Factors Affecting Embryogenic Callus Production and Plant Regeneration in Anther Culture of *Bupleurum chinense*

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Abstract: **Objective** To evaluate the influences of the genotypes, anther developmental stages, and cultural conditions on the efficiency of embryogenic callus induction and plant regeneration in the anthers culture of *Bupleurum chinense*. **Methods** The different effects such as four genotypes, plant growth regulators, and temperature condition were compared in the experiments. The histological study was performed with the process of the anther culture. **Results** The highest inducing rate of embryogenic calli were achieved for the genotypes Zhongcaiyihao (ZCYH), Z4, and Z5 at the early- to middle-uninucleate stages, except for genotype ZPM1 at the tetrad stage. Cold pretreatment increased the production of the embryogenic callus, in which 4-day cold pretreatment improved the production of embryogenic callus from 0% to 2.2% and 5.0% for genotypes ZPM1 and ZCYH, respectively. No embryogenic callus was induced in the medium containing less than 0.75 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). The highest regeneration rate (34.6%) was obtained in 1/2 MS salts regeneration medium supplemented with 0.1 mg/L 6-benzylmaminopurine (BA). The low concentration of BA was able to promote the embryogenic callus formation and subsequent plantlet regeneration via somatic embryogenesis. Chromosome counting of regenerated plantlets showed mostly diploid plant ($2n$ = 12) with only one haploid plant (n = 6). Because of the low rate of microspore embryo formation, we only tracked the process of embryogenesis from the connective tissue, instead of microspore by histological observations. **Conclusion** This study establishes an efficient system for embryogenic callus induction and plant regeneration system. This is the first report on the haploid plantlet through the anther culture of *B. chinense*.

Key words: anther culture; *Bupleurum chinense*; embryogenic callus; genotype; haploid

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Introduction

The plants in *Bupleurum* L., with around 180 species (She and Watson, 2005), contain many important medicinal species such as *B. chinense* DC., *B. scorzonerifolium* Willd., *B. falcatum* L., and *B. kao* Liu. Their dried roots (*Bupleuri Radix*), known as *Chaihu*, contain several kinds of saikosaponins and have been widely used for diuretic, antithermic, and expectorant purposes in China, Japan, and Korea (Liang *et al*, 1998; Chiang *et al*, 2003). *B. chinense* and *B. scorzonerifolium* have been recorded in *Chinese Pharmacopoeia 2010* (Pharmacopoeia Committee of P. R. China, 2010). The former is the main source of medicinal *Chaihu* in China and exports to Eastern and Southeastern Asia. *Bupleuri Radix* was obtained from wild *B. chinense*. Currently, wild resources of *Bupleurum Radix* have been sharply declined (Wang, Niu, and Qin, 2007). As a result, *B. chinense* has been widely cultivated in China. However, the production of *B. chinense* is restricted by low yield and instable quality (Pen, 1996). The essential approach to acquire high yield and good quality is genetic improvement for breeding new variety of *B. chinense*. Related studies on tissue culture and rapid propagation of *B. chinense* have progressed (Hao *et al*, 2008; 2010). But the traditional breeding method is difficult for *B. chinense* due to its high level of heterozygosity, low

