Haploid Plantlet Production through Somatic Embryogenesis in Anther-Derived Callus of *Bupleurum falcatum*

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**Abstract**: This study was carried out to verify the production of haploid plantlets through somatic embryogenesis of *Bupleurum falcatum* in anther culture (2n=16). Flowers with anthers at the uninucleate stage, less than 200 µm in anther length, were exposed to 10 ºC for 5 days (cold pretreatment) and the anthers were cultured on MS medium supplemented with 2,4-D and/or picloram at various concentrations at 30 ºC. The optimal supplement for callus formation was a mixture of 0.075 mg L⁻¹ 2,4-D + 0.075 mg L⁻¹ picloram or 0.75 mg L⁻¹ 2,4-D without picloram. Only a few calli were induced from the anthers without cold pretreatment. The calli were transplanted to MS medium without phytohormones and cultured at 25ºC for plant regeneration. Among one hundred twenty root tips of the regenerated plantlets examined, 14.2% were haploid (n=8). However, in the plantlets regenerated from anthers without cold pre-treatment only 2.5% was haploid. In both haploid and diploid regenerated plantlets, the chromosome number was fixed without variation. Among the regenerated plantlets, one was albino. Haploid plantlets were transplanted to the field after acclimation in pots filled with vermiculite under 90% humidity for a month, and haploid plant were produced. The potential of haploid plants derived from anther culture for production of high-yield and good-quality cultivars is discussed.

**Key words**: Anther culture, *Bupleurum falcatum*, Haploid, Somatic embryogenesis.

*Bupleurum falcatum* is a cross-pollinating, species genetically heterozygous according to geographical origin (2n=16, 20, 26, 32) and has a large variation in individual plant characters (Tani et al., 1988; Shimokawa et al., 1980; Kim et al., 1995). It is indispensable to clarify the characteristics of dry matter production, saikosaponin production, and solar energy utilization (Shon et al., 1998; Minami et al., 1995), for genetic improvement of *B. falcatum* and also to find a way of shortening the breeding intervals for obtaining high yield of high quality roots. Recently, anther culture is widely used to obtain haploid plants and for shortening the breeding intervals. Doubled haploid (DH) plants can be used as inbred lines in hybrid breeding. A successful system for mass production of DH plants is thus required for obtaining F1 seeds. Additionally, the complete homozygosity of DH plants facilitates genetic experiments (Collines and Sunderland, 1974; Collines and Sunderland, 1974).

Since the first haploid plant was obtained through pollen in the anther culture of *Datura innoxia* (Guha and Maheswari, 1964), many studies have been carried out on anther culture in various crop species (Collines and Sunderland, 1974; Hu et al., 1993; Amaury-M et al., 1997). In *B. falcatum*, some studies on tissue culture using other parts of plants; i.e., root, leaf and shoot apex have been reported (Ahn et al., 1999; Amano et al., 1989; Fujioka et al., 1987; Hiraoka et al., 1986; Park et al., 1994; Xia, 1992). In the anther culture system, there are usually large variations in callus formation and shoot or embryo production. Efficiency of anther culture varied with the genotype (Collines and Sunderland, 1974; Corduan and Spix, 1973; Tomes and Collens, 1976) and the kind/and concentration of phytohormone in the medium (Hu et al., 1993; Matsubara et al., 1992). Thus, evaluation of each condition is important to find the optimum procedure for somatic embryogenesis through anther culture.

In *B. falcatum*, somatic embryos were induced by the culture of tissues other than anther culture (Ahn et al., 1999; Amano et al., 1989; Fujioka et al., 1987; Hiraoka et al., 1986; Park et al., 1994; Xia et al., 1992), but we reported plantlet regeneration and haploid production through anther culture of *B. falcatum* as a short communication (Shon et al., 1997). The present study was made to verify the haploid plant production using anther donors of different geographical origins and to elucidate the induction of plant regeneration through somatic embryogenesis from anther-derived...
callus in *B. falcatum*.

**Materials and Methods**

1. **Plant materials**
   
   Three cultivars of *B. falcatum* with different cytogenetical origins, originated from China (2n=16), Japan (2n=20) and Korea (2n=26), were used. The seeds were obtained from National Crop Experimental Station, Suwon, Korea, and Tsukuba Medicinal Plant Research Station, National Institute of Health Sciences, Japan. The seeds were sown in 15-liter pots containing sand and upland soil (2:8) in late November, 1999, and the seedlings were grown in the field at Gifu Prefectural Institute of Health and Environmental Sciences. During the winter season, pots were covered with rice straw and kept under natural conditions to advance the flowering stage. The cultivation method was described previously (Shon et al., 1998). The flowers of 4 to 5 month-old plants grown under natural condition were used for the experiments.

