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ORIGINAL ARTICLE

Effects of genotypes and culture conditions on microspore embryogenesis and plant regeneration in several subspecies of *Brassica rapa* L.

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Abstract A number of factors influencing microspore embryogenesis and plant regeneration were examined in five subspecies (*rapa*, *oleifera*, *niposinica*, *perviridis*, *broccoletto*) of *B. rapa*. Addition of 6-benzylaminopurine (BA) in 1/2 NLN-10 medium improved the embryo yield by 2–12 fold. Addition of activated charcoal (AC) in the medium was not effective for microspore embryogenesis. Moreover, AC canceled the positive effect of BA, when the medium containing both BA and AC was used. Of 24 genotypes examined for microspore embryogenesis, 22 genotypes of all five subspecies produced embryos ranging from 0.02 to 15.0 per 2×10^5 microspores, but two genotypes were not responsive. Low temperature pretreatment of flower buds significantly improved the microspore embryogenesis. When cotyledonary embryos were subcultured on a filter paper placed on top of 0.8 % agar-solidified B5-2 medium and 1.6 % agar B5-2 medium, plant regenerations were increased 4–8 fold compared to 0.8 % agar medium. The ploidy levels of regenerated plants in three genotypes were determined by flow cytometry, revealing that 66–100 % of them were diploid. The results enable the advancement of breeding programs and genetic studies in *B. rapa.*

Keywords *Brassica rapa* · Genotypic variation · Microspore culture · Activated charcoal (AC) · 6-benzylaminopurine (BA) · Plant regeneration

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Introduction

The production of haploids and doubled haploids (DHs) from gametophytic cells plays an important role in plant breeding and basic science. In genus *Brassica*, since successful isolated microspore culture of *B. napus* was reported by Lichter (1982), a large amount of research has been carried out to improve this technique and to expand it to other *Brassica* species and allied genera (Takahata 1997; Palmer and Keller 1999; Ferrie and Keller 2004; Xu et al. 2007). In order to effectively obtain haploids and DHs, successful techniques on two different culture processes are needed, the first being induction of microspore embryogenesis, and the second being effective plant regeneration from microspore-derived embryos. In the former process, many factors have been clarified, such as genotypes, developmental stage of microspores, pretreatment of microspores, culture media, and culture conditions.

In *B. rapa*, genotypic differences in embryogenesis from isolated microspores have been investigated for turnip rape (ssp. *oleifera*) (Guo and Pulli 1996), Chinese cabbage (ssp. *pekinensis*) (Kuginuki et al. 1997), and pakchoi (ssp. *chinensis*) (Cao et al. 1994). However, little work has been carried out on microspore culture of turnip (ssp. *rapa*) and other relatives (ssp. *niposinica*, *perviridis*, *broccoletto*) consumed as vegetables. Although the routine protocols of microspore embryogenesis of Brassicas have been established (Swanson 1990; Ferrie 2003), surveys of high responsive genotypes and improvement of culture media and conditions are needed in novel starting materials. Exogenous plant growth regulators are not required in the microspore embryogenesis in Brassicas. However, low levels of auxin and cytokinin were included in culture media. 6-benzylaminopurine (BA) is reported to optionally increased embryo yield in *B. napus* (Charne and

Beversdorf 1988). The use of activated charcoal (AC) also improved embryogenesis in *B. rapa* ssp. *oleifera* (Guo and Pulli 1996), *B. oleracea* (Dias 1999), and *B. juncea* (Prem et al. 2008).

Frequency of direct plant regeneration from microspore-derived embryos is often low and variable. Majority embryos undergo abnormal development such as abnormal proliferation of hypocotyle and cotyledons and the formation of secondary embryos. Of Brassicas, *B. rapa* showed lowest frequency of direct plant recovery (Palmer and Keller 1999). Several pretreatments of embryos such as low temperature, ABA, and desiccation have been reported to enhance the direct plant regeneration in *Brassica* spp. (Kott and Beversdorf 1990; Huang et al. 1991; Wakui et al. 1994; Zhang et al. 2006). Culturing the embryos on filter paper placed on the top of agar medium and/or on the medium containing high concentration of gelling agent increased the frequency of regeneration (Takahata and Keller 1991; Peng et al. 1994).

In this paper, we report microspore embryogenesis and plant regeneration from five subspecies (*rapa, oleifera, niposinica*, *perviridis*, and *broccoletto*) of *B. rapa*, which have been hardly reported until now. Special attention was given to the effect of BA and AC on the medium, genotypic variation, and pretreatment of low temperature of buds. In addition, effective plant regeneration from microspore-derived embryos was examined.