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level of autogamy, and needing five to ten years. Thus, selecting a variety with high yield and consistent quality is a major challenge for *B. chinense* cultivation. Since Guha and Mahseswari (1964) reported the first *in vitro* culture anther-derived haploid from plants in *Datura* L., due to its high selection efficiency and short breeding time, haploid production obtained from *in vitro* anther culture has been widely used around the world as an effective alternative method to obtain haploid and produce homozygous double-haploid plants for developing cultivars and uniforming lines in more than 250 plant species (Maluszynski *et al*, 2003). However, in the Apiaceae species**,** previous studies showed that it was very difficult to obtain haploid or doubled haploid by anther culture. Limited studies reported plantlets being regenerated from celery (Dohya, Matsubara, and Murakami, 1997) and mitsuba (Matsubara, Dohya, and Murakam, 1995), and haploid plants being regenerated from anther culture of caraway (Smýkalová *et al*, 2009) and carrot (Andersen, Christiansen, and Farestveit 1990; Górecka *et al*, 2008). Most of the researches on the plants of *Bupleurum* L. were concentrated in *B. falacutum*. Shon, Yoshida, and Shon, (1997) reported that haploid embryogenic calli and plantlets were obtained from anther culture of *B. falacutum*. Since then, several studies had been carried out to improve the regeneration rate of embryos in *B. falacutum* (Kwon, Jeong, and Kim, 2001; Shon, Totokv, and Yoshida, 1997; Shon *et al*, 2004). In this study, we evaluated the influences of the genotypes and various conditions on the efficiency of embryogenic callus induction and haploid plant regeneration in the anther culture of *B. chinense*. The culture protocol would be applicable to micropropagation, genetic transformation, and the variety improvement.

Materials and methods

Plant material

Four genotypes of *B. chinense* were used in the experiments, including Zhongchaiyihao (abbreviated as ZCYH, a breeding variety of *B. chinense*) and ZMP1 (derived from regeneration of plantlets by anther culture of ZCYH), and the fourth generation of inbred-lines Z4 and Z5 by individual selection was selected from ZCYH population. The ZCYH and ZMP1 were used in all experiments. The seeds were sown in mid-April in the experimental plots using standard agronomic practices in the Institute of Medicinal Plant Development (Beijing, China).

Anther culture procedure

Microspore developmental stage of anthers The umbels were harvested in mid-July when the microspore was in developmental stages from the tetrads stage to the tri-nucleate stage (Table 1). The microspore development stages were identified by 4',6-diamino-2-phenylindole (DAPI) staining (Yang *et al*, 2008). The umbels were collected in a conical flask and kept in a beaker containing 100 mL of distilled water at 6 ℃ for 4 d. Anthers were cultured in the induction medium [MS (Murashige and Skoog, 1962) basal salts supplemented with 3% maltose, 1.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg/L BA, 0.4 g/L *L*-glutamine, 0.2 g/L acidic hydrolysis casein, and 0.4% agar] for eight weeks and the calli were transferred to the regeneration medium (1/2 MS medium containing 0.1 mg/L BA and 0.4% agar) at 26 ℃ for seven weeks. Data of embryogenic calli were recorded after 15-week culture. Chemicals including DAPI, 2,4-D, BA, and acidic hydrolysis casein were from Sigma Chemical Company (St. Louis, MO, USA), and other chemicals were from Sangon Biotech (Shanghai) Co., Ltd.

Cold pretreatment To study the effect of cold pretreatments on anther response, umbels of ZMP1 and ZCYH were pre-cultured at low temperature (6 ℃) for 0, 2, 4, 6, and 8 d (Table 2). Anthers were cultured in the induction medium (same as above) for eight weeks and the calli were transferred to the regeneration medium (1/2 MS medium containing 0.1 mg/L BA and 0.4% agar) at 26 ℃ for seven weeks. Data of embryogenic calli were recorded after 15-week culture.

Callus induction and plant regeneration After cold treatments of umbels, the flowers were immersed in 70% EtOH for 30 s and disinfected in 7.5% sodium hypochlorite (10% active chlorine) for 20 min, and then thoroughly washed three times in sterile distilled water. The undamaged anthers were then excided from the filament under a microscope and placed on the induction medium consisting of MS media with 1.0 mg/L 2,4-D, 0.2 mg/L BA, 3.0% maltose, 0.4 g/L *L*-glutamine, 0.2 g/L acidic hydrolysis casein, and 0.35% agar. To evaluate the effect of phytohormone BA

