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Optimization of Microspore Embryogenesis and Plant Regeneration Protocols for *Brassica napus*

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ABSTRACT

Composition of nutrient media, inflorescence length and *in vitro* growth duration of donor plant, as well as heat shock, activated charcoal and sucrose concentrations could have remarkable effects on embryogenesis. Contribution of these factors on embryogenesis and shoot regeneration in two genotypes of oilseed rape (*Brassica napus* L.) viz. ZS758 and ZS72 was evaluated in the present study. The highest normal embryo yield in both cultivars was obtained from the NLN induction medium supplemented with 0.25 mg mL^{-1} activated charcoal and 130 g L^{-1} sucrose, which was heat shocked in darkness at 30°C for 2 days. The largest shoot regeneration frequency from microspore-derived (MD) embryos of both cultivars was obtained from the embryos aged 4 weeks. Further, the maximum shoot regeneration frequency of both cultivars was achieved from MS differentiation medium supplemented with 0.3 mg L^{-1} BA, 2 mg L^{-1} NAA and 0.1 mg L^{-1} GA_3. The plants obtained from both cultivars doubled by colchicine treatment (150 mg L^{-1} for 30 h, 300 mg L^{-1} for 15 h) were successfully transplanted to soil, where plant survival rate and doubling rate were more than 95% and 75%, respectively. © 2011 Friends Science Publishers

Key Words: Colchicine; Embryo yield; Plant hormone; Oilseed rape; Shooting frequency; Survival and doubling rate
Abbreviations: MD, microspore-derived; DH, doubled haploid; MS, Murashige and Skoog; PGRs, plant growth regulators; GA, Gibberellic acid; BA, Benzyl-aminopurine; NAA, Naphthaleneacetic acid; FCM, flow-cytometer

INTRODUCTION

In the recent years, oilseed rape (*Brassica napus* L.) has become one of the most crucial field crops for oil production in China (Zhou *et al*., 2002c; Zou *et al*., 2007). Genetic modifications by gene engineering and mutagenesis have played an important role in crop improvement. Just after the successful application of microspore culture techniques to *Brassica napus* (Lichter, 1982), the isolated microspore suspension cultures have become a valuable tool for breeding applications, plant physiology, biochemistry and molecular biology (Weselake, 2005; Zhang *et al*., 2008). Compared with the traditional methods for production of genetically stable homozygous lines, which is quite laborious and time consuming, microspore culture is more effective in accelerating the breeding process and facilitating the selection of fine recessive traits (Yu *et al*., 1997; Zhou *et al*., 2002c; Zou *et al*., 2007) by producing thousands of microspore-derived (MD) embryos from a single microspore culture (Zhou *et al*., 2002a, b). Early identification of the 50% MD embryo genotypes carrying the desired transgenic traits, along with a high expression of the trait, allows their early selection for plantlet regeneration (Nath *et al*., 2007; Zhang *et al*., 2008).

However, inefficiency of microspore transgenic method leads to the lower MD shoot regeneration frequency. Consequently, there was only a modest acceptance by researchers and plant breeders who were interested in the microspore transgenic technologies (Forster *et al*., 2007). Therefore, recent scientific and technological innovations, more understanding of underlying mechanisms and an expansion of end-user applications has brought about a resurgence of interest in the production of haploids in higher plants (Zhang, 2005; Forster *et al*., 2007). There are few reports about the production of doubled haploid (DH) plants through microspore culturing in crops like oilseed rape and wheat etc. Numerous factors e.g., donor plant's genotype and physiology, microspore developmental stage, culture conditions and pretreatment affect the microspore embryogenesis (Xu *et al*., 2007). Successful application of microspore culturing techniques for *in vitro* high-frequency plant regeneration largely depends on genotype, choice of explant, hormonal combination in the medium and other culture conditions.

Keeping in view the importance and effectiveness of MD embryos, a series of experiments were conducted on

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two leading cultivars of oilseed rape (*Brassica napus* L.) to develop a more efficient protocol for *in vitro* embryogenesis, shoot regeneration and doubling efficiency. It could be beneficial to create some novel germplasm for further use in rapeseed breeding programs.

