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Comparison of anther and microspore culture in androgenic embryogenesis and regeneration of broccoli (*Brassica oleracea* L. var. *italica* P.)

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The aim of this study was to compare the efficiency of broccoli anther and microspore culture methods for doubled haploid (DH) lines production. We evaluated the main influencing factors and optimized the culture methods to improve embryo induction and plant regeneration for efficient doubled haploid production in broccoli breeding. Six broccoli hybrids were used in this study. Our results show that generally, the efficiency of androgenic embryogenesis and regeneration in microspore culture is higher than that in the anther culture. Moreover, the microspore culture eliminated the possibility of plantlets coming from diploid tissue. In this study, the four-day cold pre-treatment yielded the highest number of embryos in both anther and microspore culture methods; the embryo yield at 32.5°C for 24 h was the highest in anther and microspore culture. Optimal plating densities were 30 anthers per dish in anther culture and 4×10^5 microspores per ml in microspore culture. In androgenic embryo production, the PG-96 medium proved to be more effective than NLN medium. Sucrose concentration at 10% for anther culture and 13% (w/v) for microspore culture was recommended. A total of 70 regenerants were obtained from three genotypes including doubled haploids, haploids and aneuploids.

Key words: Anther, broccoli, doubled haploid, microspore, plant regeneration.

INTRODUCTION

Broccoli (*Brassica oleracea* L. var. *italica* P.) is an important crop. Cultivars in use today are almost exclusively F_1 hybrids (Wang et al., 1999). Conventional inbreeding is laborious and time consuming, hence double haploid (DH) production and cross-pollinated DH-progeny as an alternative can be the greatest source in variation for plant breeding or selection. The two major advantages of using DH in plant breeding are the increase of selection efficiency and shortening the time to release new cultivars (Castillo et al., 2000). Furthermore, DH lines are very valuable for quantitative genetics studies and for genome mapping and quantitative trait locus (QTL) analysis (Monforte et al., 2004; Behn et al., 2005). Anther cultures and isolated microspore cultures are two different

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methods widely used for DH production in broccoli. DH plants of broccoli were obtained from anther culture (Keller and Armstrong, 1983; Cogan et al., 2001) and isolated microspore culture (Takahata and Keller, 1991; Duijs et al., 1992). DH parental lines were already obtained using anther culture and introduced into breeding schemes (Farnham et al., 1998). Despite these improvements, anther and isolated microspore culture methods have some limitations in broccoli. One big problem is that the yield of embryo in some broccoli genotypes is still very low, and certain genotypes do not appear amenable to the process at all (Dias, 2001; Yuan et al., 2011). Meanwhile, a general problem encountered in the use of anther culture with *B. oleracea* is that resulting populations contain a mixture of variable ploidy individuals (Chauvin et al., 1993).

Isolated microspore culture is initiated by extracting microspores from anthers, and in turn the microspores are cultured free of anther tissue, which offer the opportunity of producing large amounts of plants with less effort and reduced cost (Duijs et al., 1992). Further advantage of microspore embryogenesis would be the capability of plant regeneration from a single cell, as the isolated microspore culture eliminates the possibility of plants from diploid tissue (such as septum, anther wall and tapetum), thus resulting in an efficient androgenic embryogenesis. Androgenesis and subsequent DH production are influenced by genetic, physiological, physical and chemical factors, which affect the pollen grain while entering into a new developmental pathway. In androgenic embryogenesis, the donor genotype is one of the most important influences. In broccoli, genotype is considered to be the key factor for obtaining microspore-derived embryos (Keller and Armstrong, 1983). In androgenic cell cultures, the density of anthers or isolated microspores can influence the embryogenesis and plant regeneration. Cultured anthers release endogenous hormones and certain chemicals which could regulate and affect embryogenesis (George, 1993).