and 2,4-D concentration on callus induction of ZMP1 and ZCYH, the MS medium was supplemented with 2,4-D (0.50, 0.70, 1.00, 1.25, and 1.50 mg/L) and/or BA (0, 0.1, 0.2, and 0.3 mg/L) (Table 3). After eight weeks of culture, the anthers with calli approximately 2 mm in diameter were transferred to 1/2 MS salts with 3% sucrose supplemented with different concentrations of BA (0, 0.1, and 0.2 mg/L) for callus proliferation and shoots regeneration under a cycle of 16 h light alternating with 8 h darkness at 26 ℃, and a light intensity of approximately 56 μmol/(m^2·s) (Table 4). Shoots obtained via organogenesis or somatic embryogenesis were transferred individually to 1/2 MS medium containing 0.2 mg/L NAA for rooting. For all the media, the pH value was adjusted to 5.8 with 1 mol/L NaOH prior to autoclaving at 117 ℃ for 17 min.

Ploidy analysis

Root tips were excided from the regenerated plantlets and immersed in a solution of saturated *p*-dichlorobenzene at 4 ℃ for 7 h. After fixation in a mixed solution of ethanol-acetic acid (3:1) for 12 to 24 h, the root tips were immersed in 1 mol/L HCl for 4 min, stained with Carbol fuchsin, and pressed under a coverslip for observation. Then, the chromosome numbers were counted. Chromosomes of the donor plants grown from seeds were also observed using the root tip.

Histological observation

Tissues for sectioning were collected from various stages of anther culture and were fixed with FAA solution (ration of formalin-acetic acid-50% ethanol was 5:5:9) for at least 24 h. Then they were dehydrated in a graded ethanol series and embedded in paraffin. Sections of 10 μm thick were cut using a microtome, stained with safranine o-fast green counterstaining, and observed under a light microscope.

Data analysis

The embryogenic calli data were recorded for 15 weeks. Shoot regeneration was recorded for 20 weeks. There were five replications in each experiment with 90 anthers per replication in a Petri dish (60 mm × 15 mm) containing 15 mL of the medium. The rates of embryogenic calli and regeneration were statistically analyzed using ANOVA. Mean values were separated by LSD test. The reported values reflected the mean standard error. Values within a column followed by different letters are significantly different at $P \leq 0.05$ level according to the LSD test.

Results

Effects on embryogenic callus production from microspores of anthers in different developmental stages

The highest rate of embryogenic calli was obtained from the microspore of anther at the uninucleate stage for three genotypes: ZCYH, Z4, and Z5, except for ZPM1 which produced the highest rate of embryogenic calli in the tetrad stage (Table 1). When microspores developed into the late-uninucleate and early binucleate stages, no embryogenic callus was induced in any genotypes. In addition, the results showed that all the genotypes had the same morphological characteristics in anther culture. The pale green anthers (Fig. 1A) with the early- to mid-uninucleate microspore stages and a flower bud diameter between 0.70 and 0.80 mm were suitable for callus induction for most genotypes.

Effect of cold pretreatment on embryogenic callus induction

Microspore developmental stages of original anther cultured in two genotypes ZCYH and ZPM1 were the tetrads and the mid- to late-uninucleate stage, respectively (Table 2). The results revealed that the rate of embryogenic calli of anthers with two to four days of cold treatment was significantly greater than that of the control ($P \leq 0.05$). When cold treatment duration was more than 4 d, the rate of embryogenic calli was decreased. Both ZCYH and ZPM1 to produce the most embryogenic calli were 4-day cold treatment.

Effect of phytohormone on embryogenic callus induction

Embryogenic calli from anther culture of two genotypes, ZCYH and ZPM1 were not induced with the medium containing less than 0.75 mg/L 2,4-D (Table 3). Embryogenic calli of ZPM1 reached the maximum rate of 1.8% in the medium which was supplemented with 1.0 mg/L 2,4-D. A further increase of 2,4-D concentration in medium could not significantly increase the percentage of embryogenic calli ($P \leq 0.05$). The BA in the medium made a significant difference on embryo formation of anther culture of ZCYH. The highest rate of embryogenic calli was 10.4%, obtained in the induction medium with 1.0 mg/L 2,4-D and 0.2 mg/L BA.