MATERIALS AND METHODS

Plant materials: Two oilseed rape (*Brassica napus* L.) cultivars ZS758 and ZS72, having relatively better response to microspore culture out of several genotypes tested (data not included) were selected for this study. Healthy plants of these cultivars grown in greenhouse were used for bud collection. Inflorescences (25 & 50 cm length) were picked from the healthy plants and kept in the nutrient solution (130 g L^{-1} sucrose) or water *in vitro* at 4°C in the growth chamber for prolonged supply of donor flower buds.

Microspore isolation and culture: The microspore culture protocol is based on Fletcher *et al*. (1998) and Zhou *et al*. (2002b, c) with some modifications. Ten flower buds at late uninucleate stage of pollen development were plucked randomly from inflorescences based on bud size (usually 3-4 mm). After surface sterilization in 5% sodium hypochlorite for 18 min and washing 3 times in sterile distilled water, the buds were macerated in cold NLN medium (Lichter, 1982; Keller *et al*., 1987), buffered at pH 5.8, and filtered through a 40 μm nylon mesh into a centrifuge tube. After adding activated charcoal, microspore suspension was dispensed into 60 × 15 mm Petri dishes (4 mL per plate). A microspore density of about 2×10^4 mL^{-1} was used i.e., 10 flower buds equaled 10 Petri dishes. Dishes were sealed with double layers of Parafilm and incubated in darkness.

Embryogenesis and plant regeneration: Once the embryos were visible to naked eye, cultures were transferred to a slow rotary shaker (45-60 rpm) placed in dark at 25°C. At late torpedo stage, large embryos were transferred to solid half-strength MS (Murashige & Skoog, 1962) medium (pH 5.8) supplemented with different plant growth regulators (PGRs), such as BA, NAA and GA_3. The cultures were incubated under a 16 h photoperiod (100 μE m^{-2} s^{-1}) at 25°C. When shoots developed, for rooting they were transferred to larger growth vessels containing solid half-strength MS medium. When plantlets reached 5-6 leaf stage, they were transferred to a soil-perlite mixture and kept for 2 weeks in a nursing room at 25°C, 16 h photoperiod, low light intensity (40 μE m^{-2} s^{-1}) and high relative humidity. Gradual adaptation to glasshouse conditions was followed. The regenerated plants from MD embryos were treated with colchicine to produce DH plants.

Ploidy level analysis: Analysis of ploidy levels using a flow-cytometer-FCM (*BD FACSCalibur*, Becton Dickinson, San Jose, CA, USA) was made from newly emerged young leaves of 3-week-old androgenic plants in soil. A small sample from young leaves was chopped in a detergent solution, and stained with DAPI (Zhou *et al.*, 2002a). The suspension of cell nuclei and debris was filtered through 50 μm nylon gauze and the filtrate was immediately analyzed with FCM. The instrument was calibrated against normal diploid *B. napus,* which was used as a standard throughout the experiments. Frequency curve of the sample DNA was compared to the standard peak and assigned to the ploidy level of haploid, diploid, triploid and so on.

RESULTS

Effect of *in vitro* donor inflorescence on microspore embryogenesis: Table I shows the effect of two nutrient solutions, inflorescence length and *in vitro* growth duration of donor plant on microspore embryogenesis. Data indicated that nutrient solution (130 g L^{-1} sucrose) and inflorescence length were better parameters for prolonging the physiological activity of donor plant and production of normal embryo (Table I). Irrespective of the nutrient solution and inflorescence length, there was no significant difference in embryo yield at 7 days of *in vitro* growth. As the *in vitro* growth duration was increased, the embryo yield started to decrease in sucrose containing nutrient solution; whereas, in case of water (H_2O) as a nutrient solution, the embryo yield became zero. Moreover, we also observed that when the inflorescence length was 50 cm, the donor flower bud could keep its physiological activity for longer time (Table I).