Microspores have a remarkable capacity to develop into haploid plants *via* embryogenesis *in vitro*; stress treatment triggers the induction of this sporophytic pathway. These include nitrogen starvation, short days and low temperature treatment of donor plants, which have a strong enhancing effect on microspore embryo formation in anther cultures, as well as cold or heat shock and chemical treatment of excised inflorescences, flower buds or anthers (Touraev et al., 1997). Today, heat shock is the method of choice for androgenic cell culture (Arnison et al., 1990; Duijs et al., 1992). Cold pretreatment has been used in a variety of plant species for induction of androgenesis. Such treatments given to donor materials (spike, inflorescence, flower bud etc.) before inoculation promote embryo induction, produce more embryos and enhance the green plant yield in anther and microspore cultures (Sato et al., 2002; Osolnik et al., 1993). According to Nitsch (1974), cold treatment increases the frequency of embryoid formation by increasing the number of pollen with similar nuclei and maintaining pollen in viable condition.

In plant tissue culture, the culture medium is always an important factor, providing both nutrient and osmotic environments. Many factors have been made in order to optimize medium for plant anther and microspore culture. The induction medium has not only the task to nourish the microspores but also to redirect their developmental pathway to the formation of embryos (Jähne and Lörz, 1995). All tissue culture media require the presence of a sugar(s) as a source of carbon and energy. Sugars also act as osmotic regulators in the medium. Sucrose has been the most widely used carbohydrate source in the culture medium, also commonly employed in androgenic cell culture (Pescitelli et al., 1990). The main objective of this study was to compare the efficiency of anther and isolated microspore culture for the production of DH lines from F_1 hybrids of broccoli. The study evaluated the main influencing factors and optimized the culture methods to improve embryo induction and plant regeneration for efficient practical breeding use. To our knowledge, the present comparative study of androgenic culture methods is the first one in broccoli.

MATERIALS AND METHODS

Six F_1 hybrids that came from Xiaotangshan company were investigated: namely “Mantuolu”, “Bishan”, “Luling”, “Beilu”, “Zhulu” and “Meilu118”. Seeds were germinated in a greenhouse under controlled conditions (25/20°C, day/night temperature, 16 h photoperiod at 70 µmol m^{-2} s^{-1} light intensity) in July. Seedlings were vernalised in a cold room (6°C, 12 h photoperiod at 30 µmol m^{-2} s^{-1} light intensity) for 5 to 6 weeks, after which the plants were transferred to a greenhouse under the same conditions as before. Anther and microspore cultures were performed after harvest of flower buds. For the experiments (1 to 4), only genotype “Bishan” was used as test materials.

Experimental procedure

Microspore developmental stage was checked by acetocarmine staining. Buds were surface-sterilized with 1.6% (final concentration) sodium hypochlorite solution and 0.5% Tween 20 for 15 min, then rinsed five times in sterile water before excised. For anther culture, anthers from the same plant were cultured in a 90-mm Petri dish containing 25 ml the PG-96 solid medium (Guo et al., 1999). For microspore isolation, anthers were put into the NLN liquid induction medium (Takahata and Keller, 1991) and microspores were released using a glass rod homogenizer and a microspore suspension was obtained by filtration through 45 µm nylon mesh screen. This suspension was centrifuged three times at 100 g for 3 min and resuspended in the same medium. Microspores were resuspended in the required amount of liquid induction medium, final density of microspore was determined with a haemocytometer and adjusted to required densities. The microspore suspension was incubated in a 60 mm Petri dish. Each test has five replicates.

After five to seven weeks of culture at 25°C, well-developed embryos from anther and microspore culture were transferred to MS

Table 1. Requirements of cold treatment on androgenic embryo induction of broccoli on PG-96 media (variety "Bishan" was used).