Table 1 Effect of different developmental stages of microspores on embryogenic callus formation in anthers culture of *B. chinense*

Bud length / mm	Anther color	Microspore developmental stage in anther	Embryogenic callus / %			
			ZPM1	ZCYH	Z4	Z5
0.51－0.60	white	tetrads	2.4 ± 1.9 a	0.4 ± 0.3 b	0.06 ± 0.5 a	0 ± 0.0 a
0.70－0.85	pale green	early- to mid-uninucleate	0 ± 0.0 b	4.2 ± 0.5 a	2.0 ± 0.5 a	1.5 ± 0.4 a
0.10－1.28	bright yellow green	late-uninucleate to early binucleate	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 a	0 ± 0.0 a
1.20－1.42	deep yellow	binucleate and trinucleate	0 ± 0.0 b	0 ± 0.0 b	0 ± 0.0 a	0 ± 0.0 a

Values followed by different letters are significantly different at $P \leq 0.05$ according to LSD test. Same as below

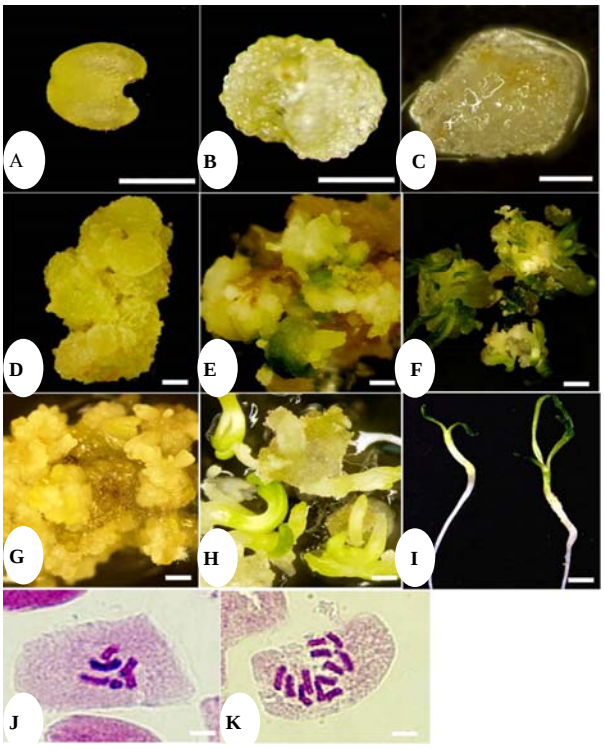

Fig. 1 Embryogenesis and plant regeneration in anther culture of *B. chinense*

A: Anther of uninucleate stage

B: Callus in anther wall began to form after three weeks of culture in induction medium with a large size increase of anther

C: Callus from anther tissues after eight weeks in induction medium

D: Development of early embryogenic callus after 7-week culture in regeneration 1/2 MS medium with 0.1 mg/L BA

E: Adventitious buds formed from embryogenic calli after 12-week culture in regeneration 1/2 MS medium with 0.1 mg/L BA

F: Shoot development from embryogenic calli via 16-week organogenesis in regeneration 1/2 MS medium with 0.1 mg/L BA

G: Somatic embryo at torpedo-shaped embryo stage developed from embryogenic callus after 12 weeks culture in regeneration 1/2 MS medium with 0.1 mg/L BA

H: Somatic embryo at cotyledon stage after 14-week culture in regeneration 1/2 MS medium with 0.1 mg/L BA

I: Plantlets with roots obtained via somatic embryos after 16-week culture in the regeneration 1/2 MS medium with 0.1 mg/L BA

J: Chromosomes (n = 6) of a root-tip cell in plants regenerated from anther cultures of genotype ZCYH

K: Chromosomes ($2n$ = 12) of a root-tip cell in parent plants of genotype ZCYH

Table 2 Effect of treatment time of initial culture at 6 ℃ on embryo formation in anther culture of *B. chinense*

Time / d	Embryogenic callus / %	
	ZPM1	ZCYH
0	0 ± 0.0 b	0 ± 0.0 b
2	1.8 ± 0.6 a	4.4 ± 0.5 a
4	2.2 ± 0.4 a	5.0 ± 0.1 a
6	1.2 ± 0.9 a	2.2 ± 0.6 b
8	0.7 ± 0.3 b	0 ± 0.0 b

