* breeding and related studies in the future.

Materials and Methods

Plant Materials

In this study, two *B. napus* cultivars Zheshuang 758 and Zheshuang 72 with good response to microspore culture were selected as donor plants. All the plants were grown in the green house of the Zhejiang University experimental station and were used for the flower bud collection. Healthy buds of two cultivars were sampled for microspore isolation.

Microspore Isolation

Modified microspore culture protocol based on Fletcher *et al.* (1998) was carried out in this study. Forty flower buds around 2–4 mm (at late uninucleate stage) were collected randomly from racemes. After sterilizing with 5% NaClO for 15 min and washing 5 times in sterile double distilled water, the buds were transferred in cold liquid NLN culture medium (pH 6.0) in the laminar flow and squeezed gently by using a glass rod (Lichter, 1982; Keller *et al.,* 1987). Then the medium with isolated microspores was filtered with a 40 μm nylon mesh and then centrifuged at 900 rpm for 15 min. Following discarding the supernatant, the precipitation was re-suspended in 40 mL fresh NLN-13 medium (liquid NLN culture medium with 13% sucrose, pH 6.0). After adding 10 drops of activated charcoal and colchicine solution (100, 200 and 300 $mg \cdot L^{-1}$) into tube, microspore suspension was divided into 60×15 mm Petri dishes with 4 mL per plate. Dishes were sealed with Parafilm® (Parafilm, U.S.A.) and incubated by a heat shock of 30°Cin darkness for one day. One day later, they were added in another 4 mL fresh NLN-13 medium and put in dark room at 25°C (Geng *et al.,* 2013).

Cytological Observations of Nucleus Development in Microspore Embryogenesis

One dish of microspore suspensions was randomly chosen and centrifuged at 900 rpmfor 15 min every day. The microspore precipitation was observed directly and after staining by 1 $\mu g \cdot mL^{-1}$ 4', 6-diamidino-2-phenylindole (DAPI) for 15 min under microscope. The DAPI staining protocol was based on the method of Zhang and Yin (2008) with some modifications.

Sample pretreatment and the method for transmission electron microscope were according to Hong *et al.* (1999). The cytological observation was preceded under the fluorescence microscope (LEICA DM IRB, German) and transmission electron microscope (JROL JEM-1200EX, Japan).

Various Concentrations of PEG 6000 as Osmoticum for Microspore Culture

Isolated microspores of *B. napus* were cultured in liquid NLN culture medium containing 0.1% sucrose as carbohydrate source with various concentrations of PEG 6000 as osmoticum. The experiment evaluated five concentrations of PEG 6000 (25, 12.5, 8.3, 6.25 and 5%), and NLN-13 culture medium (NLN culture medium containing 13% sucrose) was used as the control. The colchicine was not added in this experiment.

Plant Regeneration

After about 10 days, when some global embryos were seen by naked eyes, put into a 45–60 rpm rotary shaker and placed in dark roomat 25°C for another 10 days. The large embryos at late torpedo stage were finally transferred to solid Murashige and Skoog (MS) medium (pH 6.0, 20 $g \cdot L^{-1}$ sucrose, and 6.5 $g \cdot L^{-1}$ agar) to obtain regenerated plants, supplemented with different plant growth regulators, 6-BA (0.5, 1.0, 1.5, 2, 2.5 and 3 $mg \cdot L^{-1}$) and NAA (0.1 and 0.4

mg·L^{-1}). These were cultured into growth jars under a 16 h photoperiod (100 μE·m^{-2}·s^{-1}) at 25°C. When shoots appeared and cut from callus into another larger growth jars with solid MS medium for rooting. After reaching 5–6 leaf stage with strong roots were transplanted into soil and kept for 2 weeks in a culture room with high relative humidity (25°C, 16 h photoperiod, 40 µE·m^{-2}·s^{-1} light intensity) (Geng *et al.,* 2013).

Colchicine Treatment and Ploidy Analysis

Three colchicine concentrations (100, 200 and 300 mg·L^{-1}) were used to treat isolated microspores for doubling chromosome. Various concentrations of colchicine were added into microspore suspension. The ploidy level of MD regenerates was determined by FCM and staining the lower epidermis of leaves with FDA by observing fluorescence of guard cell chloroplasts with a microscope under ultraviolet (UV) light.