Duration of cold treatment (day)	Yield of embryo (embryos per 100 anthers) ± S. D[a]	
	Anther culture	Microspore culture
0	20.3 ± 3.2[a]	30.3 ± 4.1[b]
2	41.1 ± 9.9[b,c]	73.2 ± 12.6[c]
4	57.0 ± 13.4[c]	89.1 ± 15.0[c]
6	39.7 ± 6.3[b,c]	67.3 ± 11.5[c]
8	32.9 ± 5.6[b]	19.4 ± 2.7[a]

[a] Means followed by the same letters within columns are not significantly different according to the LSD test at the 5% level of significance. Means ± standard deviation are from five replicates.

regeneration medium (Murashige and Skoog, 1962) supplemented with 1 µM indole-3-butyric acid (IBA) and 4.4 µM 6-benzylaminopurine (BA) for four weeks and then transferred to a half-strength mineral salts MS medium for rooting. Cultures were incubated in a growth chamber with 70 µmol m^{-2} s^{-1}, and 16 h light at 25°C. Prior to transferring plantlets to soil, the roots of the plantlets were immersed in a 0.2% colchicine solution for 2 h followed by several water washes to double the chromosome. Root tip chromosomes of regenerants were counted using the Feulgen staining method (Sinkovic and Bohanec, 1988) after chromosome doubling. Every treatment was replicated five times in this study. The number of embryos and the yield of regenerants were scored and the data were analyzed with SAS 6.0 software. Statistical analyses were performed using ANOVA. Mean values were compared according to LSD test at P = 0.05.

Experiment 1: Effect of cold pre-treatment of donor materials on anther and microspore culture

Flower buds with length 3.0 to 4.0 mm were used as sources for anther and microspore culture were harvested when microspore were mostly at late uninucleate development stage, with 10 to 30% binucleate microspore and stored at 4°C in darkness with stalks in water to test the cold pre-treatment effects of different durations (0, 2, 4, 6 and 8 days). The plating densities were 30 anthers per Petri dish for anther culture and 4 × 10^5 microspores per ml for isolated microspore culture.

Experiment 2: Optimization of incubation temperature regime

In the incubation temperature regime experiment, microspores were incubated in the dark conditions at 27.5, 30 and 32.5°C for 48 h and then maintained at 25°C in the dark. The plating densities were 30 anthers per Petri dish for anther culture and 4 × 10^5 microspores per ml for isolated microspore culture. Anthers were subcultured every four weeks. For the microspore cultures, the cultures were transferred to a shaker at 60 rpm in the dark at 25°C after four weeks incubation.

Experiment 3: Optimization of plating density of anther and microspore culture

In our tests, plating density ranged from 10 to 50 anthers per dish (90 mm) for anther culture. Three microspore densities were tested for isolated microspores in liquid induction medium, including 2×10^5, 4×10^5 and 8×10^5 microspores per ml.

Experiment 4: Effects of basal medium and sugar concentrations

The PG-96 and the NLN were employed as induction media. Liquid media which were used in microspore culture were sterilized by filtration; solid media which were used in anther culture were sterilized by autoclaving at 120°C for 25 min. Additionally, α-naphthalene acetic acid (NAA) at 0.54 µM, 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.45 µM and $AgNO_3$ at 11.8 µM were filter-sterilized and added after autoclaving. For anther culture, sucrose concentration (w/v) at 6, 10 and 13% were tested on embryo induction across PG-96 and NLN media and six genotypes. For microspore culture, sucrose concentration (w/v) at 10, 13 and 17% were tested for embryo induction across both media and six genotypes. The levels of sucrose were set according to former similar study (Guo and Pulli, 1996; Guo et al., 1999). Activated charcoal was added to the culture media at 150 mg L^{-1} in anther and microspore cultures.

Experiment 5: Effect of genotypes on embryo induction

Six hybrids were tested for androgenic embryo induction under the following conditions; the stalks of donor plants were treated in sterile distilled water held at a cold temperature of 4°C in the dark for four days, incubated initially at 32.5°C for 24 h, and then transferred to 25°C in the darkness. For anther culture, plating density was 30 anthers per dish; for isolated microspore culture, plating density was 4×10^5 microspores per ml. The PG-96 with sucrose at 10% for anther culture and 13% for microspore culture was used as culture medium. The embryos bigger than 1.5 mm were counted under microscope and then transferred to MS regeneration medium.