Table 3 Effect of plant growth regulators on callus and embryo formation in anther culture of *B. chinense*

Growth regulators / ($mg \cdot L^{-1}$)	Embryogenic callus / %	
	ZPM1	ZCYH
2,4-D (0.50)	0 ± 0.0 b	0 ± 0.0 c
2,4-D (0.75)	0 ± 0.0 b	0 ± 0.0 c
2,4-D (1.00)	1.8 ± 0.8 a	4.0 ± 1.5 b
2,4-D (1.25)	1.2 ± 2.0 a	4.2 ± 1.6 b
2,4-D (1.50)	0.8 ± 0.8 a	4.8 ± 1.2 b
2,4-D (1.00) + BA (0.10)	1.3 ± 1.2 a	9.3 ± 0.6 a
2,4-D (1.00) + BA (0.20)	1.7 ± 0.5 a	10.4 ± 0.8 a
2,4-D (1.00) + BA (0.30)	0.5 ± 0.2 b	0.9 ± 0.2 c

Plantlet regeneration

The calli on anther wall began to form after three weeks culture in the induction medium (Fig. 1B). After being cultured in induction media for eight weeks (Fig. 1C), the calli of ZPM1 and ZCYH of approximately 2 mm in diameter were selected and transferred to regeneration media (Table 4) to promote embryo development and shoot regeneration. The blocks of callus continued to develop to early embryogenic calli in the regeneration media for seven weeks, and reach about 1.0 to 1.5 cm in diameter (Fig. 1D). Then, plant regeneration of embryogenic calli was observed in both pathways: organogenesis (Fig. 1E and 1F) or somatic embryogenesis (Figs. 1G and 1H). The regeneration

rate of embryogenic calli reached the highest level of 34.6% in 1/2 MS medium containing 0.1 mg/L BA after 16 weeks (Fig. 1I). Plant regeneration with somatic embryogenesis only occurred in the regeneration medium supplemented with BA (Table 4). The medium without phytohormones usually promoted root regeneration and inhibited shoot regeneration, which resulted in a relatively low regeneration rate (2.8%).

Ploidy levels of regeneration plants

The ploidy level of regenerated plants from embryogenic calli of anthers of ZPM1 and ZCYH was determined by examining chromosome numbers on root tips (Table 5). Out of the regenerated plants from ZCYH, only one was haploid (n = 6) (Fig. 1J). The remainders were diploid ($2n$ = 12) (Fig. 1K). All regenerated plants from ZPM1 were diploid (Table 5). The haploid plantlet grew weakly and its leaves were small and slender in comparison with the diploid plantlets obtained by the anther culture.

Table 4 Effect on plants regeneration from embryogenic calli of different plant hormones

Media / (mg·L^{-1})	Number of calli transferred	Regeneration frequency / %	Regeneration pathway
1/2MS	48	2.8 ± 0.8 c*	organogenesis
1/2MS + BA (0.1)	50	34.6 ± 3.8 a	somatic embryogenesis and organogenesis
1/2MS + BA (0.2)	66	10.0 ± 2.1 b	somatic embryogenesis and organogenesis

Anthers cultured in the regeneration media (1/2 MS salts with 3% sucrose supplement, different concentration of BA, and 0.4% agar). Shoot regeneration recorded after a culture period of 20 weeks

Table 5 Ploidy level of regenerated plants of two genotypes ZPM1 and ZCYH as detected by stained root tip cells

Genotypes	No. of regeneration plants tests	No. of plantlets with respective ploidy level	
		1n = x	2n = 2x
ZCYH	156	1 (0.6%)	155 (99.4%)
ZPM1	96	0 (0%)	96 (100%)