Flow Cytometry (FC) Analysis

All MD plantlets ploidy level was estimated from nuclear DNA content measured in FC (Nelson *et al*. 2009). Leaf materials (about 5 mg) of MD plantlets were collected in the growth chamber, placed into a labeled micro centrifuge tube (containing beads) on ice to the laboratory. Cold lysis buffer (500 μL) was added to the tubes which were placed on ice at -20°C for 8–10 min. The tubes were then placed in a Tissue Lyser machine in freezer-treated holders and shaken on a setting of 25 HZ for 24 sec. The macerated suspensions were filtered through 30 μM filter into fresh tubes. Two μL RNase (3 mg mL^{-1}) was added to another tube with 60 μL filtrate. Tubes were then incubated in a 37°C room for 20–30 min, and then 140 μL of propidium iodide (PI) staining solution was added at room temperature. Tubes were then placed in the dark (not on ice) and taken for analysis on FCM using single tubes.

BD FACSDiva V6.1.1 (BD Biosciences San Jose, CA, USA) was used as sample data generation. Flow Jo V7.2.5 (Tree Star Inc., Ashland, OR, U.S.A.) were used to analyze the experimental data, CV calculations and finally classified the samples as haploid, diploid, mixed or other types of ploidy plants (Cousin *et al.,* 2009; Nelson *et al.,* 2009; Geng *et al.,* 2013;). Tetraploid *Brassica* plant (cv. Zheshuang 758) was chosen as standard sample.

Guard Cell Chloroplast Observation

The lower epidermises of leaves were stained with 0.01% FDA solution by observing fluorescence of guard cell chloroplasts with a microscope under UV light (Compton *et al.,* 1999). Tetraploid *Brassica* plant (cv. Zheshuang 758) was used to establish the standard value for the number of chloroplasts per guard cell and stoma size.

Statistical Analysis

The variations in swelling and divided rate, shoot regeneration rate, embryo production, plantlet regeneration and doubling frequency in various concentrations of PEG 6000 and colchicine treatments on the MD plantlets of *B. napus*, were evaluated by analysis of variance (ANOVA) followed by the LSD test by SPSS software version 16.0 (SPSS, Chicago, IL, U.S.A.). All statistical analysis data owned three repetitions. Results were considered significant at $P \leqslant 0.05$.

Results

Cytological Observation of Microspore Embryogenesis of *B. napus*

Microspores at the late uninucleate stage were isolated and cultured in liquid NLN culture medium (Fig. 1a, 2a). A portion of microspore cells (9.7%) were observed as swollen apparently after one day culture (Fig. 1b, 2b) and these swollen microspores began to divide into 2-cell on the second day (Fig. 2c). Cell division and swelling occurred in the first three days after culture, while the first division began on the second day. The second division started after 3 days culture and divided into 2-cell, 3-cell with "T" shape (Fig. 2e, f).

Approximately 42% microspore cells at the late uninucleate stage swelled into large cells, which had the embryo genetic capability to develop into embryos (Fig. 3). The nucleus moved to center of the cell and the vacuoles partly or totally disappeared, when the late uninucleate microspore swelled into larger one (Fig. 2b, h; 4b). However, only 12.3% of the swollen cells could divide into 2-cell and then develop into embryos eventually (Fig. 3). The swelling and divided rates of microspore cells were no longer increase after about 5 days culture (Fig. 3). According to the observation, the swelling of microspore cell is the key factor to start cell division. Thus, it is quite important to increase the number of swollen microspore cells through improving the culture environment and methods.

The microspore cells further developed into multicellular proembryos after 6–8 days culture (Fig. 1c) and then globular-shaped embryos with an attached suspensor (Fig. 1d). About 2 or 3 weeks later, hearted-shaped embryos (Fig. 1e), torpedo-shaped embryos (Fig. 1f) and cotyledon embryos (Fig. 1g, h) were observed gradually. It showed that 3 weeks culture was suitable for microspore embryogenesis.