Effect of heat shock on microspore embryogenesis: Fresh isolated microspores were immediately incubated in liquid NLN-13 medium in darkness at 30 and 33°C for 2 and 5 days, respectively. For the phase of embryo differentiation, the microspores were cultured at 25°C in darkness. As soon as embryos were visible to the naked eye, the dishes were transferred to an orbital shaker (60 rpm), and cultured at 25°C in darkness until well-developed cotyledons were formed. Results indicated that normal embryo yield of both cultivars ZS758 and ZS72 at 30°C heat shocked for 2 days, was 81.5 and 74.6 embryos per bud, respectively (Table II). Embryo yield was very low as the heat shock increased up to 33°C and incubated for 5 days (31.5 & 23.0 embryos per bud of cv. ZS758 and ZS72, respectively).

Effect of activated charcoal on microspore embryogenesis: During the stage of embryo induction, microspores were cultured in NLN medium supplemented with activated charcoals at various concentrations (0-1.25 mg mL^{-1}). We observed that increased concentration of activated charcoal enhanced the yield of embryos in both cultivars. When the activated charcoal concentration was 0.25 mg mL^{-1}, the normal embryo yield of both cultivars ZS758 and ZS72 was at the highest, and it reached 82.3 and 76.5 embryos per bud, respectively (Fig. 1A).

Effect of sucrose on microspore embryogenesis: In order to fulfill the osmotic requirements, sucrose plays a key role in embryogenesis. In the present study, isolated microspores were cultured in NLN medium supplemented with different concentrations (32.5-260 g L^{-1}) of sucrose. Data showed the

Table I: Effects of nutrient solution, inflorescence length and *in vitro* growth duration of donor plant on microspore embryogenesis in *B. napus*

Variety	Nutrient solution	Inflorescence length (cm)	*In vitro* growth duration (d)	Normal embryo yield (Embryos/bud)
ZS758	Sucrose	25	7	72.6±3.07a*
			14	45.8±2.16b
			21	5.7±0.08d
		50	7	74.5±4.13a
			14	50.2±2.98b
			21	15.6±0.95c
	H_2O	25	7	69.5±3.64a
			14	0.0±0.00e
			21	0.0±0.00e
		50	7	73.1±3.45a
			14	0.0±0.00e
			21	0.0±0.00e
ZS72	Sucrose	25	7	68.4±3.62a
			14	42.5±2.47b
			21	9.3±0.08c
		50	7	64.7±5.04a
			14	49.2±3.67b
			21	12.6±0.70c
	H_2O	25	7	67.5±4.88a
			14	0.0±0.00d
			21	0.0±0.00d
		50	7	66.1±2.51a
			14	0.0±0.00d
			21	0.0±0.00d

Table II: Effect of heat shock on embryo yield from microspore embryogenesis in *B. napus*

Variety	Heat shock (°C)	Incubated duration (d)	Normal embryo yield (Embryos/bud)
ZS758	30	2	81.5±3.24a*
	30	5	44.8±1.68c
	33	2	63.0±3.46b
	33	5	31.5±2.08c
ZS72	30	2	74.6±3.71a
	30	5	38.5±1.95c
	33	2	56.8±2.64b
	33	5	23.0±1.76d

Table III: Effect of embryo age on shoot regeneration from microspore-derived embryos in *B. napus*

Variety	Embryo age (d)	No. of embryos inoculated	Shoot regeneration frequency (%)	No. of shoots per explant
ZS758	14	80	32.6c*	4.2±0.07b*
	21	80	71.0b	4.5±0.06b
	28	80	90.5a	5.6±0.10a
	35	80	86.1a	2.8±0.01c
ZS72	14	100	40.5c	3.5±0.05b
	21	100	77.2b	4.7±0.08a
	28	100	92.8a	5.1±0.07a
	35	100	83.3ab	2.0±0.03c

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

significant effect of sucrose on production of normal embryos. Sucrose concentration at 130 g L^{-1} in NLN medium, the normal embryo yield of both cultivars ZS758 and ZS72 reached to the highest count of embryos per bud (Fig. 1B).