Materials and methods

Plant material and growth condition

Twenty-four accessions of *B. rapa* including 16 accessions of turnip (ssp. *rapa*), 2 of turnip rape (ssp. *oleifera*), 2 of mizuna (ssp. *niposinica*), 2 of komatsuna (ssp. *perviridis*), and 2 of broccoletto (ssp. *broccoletto*) were kindly provided by the Institute of Plant Genetics and Crop Plant Research (IPK) in Germany, the National Institute of Agrobiological Sciences (NIAS) in Japan, the Dutch Crop Genetic Resources Center (CGN) in the Netherlands, Noguchi Seed Co. in Japan, and French Co. in France (Table 1). The donor plants were grown in 24-cm pots containing black soil and leaf mold (10:1) in an uncontrolled-environment greenhouse, and fertilized with 6:10:5 (N:P:K) Hyponex (HYPONeX JAPAN) weekly after bolting.

Microspore culture

Microspore culture was carried out as previously described (Zhang and Takahata 2001). Buds 2–3 mm in length were collected and surface-sterilized in sodium hypochlorite (1.5 % active chlorite) for 10 min, and then rinsed three times with sterile distilled water. After the buds were macerated in a mortar containing B5 medium (Gamborg et al. 1968) supplemented with 10 % sucrose at pH 6.0 (B5-10), microspores were obtained by filtration through Miracloth (CALBIOCHEM), and then washed three times with B5-10 by centrifugation at 120*g* for 3 min. The microspores were suspended at a density of 1×10^5/ml in 1/2NLN medium containing 10 % sucrose (1/2NLN-10) (Takahashi et al. 2011), supplemented with BA (0, 0.1, 0.3 mg/l) and AC (0, 150 mg/l) as shown in Table 1. Two millilitres of the microspore suspension were plated in a 60 × 15-mm plastic Petri dish. The Petri dishes were incubated at 32.5 °C for 1 day prior to maintenance at 25 °C. After 2 weeks of culture in the dark, the embryo yield was examined.

Low temperature pretreatment

Buds 2–3 mm in length were collected and were surface-sterilized as described above. Twelve buds were put into a 60 × 15-mm plastic Petri dish containing 5 ml of B5-10 medium, and then were stored for 0, 5, 10, and 20 days at 4 °C in the dark. After this pretreatment, the microspores were isolated from the buds and were cultured in 1/2NLN-10 medium supplemented with 0.1 mg/l BA as described above.

Plant regeneration and ploidy determination

Cotyledonary embryos, which were produced in 1/2 NLN-10 medium with 0.1 mg/l BA, were transferred: to 0.8 % agar-solidified B5 medium supplemented with 2 % sucrose at pH 5.8 (B5-2); to a filter paper placed on top of the 0.8 % agar-solidified B5-2 medium; and to 1.6 % agar-solidified B5-2 medium. Seven embryos were cultured in a 90 × 20-mm plastic Petri dish, and incubated at 25 °C with a 16 h/day photoperiod of cool white illumination (50 μmol m^{-2} s^{-2}) for 1 month.

Ploidy level of the plant regenerated in 1.6 % agar-solidified B5-2 medium was estimated by flow cytometry using Ploidy Analyzer (Partec, Germany). Sample preparation and measurement of the ploidy level were performed according to the manufacturer's instructions and Doi et al. (2010).

Statistical analyses

The microspore cultures were performed on at least 10 plates per experiment, and each experiment had at least 3 independent replicates. Statistical analyses were performed using the computer program JMP 8.0 (SAS Institute, USA).

Table 1 Effects of genotype, 6-benzylaminoprine (*BA*) and activated charcoal (*AC*) on microspore culture of *B. rapa*