Cytological Observation of Nucleus Development during Microspore Culture

Cells at each stage of microspore development were stained by DAPI solution and observed under fluorescence microscope. The freshly isolated microspore cells were at late uninucleate stage with a nucleus extruded to the

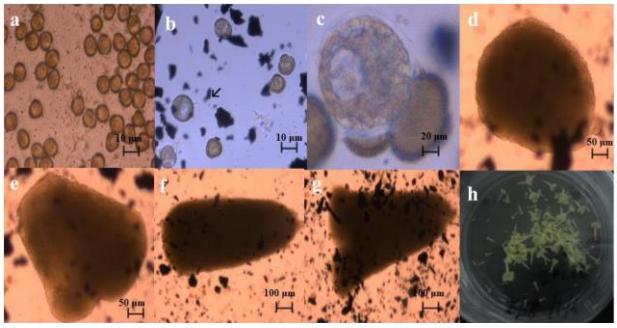

Fig. 1: Cytological observation during microspore embryogenesis in *Brassica napus*. **a:** The isolated microspore cells. **b:** Swollen microspore cells after one day heat treatment. **c:** Microspore cell developed into multicellular proembryo after several divisions. **d:** Globular embryo. **e:** Heart-shaped embryo. **f:** Torpedo embryo. **g, h:** Cotyledon embryo

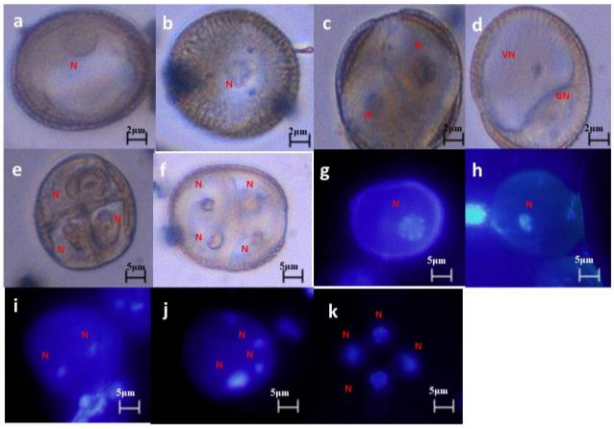

Fig. 2: Cytological observation of nucleus (N) development in microspore cells of *Brassica napus* directly under microscope (a-f) and stained by 4',6-diamidino-2-phenylindole (DAPI) solution (g-k) respectively during microspore culture. **a, g**: Late uninucleate stage microspore cell. **b, h**: Swollen microspore cell after 1 or 2 days heat treatment. **c, i**: First symmetric division of cell after two days culture. **d**: Asymmetric division. **e, j**: 3-cell proembryo after second division. **f, k**: 4-cell proembryo after second division

cell wall (Fig. 2g). After one day heat treatment, the nucleus moved into the center of the swollen cell (Fig. 2h). About 2–3 days culture, the microspore cells divided symmetrically to form two similar nucleuses (Fig. 2i). Four-five days later, the microspore cells began its second division and then formed into 3-cell and 4-cell proembryos at 6–8 days (Fig. 2j, k).

Ultrastructure Observation during Microspore Embryogenesis

Large numbers of cells at the late uninucleate stage had been observed under electron microscope when the microspores were isolated from the young buds (Fig. 4a). The nucleus

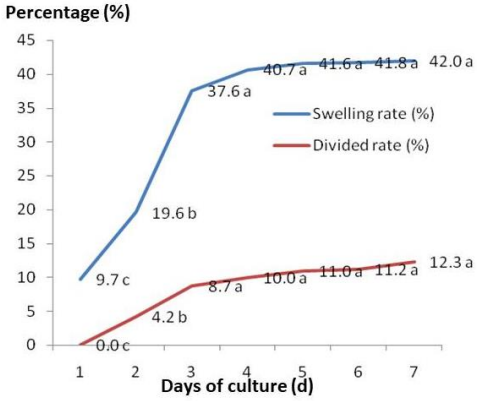

Fig. 3: The swelling and divided rates of microspore cells during 7 days culture in *Brassica napus* by observing 500 cells every day

* Means followed by the same lowercase letters are not significantly different at $P \leq 0.05$ by the LSD test