Table IV: Effects of BA and NAA on shoot regeneration from microspore-derived embryos in *B. napus*

Variety	BA (mg/L)	NAA (mg/L)	No. of embryos inoculated	Shoot regeneration frequency (%)	No. of shoots per explant
ZS758	0.1	0.5	80	31.2d*	2.3±0.01c*
	0.1	1	80	42.6cd	3.6±0.03b
	0.1	2	80	62.1bc	3.5±0.04b
	0.1	4	80	53.5c	3.1±0.02bc
	0.3	0.5	80	73.8b	4.2±0.03b
	0.3	1	80	81.4ab	5.0±0.02a
	0.3	2	80	91.6a	5.6±0.04a
	0.3	4	80	88.2a	5.3±0.03a
ZS72	0.1	0.5	100	28.4e	2.3±0.01c
	0.1	1	100	39.6d	2.9±0.03bc
	0.1	2	100	51.0c	3.4±0.02b
	0.1	4	100	43.5cd	3.6±0.01b
	0.3	0.5	100	73.5b	4.0±0.05ab
	0.3	1	100	82.4ab	4.5±0.03a
	0.3	2	100	92.5a	5.1±0.04a
	0.3	4	100	86.3a	4.8±0.05a

Table V: Effect of GA_3 on shoot regeneration from microspore-derived embryos in *B. napus*

Variety	GA_3 concentration (mg/L)	No. of embryos inoculated	Shoot regeneration frequency (%)	No. of shoots per explant
ZS758	0.05	80	73.6b*	1.3±0.02b*
	0.1	80	82.5a	2.8±0.03a
	0.2	80	48.0c	1.5±0.01b
	0.4	80	33.2d	1.6±0.02b
ZS72	0.05	100	67.5b	2.5±0.03b
	0.1	100	79.4a	3.0±0.02a
	0.2	100	52.1c	1.6±0.01c
	0.4	100	28.3d	1.2±0.01c

Table VI: Effect of colchicine treatment on plant survival rate and diploidization in *B. napus*

Variety	Colchicine concentration (mg/L)	Treatment duration (h)	Plant survival rate (%)	Doubling efficiency (%)
ZS758	150	30	100a*	76.8a*
	300	15	95a	59.3b
ZS72	150	30	99a	81.5a
	300	15	96a	63.7b

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

Effect of embryo age on shoot regeneration from MD embryos: In the phase of embryo differentiation (Fig. 2), we studied the effect of embryo age on shoot regeneration frequency and number of shoots per explant from MD embryos. Embryo age means the physiological condition or the time from the day visible to naked eye. We observed that the embryo age has significant effects on shoot regeneration frequency and number of shoots per explant (Table III). As the embryo age reached 4 weeks (cotyledonary stage), the shoot regeneration frequency and number of shoots per explant of both cultivars ZS758 and ZS72 were 90.5% and 5.6, and 92.8% and 5.1, respectively. The study indicated that shoot regeneration frequency was very low when the embryo age was 2 weeks (late torpedo stage). Similarly,

number of shoots per explant was relatively low when the embryo age was too much (5 weeks).

Effects of BA and NAA on shoot regeneration from MD embryos: Four week-old cotyledonary embryos were transferred onto a solid MS medium (20 g L^{-1} sucrose, 8.25 g L^{-1} agar) supplemented with different concentrations of BA (0.1 and 0.3 mg L^{-1}) and NAA (0.5-4.0 mg L^{-1}) and cultured in conical flasks at 25°C with a 16-h photoperiod under a photosynthetic photon-flux density of 100 μE m^{-2} s^{-1} emitted by white tubular florescent lamps. As shoots developed from the embryos, they were excised from callus or hypocotyls tissues and transferred to fresh 1/2 MS medium (Fig. 2). We observed that if the medium was supplemented with 0.3 mg L^{-1} BA and 2 mg L^{-1} NAA, the shoot regeneration frequency and number of shoots per explant of both cultivars ZS758 and ZS72 reached 91.6% and 5.6, and 92.5% and 5.1, respectively (Table IV).