Subspecies	Genotype	Origin	Source (accession no.)	BA (mg/l)	AC[a]	No. of embryos per Petri dish[b]
rapa	Nagasaki Aka Kabu	Japan	Noguchi Co.	0.0	−	3.03 ± 1.04 cdef
				0.1	−	13.20 ± 2.07 ab
				0.3	−	10.78 ± 0.77 b
				0.0	+	2.07 ± 0.15 cdef
				0.1	+	2.19 ± 1.81 cdef
				0.3	+	2.13 ± 0.67 cdef
	Yorii Kabu	Japan	Noguchi Co.	0.0	−	0.05 ± 0.05 f
				0.1	−	0.23 ± 0.03 ef
				0.3	−	0.60 ± 0.35 def
				0.0	+	0.03 ± 0.06 f
				0.1	+	0.00 ± 0.00 f
				0.3	+	0.04 ± 0.03 f
	Shogoin Kabu	Japan	Noguchi Co.	0.0	−	0.47 ± 0.55 ef
				0.1	−	0.87 ± 0.31 cdef
				0.3	−	0.77 ± 0.38 cdef
				0.0	+	1.43 ± 0.21 cdef
				0.1	+	1.21 ± 0.26 cdef
				0.3	+	2.37 ± 0.32 cdef
	Kanamachi Ko Kabu	Japan	Noguchi Co.	0.0	−	0.78 ± 0.95 cdef
				0.1	−	1.96 ± 1.71 cdef
				0.3	−	1.63 ± 1.45 cdef
				0.0	+	0.20 ± 0.30 ef
				0.1	+	0.15 ± 0.13 ef
				0.3	+	0.11 ± 0.05 f
	Early White Flat Dutch	Unknown	IPK (BRA2209)	0.0	−	3.32 ± 2.20 cdef
				0.1	−	15.00 ± 3.36 a
				0.3	−	14.47 ± 0.95 ab
				0.0	+	2.09 ± 0.28 cdef
				0.1	+	1.73 ± 0.70 cdef
				0.3	+	1.19 ± 0.62 cdef
	Italiaanse Witte Roodkop	Italy	IPK (BRA1115)	0.0	−	1.03 ± 0.96 cdef
				0.1	−	3.40 ± 1.25 cdef
				0.3	−	3.10 ± 0.92 cdef
				0.0	+	0.70 ± 0.72 def
				0.1	+	1.10 ± 0.72 cdef
				0.3	+	0.33 ± 0.40 ef
oleifera	Kalyania	Bangladesh	CGN (CGN06839)	0.0	−	4.03 ± 3.90 cde
				0.1	−	12.7 ± 2.71 ab
				0.3	−	11.33 ± 2.06 ab
				0.0	+	3.57 ± 1.58 cdef
				0.1	+	4.45 ± 1.13 cd
				0.3	+	2.80 ± 0.70 cdef
niposinica	Maruba Mibuna	Japan	Noguchi Co.	0.0	−	1.00 ± 0.74 cdef
				0.1	−	4.07 ± 0.92 cde
				0.3	−	3.54 ± 0.82 cdef
				0.0	+	0.40 ± 0.35 ef
				0.1	+	0.40 ± 0.49 ef
				0.3	+	0.33 ± 0.41 ef

Table 1 continued

Subspecies	Genotype	Origin	Source (accession no.)	BA (mg/l)	AC[a]	No. of embryos per Petri dish[b]
perviridis	Wase Maruba Komatsuna	Japan	Noguchi Co.	0.0	−	1.10 ± 1.15cd ef
				0.1	−	4.67 ± 1.65 c
				0.3	−	3.77 ± 1.25 cdef
				0.0	+	0.67 ± 0.47 def
				0.1	+	0.80 ± 0.30 cdef
				0.3	+	0.53 ± 0.49 def
broccoletto	Qarantina	Italy	French Co.	0.0	−	0.00 ± 0.00 f
				0.1	−	0.02 ± 0.03 f
				0.3	−	0.05 ± 0.05 f
				0.0	+	0.08 ± 0.06 f
				0.1	+	0.04 ± 0.06 f
				0.3	+	0.07 ± 0.08 f

Values tabulated are mean ± SD at three replications. Different letters are significantly different using Tukey–Kramer's HSD test ($P < 0.05$)

[a] + present; − absent

[b] 2×10^5 microspores were cultured in each Petri dish

Results

Effects of BA, AC and genotypes for microspore embryogenesis

Effects of BA and AC for microspore embryogenesis of *B. rapa* were examined using 10 cultivars (Table 1). Although microspore embryogenesis was found in the 1/2NLN-10 medium without BA and AC for all genotypes except for 'Quarantina,' addition of BA in the medium without AC significantly improved embryo production in all genotypes (Tables 1 and 2). In particular, 0.1 mg/l BA was more effective than 0.3 mg/l BA, although there was no significant statistical difference between them (Table 2). Addition of AC in the medium without BA was not effective for microspore embryogenesis in all genotypes (Table 1). In addition, AC canceled the positive effect of BA, when microspores were cultured in the medium containing both BA and AC.