RESULTS

Influence of cold per-treatment on anther and microspore culture

Our results reveal that androgenic embryogenesis was generally enhanced by the cold pre-treatment in broccoli. Table 1 shows the yield of embryo in genotype "Bishan" significantly improved by 2 to 8 days cold pre-treatment to anther culture and 2 to 6 days to isolated microspore culture.

Effect of cold pre-treatment on embryo induction in

Table 2. Effects of heat shock on embryo induction of anther and microspore culture of broccoli on PG-96 media (variety "Bishan" was used).

Heat shock treatment	Number of embryos per 100 anthers ± S. D.[a]	
	Anther culture	Microspore culture
25°C (control)	15.4 ± 7.2[a]	20.2 ± 7.0[a]
27.5°C (48 h)	20.4 ± 11.2[a]	24.9 ± 12.3[a]
30°C (48 h)	31.6 ± 13.5[a,b]	67.2 ± 14.5[b]
32.5°C (24 h)	54.9 ± 19.9[b]	83.5 ± 20.4[b]
32.5°C (48 h)	45.1 ± 12.9[b]	59.7 ± 12.4[b]

[a]Means followed by the same letters within columns are not significantly different according to the LSD test at the 5% level of significance. Means ± standard deviation are from five replicates.

Table 3. Effects of plating density on embryo yield on PG-96 media in anther and microspore culture of broccoli (Variety "Bishan" was used).

Plating density	Yield of embryo (embryos per 100 anthers) ± S. D[a]	
	Anther culture	Microspore culture
10 anther/dish	12.1 ± 6.2[ab]	
20 anther/dish	26.4 ± 8.2[b]	
30 anther/dish	49.9 ± 14.2[c]	
40 anther/dish	29.6 ± 11.5[b,c]	
50 anther/dish	6.8 ± 2.8[a]	
2×10^5 microspores/ml		57.5 ± 13.7[b]
4×10^5 microspores/ml		77.8 ± 20.8[b]
8×10^5 microspores/ml		23.1 ± 9.8[a]

[a]Means followed by the same letters within columns are not significantly different according to the LSD test at the 5% level of significance. Means ± standard deviation are from five replicates.

microspore cultures was greater than that in anther cultures. In our test, a 4-day cold treatment gave the best results for both anther and microspore cultures; the embryo yields of anther and microspore cultures were 57.0 per 100 anthers and 89.1 per 100 anthers for genotype "Bishan", respectively (Table 1).

The effect of heat shock regime on anther and microspore culture

In this study, a heat shock pre-culture at 32.5°C given at the beginning of culture initiation, instead of normal dark culture at 25°C, resulted in an increase of micro embryo formation.

The best result was achieved from a heat shock at 32.5°C for 24 h; the number of embryo per 100 anthers were 54.9 and 83.5 in anther and microspore culture, respectively (Table 2). The influence of heat shock regime on microspore culture was more important than that of anther culture (Table 2).

Effects of the plating density on anther and microspore culture

In our current test, culture density significantly influenced embryo induction efficiency in anther and microspore cultures. For anther culture, the density of 30 anthers per dish gave the maximum yield, while for microspore culture, the density of 4×10^5 gave the best result (Table 3).

Influences of basal media, sugar concentration on anther and microspore culture

In the current experiments, the PG-96 and the NLN used as embryo and shoot induction media were employed for both anther and microspore cultures. In the present tests, the highest embryo yields of genotype "Bishan" on the PG-96 media were 53.9 per 100 anthers and 87.6 per 100 anthers in the anther and microspore culture, and those in the NLN medium were 46.1 per 100 anthers and 64.5 per 100 anthers, respectively (Table 4). Results of the present

Table 4. Effects of sugar concentration and culture media on embryo induction of anther and microspore culture in broccoli (variety "Bishan" was used).