Histological observation

Histological observation of primary somatic embryos in anther culture revealed that enlarged cells of anther wall had vacuolation in certain degrees and began to degenerate after 3-week culture in the uninuclear microspore (Fig. 2A), but the microspore still survived at the uninucleate stage (Fig. 2B). Calli emerged synchronously from connective tissues (Fig. 2B), and subsequently mass-cell proliferated after 5-week culture while the anther wall tissue partially degenerated (Fig. 2C). Some microspores developed into the early binucleate stage and some were dead (Fig. 2C). The mass somatic callus formation, degeneration of anther wall, and the lower rate of microspore embryo-genesis eventually led to difficulties in the observation of the microspore embryogenic process. On the other hand, after 16-week culturing anthers of ZPM1 at the tetrad stage, the microspore still survived and stayed at the tetrad stage. Meanwhile embryogenic calli from connective tissues differentiated into green spots in the regeneration medium (Fig. 2D). The regenerated plants of the genotype of ZPM1 were all diploid (Table 5) after

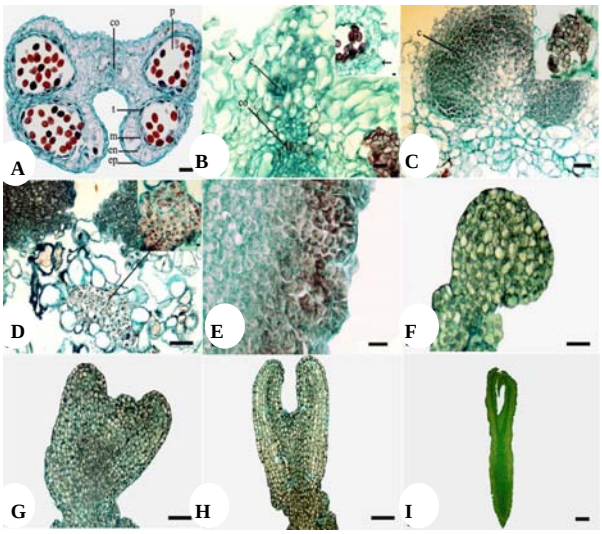

Fig. 2 Histological observation of primary somatic embryos of anther culture of *B. chinense*

A: Longitudinal section of an anther of microspore at uninuclear stage. P: Pollen sac, co: connective tissue; ep: epidermins; en: endothecium; m: middle layer; t: tapetum; c: callus

B: After 3-week culture, enlarged anther wall cells (arrow) had vacuolation in a certain degree and began to degenerate. Meanwhile calli emerged from connective tissues

C: Mass cell production from connective tissues after 5-week culture while other parts of anther degenerating. Inset: Some microspores developed into early binucleate stage (arrow)

D: When anthers at tetrads stage were cultured, microspores could still stay at microspore tetrad stage after 16-week culture, while embryogenic calli differentiated into green spots in regeneration medium

E: Development of early globular somatic embryos

F: Development of late globular embryos

G: Embryo at heart-shape stage

H: Embryos at torpedo-shape stage

I: Embryo at cotyledonary-shape stage

ploidy check. Embryogenic calli in the regeneration medium were regenerated by developing from the globular shape to heart shape, torpedo shape, and cotyledonary stages via somatic embryogenesis (Fig. 2E—I). All these results indicated that embryogenic calli were mostly derived from the connective tissues, and the anther at the mid- to late-uninucleate microspore stages was probably more appropriate than that at the tetrad to early uninucleate stages for embryogenesis.

Discussion

Our study revealed that the genotypes, anther development stages, anther cold pretreatment, and culture medium all had significant effects on embryogenic callus and plant production from the anthers of *B. chinense*.

Our study showed that different genotypes had different suitable culture stages. The suitable stage of anther culture of ZMP1 was the tetrad stage, but the early- to mid-uninucleate stage was suitable for the other three genotypes. Furthermore, the cultivar ZCYH had the highest rate of embryogenic calli and produced the haploid plant. The result revealed that the genotypes played an important role in the process of anther culture of *B. chinense*. Shon *et al* (2004) reported the difference in the rates of callus formation in three *B. falcatum* cultivars and acquired haploid plants from the genotype ($2n = 16$). In the process of androgenesis in carrot anther culture, only one of five cultivars turned out to be embryogenic calli, and considerable differences in the capacity for androgenesis were observed among individual donor plants (Górecka, Krzyżanowska, and Górecki, 2005).