One half NLN-10 medium supplemented with 0.1 mg/l BA was used to test microspore embryogenic ability in 14 other cultivars. Table 3 shows genotypic variation on embryogenesis in 24 cultivars of five subspecies. Of 24 cultivars, 22 from five subspecies showed embryogenesis. The embryo yield ranged from 0.02 per 2×10^5 microspores for three cultivars (Hinona, BRA1901, Qarantina) to 15.0 for 'Early White Flat Dutch.' Three cultivars such as 'Nagasaki Aka Kabu,' 'Early White Flat Dutch' and 'Kalyania' showed significantly higher embryo yields than other cultivars. On the other hand, two cultivars, 'Okute Mizuna' and 'Centoventina,' did not exhibit any microspore embryogenesis.

Table 2 Effects of activated charcoal (*AC*) and 6-benzylaminoprine (*BA*) on microspore culture of *B. rapa*; the values are means of 10 genotypes shown in Table 1

AC[a]	BA (mg/l)	No. of embryos per Petri dish[b]
−	0.0	1.48 ± 1.91 b
−	0.1	5.61 ± 5.76 a
−	0.3	5.00 ± 5.10 a
+	0.0	1.12 ± 1.22 b
+	0.1	1.21 ± 1.46 b
+	0.3	0.99 ± 1.09 b

Values tabulated are mean ± SD. Different letters are significantly different using Tukey–Kramer's HSD test ($P < 0.05$)

[a] + present; − absent

[b] 2×10^5 microspores were cultured in each Petri dish

Low temperature pretreatment

The effect of low temperature pretreatment on embryogenesis was examined in the most responsive three cultivars, i.e. 'Nagasaki Aka Kabu,' 'Early White Flat Dutch' and 'Kalyania.' Low temperature pretreatment for 10 days produced more embryos than non-treatment in all three genotypes, and especially significant increase was shown in 'Early White Flat Dutch' (Table 4). On the other hand, embryo yields of 5 and 20 days pretreatment were similar to those of non-treatment.

Plant regeneration and ploidy level determination

Plant regeneration from embryos varied between subculture conditions and between genotypes (Table 5). When

Table 3 Genotypic variation on microspore embryogenesis in *B. rapa*

Subspecies	Genotype	Origin	Source (accession no.)	No. of embryos per Petri dish[a]
rapa	Nagasaki Aka Kabu	Japan	Noguchi Co.	13.20 ± 2.07 a
	Yorii Kabu	Japan	Noguchi Co.	0.23 ± 0.03 cd
	Shogoin Kabu	Japan	Noguchi Co.	0.87 ± 0.31 bcd
	Kanamachi Ko Kabu	Japan	Noguchi Co.	1.96 ± 1.71 bcd
	Early White Flat Dutch	Unknown	IPK (BRA2209)	15.00 ± 3.36 a
	Italiaanse Witte Roodkop	Italy	IPK (BRA1115)	3.40 ± 1.25 bcd
	Hinona	Japan	Noguchi Co.	0.02 ± 0.03 d
	Sugukina	Japan	Noguchi Co.	0.29 ± 0.33 cd
	Kanazawa Ao Kabu	Japan	Noguchi Co.	0.07 ± 0.03 d
	Onaga	Japan	NIAS (26813)	1.90 ± 1.11 bcd
	Kisobeni Kabu	Japan	Noguchi Co.	0.28 ± 0.13 cd
	Atsumi Kabu	Japan	Noguchi Co.	0.35 ± 0.31 cd
	Kuretsubo Kabu	Japan	Noguchi Co.	0.15 ± 0.10 d
	Pusa Chandrina	India	CGN (CGN06711)	1.95 ± 0.77 bcd
	YUEN QIN CAI	China	NIAS (76706)	0.92 ± 0.69 bcd
	Unknown	Afghanistan	IPK (BRA2291)	0.57 ± 0.45 cd
	Unknown	Iraq	IPK (BRA1901)	0.02 ± 0.03 d
oleifera	Kalyania	Bangladesh	CGN (CGN06839)	12.70 ± 2.71 a
	Unknown	Pakistan	CGN (CGN07217)	3.57 ± 1.89 bcd
niposinica	Maruba Mibuna	Japan	Noguchi Co.	4.07 ± 0.92 bc
	Okute Mizuna	Japan	Noguchi Co.	0.00 ± 0.00 d
perviridis	Wase Maruba Komatsuna	Japan	Noguchi Co.	4.67 ± 1.65 b
	Sin Bansei Komatsuna	Japan	Noguchi Co.	0.12 ± 0.03 d
broccoletto	Qarantina	Italy	French Co.	0.02 ± 0.03 d
	Centoventina	Italy	French Co.	0.00 ± 0.00 d