Method	Sucrose (%)	Number of embryo per 100 anthers ± S. D[a]	
		NLN	PG-96
Anther culture	6	34.2 ± 10.6[a,b]	46.7 ± 18.6[a]
	10	46.1 ± 17.3[b]	53.9 ± 16.9[a]
	13	20.4 ± 8.1[a]	43.5 ± 14.8[a]
Microspore culture	10	44.3 ± 6.9[a]	51.8 ± 9.0[b]
	13	64.5 ± 24.9[a]	87.6 ± 11.2[c]
	17	49.3 ± 13.9[a]	24.9 ± 6.1[a]

[a]Means followed by the same letters within columns are not significantly different according to the LSD test at the 5% level of significance. Means ± standard deviation are from five replicates.

Table 5. Effects of genotype on androgenic embryo production using PG-96 media in anther and microspore culture of broccoli.

Genotype	Number of embryo per 100 anthers ± S. D[a]	
	Anther culture	Microspore culture
Bishan	50.1 ± 18.4[a]	78.3 ± 20.1[a]
Mantuolu	29.9 ± 7.6[a]	56.9 ± 19.9[a,b]
Luling	32.1 ± 12.0[a]	41.5 ± 14.3[b,c]
Meilu 118	45.3 ± 16.1[a]	38.3 ± 6.9[b,c]
Beilu	12.6 ± 6.0[b]	23.5 ± 11[c]
Zhulu	3.9 ± 0.9[c]	5.6 ± 2.4[d]

[a]Means followed by the same letters within columns are not significantly different according to the LSD test at the 5% level of significance. Means ± standard deviation are from five replicates.

study demonstrate the advantage of the PG-96 to the NLN medium for embryo induction. In the present experiments, anther cultures produced the highest embryo yields in media with 10% of sucrose, which produced the lowest ones in 6% sucrose concentration; microspore cultures produced the highest embryo yields in media with 13% of sucrose, which produced the lowest ones in 10% sucrose concentration (Table 4).

Effects of genotype on anther and microspore culture

In the case of broccoli, anther and microspore culture ability is genetically controlled and that culture efficiency is thus genotype dependent (Takahata and Keller, 1991). This study shows that genetic order, from the highest to the lowest embryo producer in microspore culture, was "Bishan" (78.3 embryos per 100 anthers), "Mantuolu" (56.9 embryos per 100 anthers), "Luling" (41.5 embryos per 100 anthers), "Meilu118" (38.3 embryos per 100 anthers), "Beilu" (23.5 embryos per 100 anthers) and "Zhulu" (5.6 embryos per 100 anthers), respectively (Table 5).

Plant regeneration and ploidy levels of regenerant

Regeneration was relatively difficult in the present study. Many androgenic micro embryos stopped growth or developed into plantlet-like structures with vitrification. Approximately 3 to 5% of androgenic embryos developed into regenerated plants. A total of 70 regenerants had been obtained so far including doubled haploids (2n = 18), haploids (n = 9) and aneuploids (Table 6). Ploidy levels of regenerant were estimated by chromosome counting. Frequencies of chromosome doubling are shown in Table 6.

DISCUSSION

Some important factors, such as stress treatments, optimal plating densities, the basal media, sugar concentrations as well as the genotypes of donor plants strongly influence the induction of androgenic embryogenesis and the regeneration of broccoli. Cold pre-treatment of anthers is commonly employed to enhance the frequency of androgenic embryogenesis. It has been used extensively

Table 6. Ploidy levels of regenerant in anther and microspore culture of broccoli.