Cold shock (Nitsch and Norreel, 1974) has long been known to increase embryo, callus or haploid formation in anther cultures. The present study further indicated that cold treatment at 6 ℃ for 2—6 d resulted in better embryo formation compared to that of the control. Shon *et al* (1997; 2004) reported similar results that cold pretreatment at 5 or 10 ℃ for 72 to 120 h was effective in the formation of anther calli of *B. falcatum* populations. In caraway anther culture, a cold pretreatment to both donor plants (at 22 ℃ for 9 d, then at 6 ℃ for 19 d) and anthers (cultured at 6 ℃) significantly increased the rate of the responsive anther (Smýkalová *et al*, 2009). In addition, cold pretreatment also increased the rate of haploid plantlet production of *B. falcatum* from 2.5% to 14.2% (Shon *et al*, 2004).

Plant growth regulators in the culture medium also affected the embryogenesis and plantlet regeneration. In *B. falacatum* anther culture, 0.75 mg/L 2,4-D alone was effective to all cultivars examined (Shon *et al*, 2004). Our study revealed that the combination of 1.0 mg/L 2,4-D and low concentration of 0.2 mg/L BA was most effective on the induction of callus and embryo regeneration. The effects of BA on induction of somatic embryogenesis were further supported by similar reports on the study of Chinese narcissus (Chen *et al*, 2005) and wheat (Kim and Baenziger, 2005). In addition, our results showed that the regeneration medium supplemented with BA enhanced further development of embryogenic calli and embryo regeneration via somatic embryogenesis to obtain regenerated plantlets with high quality. Similar results had been reported in anther culture of sunflower (Saji and Sujatha, 1998), niger (Hema and Murthy, 2007), and *B. falacatum* (Kwon, Jeong, and Kim, 2001).

In general, diploid plants account for a large proportion of regeneration plants in anther culture of many plants. Somatic plants come from the anther somatic tissue (Zhong, Nichaux-Ferrier'e, and Coumans, 1995). Histological observation of anther cultures of *B. chinense* in our study showed that embryogenic calli were mostly derived from the connective tissues, and the somatic calli occurring earlier and growing faster may be detrimental to the microspore development process (Rodrigues *et al*, 2005). In addition, our results showed that the process of microspore embryogenesis was not able to be detected continuously due to the lower rate of microspore embryogenesis (0.6%). As a result, there was only one haploid plant in the regenerated plants with the rest being all diploid plants. Moreover, our study found that the microspore at the uninucleate stage could develop into the binucleate stage after five weeks in the induction medium. By contrast, the microspore tetrads enveloped in the callose wall were not able to further develop into single microspores in the induction medium. The possible reason was that tapetum gradually degenerated in the process of anther culture (Rodrigues *et al*, 2005), consequently the callose was not able to absolutely dissolve the callose wall and microspores could not be released from tetrads. These results indicated that the anther was suitable for culturing at the

uninucleate stage. In *B. falacatum* anther culture, the anther containing uninucleate microspores was the most effective in inducing haploid regenerated plants (Shon *et al*, 2004).

In conclusion, our study showed that the early- to mid-uninucleate stage was suitable for the most genotypes for 4-day cold treatment of umbels. The importance of 2,4-D in combination with BA in culture medium was emphasized for significantly increasing the embryogenic callus frequency. Furthermore, the culture medium containing low concentration of BA could significantly improve the plant regeneration rate. We described for the first time an efficient embryogenic callus induction and plant regeneration system for anther culture and haploid plant production in *B. chinense*. However, in order to apply this system to general breeding of *B. chinense* and to improve the rate of regenerated haploid plants, further studies and work need to be done, with avoiding the effect of diploid cells, expanding the range of genotypes and optimizing hormonal conditions.

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