Effect of GA_3 on shoot regeneration from MD embryos: Table V shows that GA_3 has significant effect on shoot regeneration frequency and number of shoots per explant. Four week-old cotyledonary embryos transferred onto a solid MS medium (20 g L^{-1} sucrose, 8.25 g L^{-1} agar) supplemented with GA_3 (0.1 mg L^{-1}) differentiated the highest number of shoots (Table V). Shoot regeneration frequency and number of shoots per explant of both cultivars ZS758 and ZS72 reached 82.5% and 2.8, and 79.4% and 3.0, respectively.

Effect of colchicine treatment on plantlet survival and doubling efficiency: According to our previous studies, we used two colchicine concentrations (150 & 300 mg L^{-1}) and two treatment durations (15 & 30 h) to treat the plant roots for doubling the chromosome number. Doubling frequency reached 76.8% in cv. ZS758 and 81.5% in cv. ZS72 (Table VI) when the colchicine concentration was 150 mg L^{-1} and treated for 30 h. Plantlet survival rate was beyond 95% for all combinations of colchicine concentrations and treatment durations.

Ploidy level of regenerated plants: A large number of regenerated plants being relatively stable and consistent on ploidy level were haploids and diploids (Fig. 3). More than 76.8% of regenerated plants in *B. napus* cv. ZS758 were double haploids, less than 11% were haploids, and 12.2% of the plants tested were polyploids such as triploids, tetraploids, pentaploids, hexaploids or chimerics with haploid and diploid cells, and diploid plus tetraploid cells. Further, more than 74.3% of regenerated plants in *B. napus* cv. ZS72 were double haploids, less than 13% were haploids, and 12.6% of the plants tested belonged to polyploids such as triploids, tetraploids, pentaploids, hexaploids or chimerics with haploid and diploid cells, and diploid plus tetraploid cells.

DISCUSSION

These studies investigated the effects of various factors on microspore embryogenesis and shoot regeneration from MD embryos in *B. napus*. Results showed that heat shock, incubation time, activated charcoal and sucrose concentration had significant effect on microspore embryogenesis (Tables I, II; Figs. 1A, B). Moreover, embryo age, BA and NAA combination and GA_3 had profound effect on shoot regeneration frequency (Tables III, IV & V).

Previous studies have shown that heat shock plays a key role to affect the microspore embryogenesis (Zhou *et al.*, 2002b). In this experiment, microspores were incubated

Fig. 1: Effects of activated charcoal (A) and sucrose concentrations (B) on normal embryo yield from microspore embryogenesis in *B. napus*

The same variety means followed by the same lowercase letters are not significantly different at $P \leq 0.05$ by DMR test

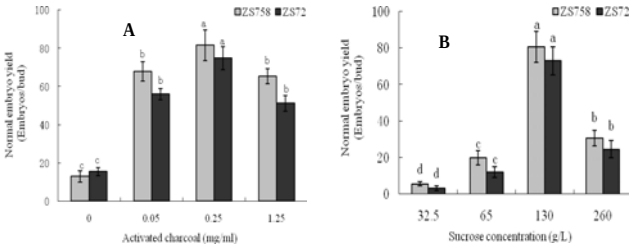


Fig. 2: Microspore embryogenesis, plant regeneration and growth in *B. napus*, (A) Microspore-derived embryos; (B) Embryos were transferred to solid half-strength MS medium; (C) Embryo differentiation; (D) Shoot regeneration; (E) Rooting; and (F) Nursing