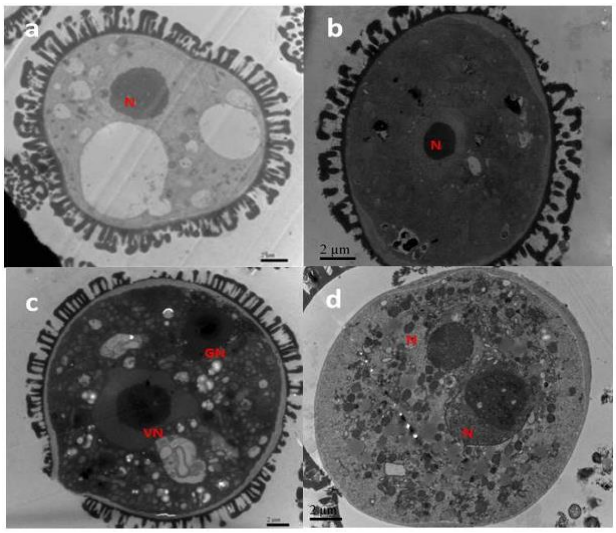

Fig. 4: The ultrastructure observation of microspore cells during microspores embryogenesis in *Brassica napus*. a Microspore cell at the late uninucleate stage with a large vacuole. b A swollen cell with a central nucleus after two days heat treatment. c Asymmetric division formed one vegetative nucleus (VN) and one generative nucleus (GN). D Symmetric division formed two similar nucleus (N)

was extruded to the cell wall due to presence of large central vacuole. The cytoplasm was dense and had abundant endoplasmic reticulum ribosomes and mitochondria.

After one day heat treatment, several microspore cells began to swell. The large central vacuoles in swollen cells were completely or partly disappeared and the nucleus moved to the center of the cells. Meanwhile, the mitochondria, ribosomes and golgibodies were heavily accumulated in the cytoplasm. The starch grains were richly attached to the nucleus at this stage and different from the late uninucleate stage of the microspores (Fig. 4b). Two days later, first division was started in microspore cells.

Table 1: Effects of different concentrations of 6-benzyl-aminopurine(6-BA) and naphthaleneacetic acid (NAA) on shoot regeneration rate of microspore-derived embryos in *Brassica napus* cultivars Zheshuang 758 and Zheshuang 72

Cultivar	6-BA (mg·L^{-1})	NAA (mg·L^{-1})	Total number of embryos	Shoot regeneration rate (%)
Zheshuang758	0.5	0.1	100	23.1 g
		0.4	100	32.6 f
	1	0.1	100	43.2 e
		0.4	100	56.7 c
	1.5	0.1	100	49.2 cde
		0.4	100	53.4 cd
	2	0.1	100	48.7 de
		0.4	100	69.2 b
	2.5	0.1	100	46.9 de
		0.4	100	87.4 a
	3	0.1	100	50.8 cde
		0.4	100	80.6 a
Zheshuang72	0.5	0.1	100	37.5 d
		0.4	100	26.2 e
	1	0.1	100	30.5 de
		0.4	100	50.9 c
	1.5	0.1	100	46.3 c
		0.4	100	60.5 b
	2	0.1	100	37.5 d
		0.4	100	77.6 a
	2.5	0.1	100	50 c
		0.4	100	83.4 a
	3	0.1	100	25.5 e
		0.4	100	79.3 a

*Within columns for each genotype, means followed by the same lowercase letters are not significantly different at $P \leq 0.05$ by the LSD test

Table 2: Embryo production, plantlet regeneration rate and spontaneous doubling frequency in the liquid NLN culture medium with various concentrations of polyethylene glycol (PEG 6000) during microspore culture in *Brassica napus* cultivars Zheshuang 758 and Zheshuang 72

Cultivar	Concentration of PEG 6000 (%) [a]	Number of embryos per bud	Plantlet regeneration rate (%)[b]	Spontaneous doubling frequency (%)[c]
Zheshuang 758	25	5.7 d	97.0 e	60.5d
	12.5	7.3 d	89.5de	41.5c
	8.3	10.0 cd	80.1cd	40.6c
	6.25	21.3 b	75.5c	24.0 b
	5	12.3 c	67.4b	17.1b
	CK[d]	63.0 a	53.0 a	5.5a
Zheshuang 72	25 .0	4.3 e	95.4e	58.4d
	12.5	8.3 de	87.6d	39.5c
	8.3	14.3 c	79.2c	38.6c
	6.25	21.7 b	72.9c	21.0 b
	5 .0	11.7 cd	58.7a	14.8b
	CK[d]	55.3 a	54.3a	6.7a