Values tabulated are mean ± SD at three replications. Different letters are significantly different using Tukey–Kramer's HSD test ($P < 0.05$)

[a] 2×10^5 microspores were cultured in each Petri dish

cotyledonary embryos were subcultured onto a filter paper placed on top of 0.8 % agar-solidified B5-2 medium and 1.6 % agar-solidified B5-2 medium, the frequency of normal plant regenerations was 4–9 fold higher than 0.8 % agar B5-2 medium in 'Nagasaki Aka Kabu' and 'Early White Flat Dutch.' The highest frequency of normal development was obtained in 1.6 % agar B5-2 medium in all genotypes. On the other hand, the frequency of normal development from embryos of 'Kalyania' was very low, and almost all their embryos showed abnormal development with thickened hypocotyls and cotyledons.

Ploidy levels of plantlets regenerated on 1.6 % agar B5-2 medium were determined by flow cytometry (Table 6). The higher frequency of diploids was obtained in all three cultivars examined. In particular, all regenerants were diploids in 'Nagasaki Aka Kabu.' In 'Early White Flat Dutch' and 'Kalyania,' 21 and 29 % haploids were also obtained, respectively. Besides diploid and haploid, low frequency of triploids were found in these two cultivars.

Discussion

We found that addition of BA significantly improved microspore embryogenesis of several subspecies of *B. rapa*. Such positive effect of BA is in agreement with previous results in *B. napus* (Charne and Beversdorf 1988). Ferrie (2003) recommended the addition of 0.1 mg/l BA in microspore culture of *B. rapa*, though the comparison between with and without BA was not shown. Cao et al. (1994) reported the promotive effect of plant growth regulators (0.5 mg/l NAA and 0.05 mg/l BA) in microspore embryogenesis of *B. rapa* ssp. *chinensis*. However, it was unclear which plant growth regulators were effective. The present study demonstrates that BA is effective on microspore embryogenesis in several subspecies of *B. rapa*.

The use of AC has been reported to improved microspore embryogenesis in *B. rapa* (Guo and Pulli 1996), *B. oleracea* (Dias 1999), *B. nigra* (Margale and Chevre 1991), and *B. juncea* (Prem et al. 2008). On the other hand, Prem et al. (2005) reported that AC gave a negative effect

Table 4 Effect of duration of low temperature pretreatment on microspore culture of *B. rapa*

Genotype	Duration (days)	No. of embryos per Petri dish[a]
Nagasaki Aka Kabu	0	11.9 ± 5.6 bcd
	5	12.9 ± 3.5 bcd
	10	17.5 ± 4.3 b
	20	13.6 ± 3.4 bc
Early White Flat Dutch	0	14.6 ± 3.5 bc
	5	15.1 ± 4.1 bc
	10	30.7 ± 8.3 a
	20	18.0 ± 8.7 b
Kalyania	0	9.7 ± 3.0 cd
	5	8.3 ± 4.0 cd
	10	14.7 ± 4.2 bc
	20	4.7 ± 2.3 d

Values tabulated are mean ± SD at three replications. Different letters are significantly different using Tukey–Kramer's HSD test ($P < 0.05$)

[a] 2×10^5 microspores were cultured in each Petri dish

Table 5 Percentage of normal shoot development of microspore-derived embryos under three subculture conditions in *B. rapa*

Genotype	Condition of embryo subculture	No. of total embryos subcultured[a]	% Normal developing embryos per Petri dish ± SD[b]
Nagasaki Aka Kabu	Agar 0.8 %	56	5 ± 7 de
	Agar 0.8 % + FP[c]	56	20 ± 11 cd
	Agar 1.6 %	56	45 ± 16 ab
Early White Flat Dutch	Agar 0.8 %	56	9 ± 11 de
	Agar 0.8 % + FP	56	32 ± 18 bc
	Agar 1.6 %	56	57 ± 19 a
Kalyania	Agar 0.8 %	56	0 ± 0 e
	Agar 0.8 % + FP	56	0 ± 0 e
	Agar 1.6 %	56	2 ± 5 de