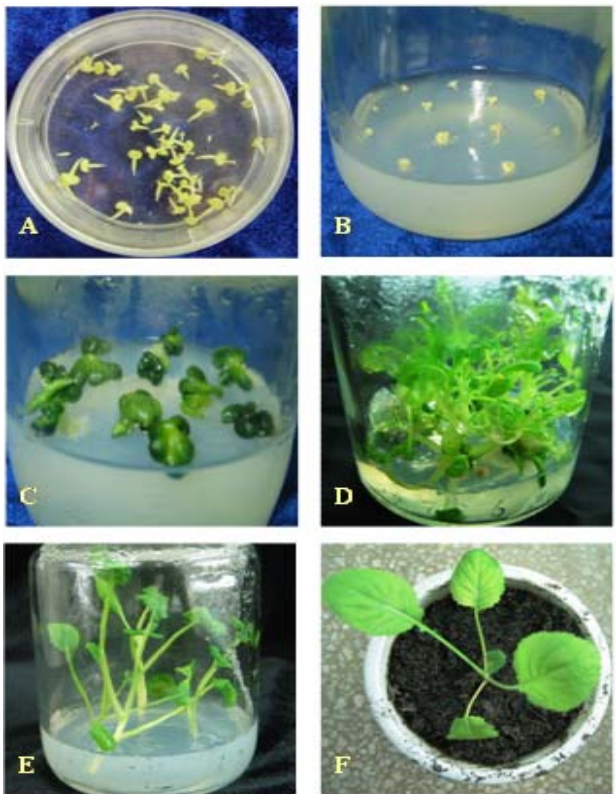
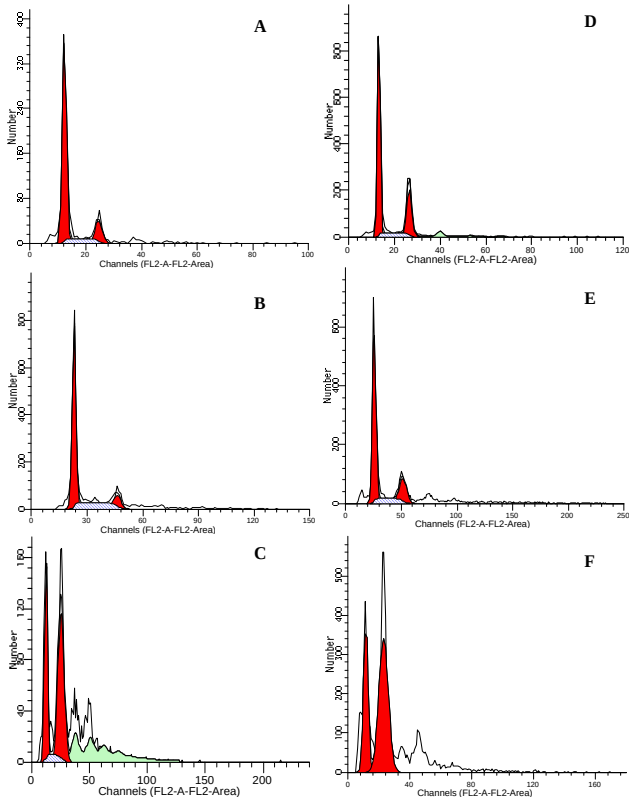


Fig. 3: Flow-cytometric histograms of haploid (A), diploid (B) and chimerics (C) of microspore-derived plants from *B. napus* cv. ZS758, and haploid (D), diploid (E) and chimerics (F) of microspore-derived plants from *B. napus* cv. ZS72



in liquid NLN-13 medium in darkness at 30 and 33°C for 2 d and 5 d, respectively. With the heat shock at 30°C and incubation for 2 days, the normal embryo yield of the two cultivars reached 81.5 and 74.6 embryos per bud, respectively (Table II). Yu and Liu (1997) and Zhou *et al*. (2002b) reported that temperature shock plays an important role in embryo induction.

During the phase of embryo induction, microspores were cultured in NLN medium supplemented with activated charcoal at various concentrations (0-1.25 mg mL^{-1}). It was noted that when the activated charcoal was 0.25 mg mL^{-1}, the normal embryo yield of both cultivars reached the highest count of embryos per bud (Fig. 1A). Results are in conformation with that observed in other cultivars of oilseed rape (Huang *et al*., 1999; Niu *et al*., 2002), indicating that added activated charcoal could improve the normal embryo yield. This encouraging effect of activated charcoal on embryogenesis could be due to its absorption of toxic compounds secreted from anther and culture medium (Tang, 2004).