*Within columns for each cultivar, means followed by the same lowercase letters are not significantly different at $P \leq 0.05$ by LSD test
[a] Microspore embryogenesis was developed in the liquid NLN culture medium containing 0.1%sucrose and various concentrations of polyethylene glycol (PEG 6000)
[b] Embryos derived from NLN medium with various concentrations of PEG 6000 were transferred into solid MS medium to regenerate shoot
[c] The ploidy level of all the plantlets derived from NLN medium with various concentrations of PEG 6000 without cochicine treatment was analyzed by flow cytometry
[d]CK means liquid NLN culture medium containing 13%sucrose

Two kinds of division were observed under electron microscope: symmetrical division and asymmetrical division. Two similar cells without cell walls were formed during symmetric division, both of which had embryogenetic capability and could continue embryo development (Fig. 4c). While asymmetric division produced one vegetative cell and one reproductive cell (Fig. 4d), *in vitro* vegetative cell would disappear, and reproductive cell would continue to divide into tetrad and finally develop into pollen.

Influences of 6-BA and NAA on Shoot Regeneration of MD Embryos

The cotyledon embryos were put to the solid MS culture medium with different concentrations of 6-BA (0.5, 1.0, 1.5, 2.0, 2.5 and 3 mg·L^{-1}) and NAA (0.1 and 0.4 mg·L^{-1}) for shoot regeneration. When MS medium supplemented with 6-BA (2.5 mg·L^{-1}) and NAA (0.4 mg·L^{-1}), the shoot regeneration frequency of the two cultivars Zheshuang 758 (87.4%) and Zheshuang 72 (83.4%) reached at highest value (Table 1). The optimal concentration of 6-BA and NAA for shoot regeneration was 2.5 mg·L^{-1} and 0.4 mg·L^{-1}, respectively.

Impact of PEG 6000 as an Osmoticum in NLN Medium for Microspore Culture

There were significantly different numbers of embryos produced between PEG 6000 and the sucrose as an

osmoticum. Number of embryos cultured from NLN medium with 6.25% PEG 6000 as the osmoticum was consistently higher than other concentrations in two *B. napus* cultivars (Zheshuang 758 and Zheshuang 72). Embryos from NLN medium with 5% PEG 6000 showed better quality, although the number of embryos was less than NLN-13 medium (Table 2). The embryos cultured from PEG 6000 treatment showed better quality zygotic embryos, plant regeneration capabilities (58.7–97.0%) and spontaneous doubling frequency (17.1–60.5%) than by using sucrose in the NLN culture medium in Zheshuang 758 and Zheshuang 72 (Fig. 5a, c; Table 2).

Effect of Various Concentrations of Colchicine on Isolated Microspores

Results demonstrated that haploid occupied 94% of the total MD plantlets and only 6% plantlet could be spontaneously doubled in the two *B. napus* cultivars without colchicines treatment (Table 2). Three colchicine concentrations (100, 200 and 300 $mg \cdot L^{-1}$) were used to treat isolated microspores for chromosome doubling. The results showed that the doubling frequency was significantly increased after colchicine treatment (Fig. 6). The doubling frequencies were approximately 78% in Zheshuang 758 and 73% in Zheshuang 72, when 200 $mg \cdot L^{-1}$ colchicine was applied (Fig. 6). There was no significantly difference between 200 and 300 $mg \cdot L^{-1}$ colchicine treatments in two *B. napus* cultivars.

Ploidy Level of MD Plantlets Observing via Guard Cell Chloroplast

Ploidy level of plantlets was estimated by staining the lower epidermis of intact *in vitro*-derived leaves with FDA and observing fluorescence of GCC with a microscope under UV light (Fig. 7a, b). Number of chloroplasts per guard cell and stoma size of tetraploid *Brassica* plant (cv. Zheshuang 758) were used to establish the standard value. Then, the GCC of unknown ploidy plants were observed. This study indicated that the standard tetraploid *Brassica* of Zheshuang 758, doubled haploid and haploid showed approximately 16, 16 and 10 chloroplasts per guard cell, respectively. The stoma sizes of tetraploid *Brassica*, doubled haploid and haploid plants reached at the length of 240 µm, 240 µm and 160 µm, and the width of 180 µm, 180 µm and 137.5 µm, respectively.