Different letters are significantly different using Tukey–Kramer's HSD test ($P < 0.05$)

[a] Seven embryos were subcultured in each Petri dish

[b] Values tabulated are means ± SD for eight replications

[c] Embryos were subcultured onto a filter paper placed on top of 0.8 % agar-solidified B5-2 medium

in embryogenesis of *B. juncea*. Our results also showed that AC was deleterious for microspore embryogenesis. It is possible that this discrepancy in the effect of AC may be due to differences in the treatment method of AC and the strength of major salts of the culture medium. In all reports

Table 6 Ploidy level of regenerated plants from microspore culture of *B. rapa* determined using flow cytometry

Cultivars	No. of regenerated plants[a]		
	Haploids	Diploids	Triploids
Nagasaki Aka Kabu	0	100	0
Early White Flat Dutch	21	77	2
Kalyania[b]	29	66	5

[a] 100 plants were analyzed

[b] Abnormally developed plants were included

except for Guo and Pulli (1996), which showed a positive effect, addition of AC with agarose was used according to Gland et al. (1988). On the other hand, in studies showing negative effect (Prem et al. 2005; present study), AC was used without agarose. Prem et al. (2005) suggested that sticking the AC to the microspores and developing embryos hinders their growth and development. NLN medium was used in all research in which addition of AC promoted embryogenesis. We used 1/2NLN medium, which is the strength of major salts reduced to half. Wang et al. (2009) reported that 1/2NLN medium induced significantly more embryo production than NLN medium. It is believed that AC is a non-selective absorbent and may be absorbing inhibitory substances released from microspores into culture medium (Babbar and Gupta 1986; Babbar et al. 2004). It is considered that AC in NLN medium also reduced concentration of major salts resulting in optimum conditions for embryo induction, but AC in 1/2NLN medium absorbed optimal concentration of major salts to lower levels resulting in reduction of embryogenesis.

Low temperature pretreatment significantly enhanced microspore embryogenesis. These results are in agreement with earlier studies in *B. napus* (Lichter 1982; Gu et al. 2004) and Chinese cabbage (Sato et al. 2002). In addition to such positive effects, the storage of buds in low temperature for certain periods can avoid the concentration of work in limited time (Sato et al. 2002; Gu et al. 2004).

As shown in previous reports of microspore embryogenesis in *B. rapa* ssp. *chinensis* (Cao et al. 1994), *oleifera* (Guo and Pulli 1996), and *pekinensis* (Kuginuki et al. 1997), and in *Brassica* spp. (Chuong and Beversdorf 1985; Takahata and Keller 1991), our study indicated large genotypic variations in embryo yield. Zhao et al. (2010), who examined microspore culture of many accessions, reported that Chinese cabbage (ssp. *pekinensis*), pakchoi (ssp. *chinensis*), and winter oil rape (ssp. *oleifera*) had good responses, but ssp. *broccolette* and other ssp. had no responses. In this study, all five subspecies produced embryos, and to the best of our knowledge, this is the first report on the embryogenesis from ssp. *niposinica*, *perviridis*, and *broccoletto*.

Plant regeneration frequencies from microspore-derived embryos are often low and variable (5–70 %) depending on species and genotypes, and especially with *B. rapa*, plant recovery was lower (5–20 %) in comparison with other *Brassica* spp. (Palmer and Keller 1999). Our results demonstrate that culturing embryos on media solidified with a higher concentration (1.6 %) of gelling agent and/or on filter paper placed on top of 0.8 % agar medium enhanced plant regeneration. These results are in agreement with those of *B. oleracea* (Takahata and Keller 1991) and *B. napus* (Peng et al. 1994). Peng et al. (1994), working on various concentration of agar in the medium, reported that optimum matric potential for plant regeneration from microspore-derived embryos was −3 to −4 kPa, which was caused by 1.6 % agar. These results indicate that direct plant regeneration is promoted by osmotic stress.

The majority (66–100 %) of regenerated plants were diploids. This is consistence with Takahata (1997), who indicated a higher frequency of diploids in *B. rapa* than in *B. napus* and *B. oleracea*. Although the exact mechanism of how the diploids are induced in microspore culture is unknown, spontaneous diploidization is advantageous for haploid breeding as it omits the need for doubling treatment. Until now, we have obtained DHs of 17 cultivars in five subspecies of *B. rapa* (data not shown). The results in the present study are useful for breeding programs and genetic studies in *B. rapa*.

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