Sucrose plays two crucial roles, as carbon source to support plant growth, and it also regulates osmotic pressure. In this experiment, we studied sucrose at different concentrations (32.5-260 g L^{-1}) for embryogenesis. Data showed that when the sucrose concentration was 130 g L^{-1}, the normal embryo yield of both cultivars reached the highest embryos count per bud (Fig. 1B). Accordingly, effects of sucrose on normal embryo yield in *B. napus* were significant. However, Ilic-Grubor *et al*. (1998) and Ferrie & Keller (2007) observed that the quality and quantity of MD embryos of cruciferous species can be enhanced by using polyethylene glycol (PEG-4000) to replace sucrose in the culture medium. Benefit of using PEG is that embryos are morphologically similar to zygotic embryos and have enhanced germination capabilities (Ferrie & Keller, 2007). Therefore, it is worthwhile to replace sucrose to PEG as osmoticum for efficient embryogenesis.

Embryo age is one of the most important factor for affecting shoot regeneration frequency and number of shoots per explant from MD embryos, but seldom reported in previous studies. When the embryo age reached 4 weeks, the shoot regeneration frequency and number of shoots per explant of both cultivars produced the highest frequency (Table III). The study showed that shoot regeneration was very low when the embryo age was too small (2 weeks) and number of shoots per explant was lower when the embryo age was too much (5 weeks). We speculated that if the embryo is too young, they might not adapt to new environment and differentiate shoots; whereas, if embryo is too old, culture media (NLN medium) could become exhausted and can not support nutrition to maintain its physiological activity.

If the culture media was supplemented with higher concentration of BA (0.3 mg L^{-1}) and medium concentration of NAA (2.0 mg L^{-1}), the shoot regeneration frequency and number of shoots per explant of both cultivars gave the best response. These results are consistent with those of Zhang (2005) in oilseed rape. However, Waseem *et al.* (2009) reported that the best regeneration of chrysanthemum plantlets was at the lowest NAA concentration (0.5 mg L^{-1}). Similarly, Panjaitan *et al.* (2007) obtained the highest shoot count per explant of papaya in MS medium having 1.0 mg L^{-1} BAP along with 0.05 mg L^{-1} NAA. A decrease in auxin concentration and increase in cytokinin concentration is reported to improve the shoot count in differentiation tissues (Misra, 1996; Misra *et al.*, 1994a, b). Further, we observed the effects of GA_3 treatment on shoot regeneration frequency and number of shoots per explant from MD embryos. Shoot regeneration frequency and number of shoots per explant of both cultivars got better response when GA_3 concentration reached 0.1 mg L^{-1}. Rustaei *et al.* (2009) got significant positive results even with 2 mg L^{-1} GA in both MS and N6 media used for shoot regeneration and proliferation in apple. However, added GA_3 can cause vitreous in shoot regeneration, which was also observed by Tang *et al*. (2003) and He *et al*. (2006). Further studies using different GA_3 concentrations in differentiation medium need to be done to explain this.

It is generally assumed that occurrence of spontaneous diploidy is primarily controlled by genetic

factors (Keller & Armstrong, 1978; Chen & Beversdorf, 1992). Basic diploid *Brassica* types such as *B. rapa* and *B. oleracea* might be more prone to spontaneous diploidization (Gu *et al.*, 2003). While, spontaneous diploidization for the autotetraploid *B. napus* cultivars ZS758 and ZS72 was only 36.5% and 47.1%, respectively. This is insufficient for exploitation in actual DH breeding program, and thus requires an additional treatment with chromosome doubling agents such as colchicine. However, the real mechanism of spontaneous diploidization in microspore cultures still remains unclear, and thus requires further research.

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