Ploidy Level of MD Plants using Flow Cytometry

The peak of nuclear DNA content (PI_DNA-A) by flow cytometry for standard tetraploid *Brassica* plant (cv. Zheshuang 758) was found to be at approximately 25,000 units. The PI_DNA-A of doubled haploid MD progeny was about 25,000 units (Fig. 7c) and haploid MD progeny was about 12,500 units (Fig. 7d). After analyzing the ploidy level, DH plants were transferred into soil and the other plants were discarded.

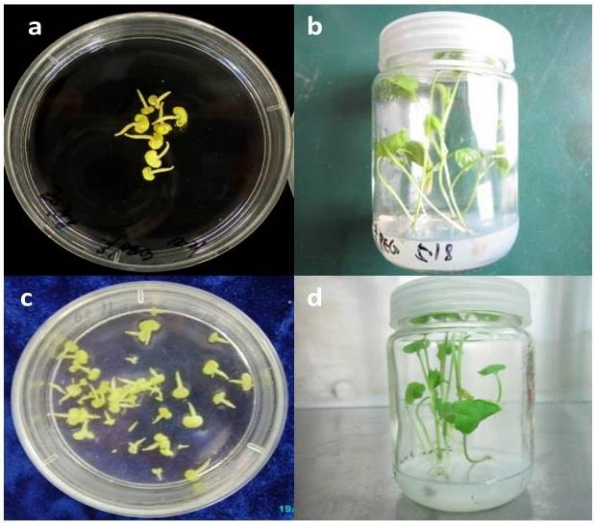

Fig. 5: Embryos and plantlets derived from microspores cultured in the liquid NLN culture medium containing 13% sucrose (NLN-13 medium) and 0.1% sucrose and 25% polyethylene glycol (PEG 6000) in *Brassica napus*. **a:** Microspore embryos from NLN medium with 25 % PEG 6000. **b:** Microspore-derived plantlets from NLN medium with 25% PEG 6000. **c:** Microspore embryos from NLN-13 medium. **d:** Plantlets from NLN-13 medium

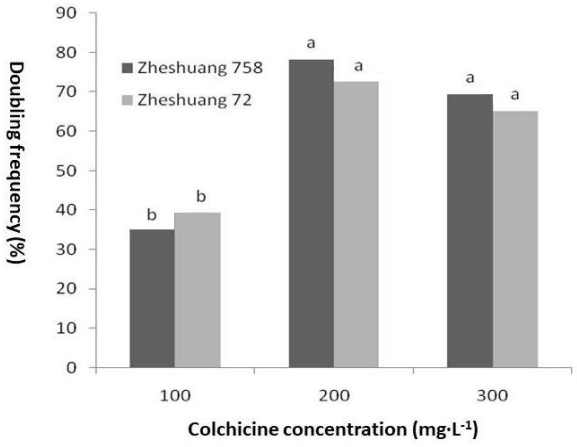

Fig. 6: Effects of various colchicine concentrations on isolated microspores for chromosome doubling in *Brassica napus* cultivars Zheshuang 758 and Zheshuang 72

*Within columns for each genotype, means followed by the same lowercase letters are not significantly different at $P \leq 0.05$ by the LSD test

Discussion

The mechanism of microspore embryogenesis has not been well explained in *Brassica napus*. In this study, cell morphology and embryogenesis of Zheshuang 758 and Zheshuang 72during microspore culture were carried out. Our results showed that cell expansion and division mainly occur in the first three days after culture, and the division starts from the second day, which were consistent with some previous studies (Maraschin *et al.*,