Genotype	Anther culture			Isolated microspore culture		
	DH (%) 2n = 18	Haploid (%) n = 9	Others (%)	DH (%) 2n = 18	Haploid (%) n = 9	Others (%)
Bishan	11 (68.8)	3 (18.8)	2 (12.5)	15 (62.5)	4 (16.7)	5 (20.8)
Mantuolu	4 (50.0)	3 (37.5)	1 (12.5)	7 (63.6)	2 (18.2)	2 (18.2)
Meilu118	3 (37.5)	3 (37.5)	2 (25.0)	1 (33.3)	2 (66.7)	0 (0.0)

in many crops such as *Brassica campestris* (Guo and Pulli, 1996) and *Triticum aestivum* (Li et al., 1988). Instead of asymmetry of normal first pollen mitosis, more symmetric divisions have been reported following cold pretreatment (Kiviharju and Pehu, 1998) likely due to blocking the normal gametophytic development. Stress treatments including low temperature treatment and heat shock act as triggers for promoting the sporophytic pathway (Touraev et al., 1996, 1997). Synthesis of proteins has been reported as a result of stress treatments. Moreover, the involvement of cytoskeletal elements or the delay of pollen or anther wall senescence has been suggested (Cordewener et al., 1995; Kiviharju and Pehu, 1998). For isolated microspore culture, the swollen microspores have specific cytological characters, comprising a large central vacuole, thin tonoplast, parietal cytoplasm and peripheral nucleus. Many microspores between very late uninucleate and early binucleate stages will “swell” after the heat shock, whereas younger and older microspores will not. Only such “swollen” microspores have the potential to continue to develop, divide and finally form microcalluses. Microspores have a remarkable capacity to develop into haploid plants *via* embryogenesis *in vitro*; stress treatment can further trigger the induction of this sporophytic pathway. Deviation from a gametophytic to a sporophytic developmental pathway has been induced in microspores by applying various pretreatments either *in vivo* or *in vitro* (Touraev et al., 1997). Currently, the method of heat shock is commonly used for androgenic cell culture in *Brassica oleracea* (Takahata and Keller, 1991; Duijs et al., 1992; Fabijanski et al., 1991). Furthermore, the combination of cold pretreatment and heat shock significantly enhances microspore embryogenesis efficiency and results in higher diploid frequency of the regenerated population compared to traditional microspore culture protocol which typically uses heat shock in broccoli (Yuan et al., 2011).

Plating density is an important factor influencing androgenesis since cultured anthers and microspores release endogenous hormones and other metabolic by-products which not only affect embryogenesis, but also competitively exploit nutrients and oxygen supply as well. Meanwhile, dead microspores are likely to release toxic substances into the culture medium. Those toxic substances tamper with the development of viable microspores (George, 1993). Constituents of the basal medium are important factors in eliciting successful androgenesis. By now, the NLN medium is recognized as a suitable medium for broccoli androgenesis (Takahata and Keller, 1991; Duijs et al., 1992). The PG-96 medium composed of relatively complex organic acids and vitamin compounds. In previous studies, the modified PG-96 induction medium promoted androgenic embryogenesis and regeneration in timothy (Guo and Pulli, 1999). Sugar as the source of carbon and energy also functions as an osmotic regulator in culture media. Carbon source in induction medium has a profound effect on anther culture response. In the case of *Brassica*, sucrose has proved effective for increasing embryo induction or improving embryo development and plant regeneration (Takahata and Keller, 1991; Duijs et al., 1992; Na et al., 2011).

Auxin and cytokinin as a whole are essential in embryo induction and shoot formation. Low level or free of growth regulator has been proven suitable for *Brassica* androgenesis (Arnison et al., 1990; Takahata and Keller, 1991; Duijs et al., 1992). In broccoli androgenic cell cultures, spontaneous diploidization has occurred with great variation. A general problem encountered in the use of anther and microspore culture with *B. oleracea* results in the mixture of regenerants with variable ploidy (Chauvin et al., 1993). The anther and microspore culture methods for the induction of androgenic embryogenesis and requirements for successful regeneration of broccoli were compared in this paper. All the important factors mentioned above had been optimized to improve embryo induction and green plant regeneration for efficient practical breeding use. The efficiency of androgenic embryogenesis and regeneration in isolated microspore culture was higher than that in the anther culture. Both anther culture and isolated microspore culture methods could be used for DH breeding in broccoli.

Conflict of interests

The author(s) did not declare any conflict of interest.

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