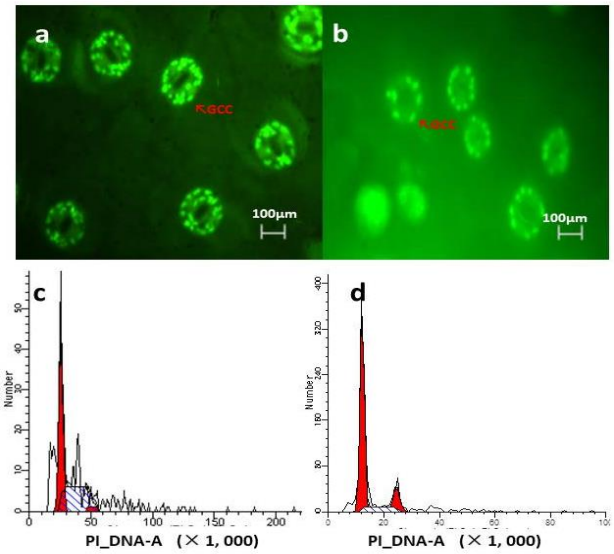

Fig. 7: Ploidy level of microspore-derived (MD) plantlets tested through guard cell chloroplasts (GCC) stained by fluorescein diacetate and flow cytometry in *Brassica napus*. **a:** MD doubled haploid plant (the length of stoma is 240 μm, the width of stoma is 180 μm, number of chloroplasts per guard cell is 16). **b.** MD haploid plant (the length of stoma is 160 μm, the width of stoma is 137.5 μm, number of chloroplasts per guard cell is 10). **c:** MD doubled haploid plant (the peak of nuclear DNA content is 25,000 units). **d:** MD haploid plant (the peak of nuclear DNA content is 12,500)

2005; Takahata *et al.*, 2013). Cordewener *et al.* (1995) reported that heat treatment can change the developmental fate of isolated *Brassica napus* microspores from pollen development to embryogenesis that might be due to a number of heat-shock proteins (HSPs) of the 70-kDa class: HSP68 and HSP70, which caused an altered pattern of cell division. Takahata *et al.* (2013) observed that the vegetative cell of binucleate microspores highly contributed to embryogenesis. Studies on the morphological changes and embryogenesis of isolated microspore cultured in *Brassica napus* will lay a cytological foundation for further improving the microspore embryo yield in *Brassica* crops and enrich the content of plant somatic embryology.

PEG is a highly water-soluble, non-toxic and neutral compound. Molecular weight of PEG is too large to penetrate into plant cell walls, which is conducive to creating a low water potential culture environment. In this study, PEG 6000 was used to replace sucrose in NLN-13 liquid medium as osmotic regulator for microspore culture. Reducing sucrose content in NLN-13 liquid medium and adding a certain amount of PEG as osmotic regulator resulted in larger embryos than those cultured entirely with sucrose but were more morphologically close to embryos produced by normal fertilization of plants and owned higher regenerative ability. However, in contrast to present study results, Ilic-Grubor *et al.* (1998) used 25% polyethylene glycol (PEG) as osmoticum to induce microspore-derived embryoids in *Brassica napus* Topas 4079 and the embryo were smaller than those induced in the NLN culture medium with sucrose. As to the results of present study, Ferrie and Keller (2007) also found that the embryos induced by PEG were morphologically more similar to zygotic embryos, germination capabilities and spontaneous chromosome doubling ability were both enhanced. Unfortunately，compared with above previous studies, this present study showed that 24% PEG could induce embryos, but the induction rate was significantly lower than control (13%). It might because the stress caused by high concentration of PEG 6000 in microspore culture environment decreased the germination rate of microspore at PEG concentration of 6.25, 12.5 and 25% respectively. By optimizing the ratio of PEG to sucrose, 6.25% PEG could induce a large number of embryoids, the regeneration ability and spontaneous doubling rate of embryoids were significantly improved compared with the control (13% sucrose). Because of the use of NLN liquid medium containing PEG 6000 24.0–60.5% of the plants developed from microspore embryos spontaneously doubled. Under the same conditions, only 5.5% of the plants cultured on NLN medium. When the microspore plants were doubled by colchicine at the later stage, the concentration needed was greatly reduced, which not only saved the cost, but also greatly reduced the harm of toxic drugs to human body and environmental pollution (Gu *et al.*, 2003; Cousin *et al.*, 2009). When the microspore plants cultured with PEG as osmotic regulator were doubled, colchicine solution had less dosage, higher doubling rate and less pollution. The microspore culture with different concentrations of PEG 6000 instead of sucrose as osmotic regulator has good quality, high regeneration rate and high doubling rate. However, the problem of low recovery rate has not been solved.

Conclusion

This study investigated the cytological characterization of nucleus development in microspore embryogenesis at each stage of microspore cells and developed some efficient protocols to improve the microspore embryogenesis, thus increasing the shoot regeneration rate and enhancing the doubling frequency. The results showed that symmetric division and swelling of microspore cell are the key factors to start microspore embryogenesis and polyethylene glycol (PEG) as an osmoticum could produce healthy embryos in microspore culture. These techniques could be useful for optimization of microspore culture, DH production, genetic mapping establishment, plant breeding and other genetic programs in the future.

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