2. **Anther culture and induction of haploid plantlet**

   Flowers having anthers at the uninucleate stage, less than 200 µm in length, were collected in the morning every three days for a month with three replications. The flowers were placed in a cold storage cabinet at 10°C for 5 days (cold pretreatment), which is suitable for callus formation in anther culture, following the method of Shon et al. (1997). After the cold pretreatment, flowers were immersed in 70% EtOH

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**Fig. 1.** Percentage of anther-derived calli after 5 weeks of anther culture (cultivar 2n=16) on MS medium supplemented with 2,4-D and or picloram at various concentrations.
A few seconds and in sodium hypochlorite solution with 0.5% active chlorine with a drop of Tween 20 for 15 min. for surface sterilization, and then rinsed three times with sterile distilled water. After removal of the petals under a microscope, anthers were cultured as described below. MS medium purchased from Wako Co. Japan, supplemented with 30 g L\(^{-1}\) sucrose and 7 g L\(^{-1}\) agar, pH 5.8, was used in this study. For callus induction, 0.05 to 1.5 mg L\(^{-1}\) 2,4-D and/or 0.05 to 1.0 mg L\(^{-1}\) picloram (4-amino-3,5,6- trichloropyridine-2-carboxylic acid, Sigma) were added. The medium was autoclaved at 115°C for 15 minutes. Thirty anthers from three plants were cultured on 30 mL of the solidified medium in each 85 × 20 mm petri dish covered with Parafilm and incubated at 30°C in the dark or light of 60 µmol m\(^{-2}\) s\(^{-1}\) for (16-hour photoperiod). After 5 weeks of culture, callus formation was examined, and the calli were transferred to the regeneration medium without phytohormones. To confirm the effect of cold pretreatment, anthers from the flowers without cold pretreatment (control) were also cultured in the same way.

After 5 weeks of incubation at 30°C, callus block 5 mm in diameter was transplanted to a petri dish with 30 mL regeneration medium containing 3% sucrose without phytohormones, and cultured to regenerate plantlets at 25°C under a 16-hour photoperiod of 400 µmol m\(^{-2}\) s\(^{-1}\) photosynthetically active radiation (PAR) following the method used for carrots (Hu et al., 1993).

### Table 1. Callus induction in the anthers of *B. falcatum* cultured in light and dark conditions.

| Genotype | No. of anthers inoculated | % callus induction | No. of anthers inoculated | % callus induction |
|----------|--------------------------|-------------------|--------------------------|-------------------|
| 2n=16    | 220                      | 4.1 ± 1.26a       | 240                      | 24.4 ± 6.20a      |
| 2n=20    | 260                      | 3.6 ± 1.94a       | 220                      | 28.2 ± 5.12a      |
| 2n=26    | 260                      | 3.2 ± 2.07a       | 250                      | 37.3 ± 8.21b      |
| Mean     |                          | 3.7               |                          | 30.0              |

Anthers were cultured on MS medium supplemented with 0.075 mg L\(^{-1}\) 2,4-D + 0.075 mg L\(^{-1}\) picloram or 0.75 mg L\(^{-1}\) 2,4-D. Values within a column followed by the same letter are not significantly different at the 5% level by DMRT.
Cytological analysis

Root tips were excised from regenerated plantlets, and immersed in a solution of 0.1% colchicine for 2 hours at 20°C. After fixation in a mixed solution of ethanol and acetic acid (3 : 1 v/v) for 30 min. at 5°C, they were immersed in 1N HCl for 5 min. and stained with 1% aceto-orcein solution. The stained root tips were squashed in 45% acetic acid under a cover slip and chromosome number was counted. Chromosomes of plants grown from seed were also observed using the root tips.

Results

1. Callus induction through anther culture

Fig. 1 shows the frequency of callus formation in the anthers of B. falcatum cultured on MS medium supplemented with 2,4-D or 2,4-D + picloram, each at various concentrations. MS medium supplemented with 0.075 mg L⁻¹ 2,4-D or 0.075 mg L⁻¹ picloram induced callus formation in about 50% of the anthers. The MS medium supplemented with 0.1 mg L⁻¹ 2,4-D + 0.1 mg L⁻¹ picloram or 0.075 mg L⁻¹ 2,4-D + 0.075 mg L⁻¹ picloram also induced callus formation in about 50% of anthers. The results were similar in all cultivars examined.

Table 2. Effect of cold pretreatment on embryo induction in anther culture of B. falcatum.

| Temp.          | No. of anthers inoculated | No. of anthers producing calli | No. of anthers producing embryogenic calli | No. of anthers producing plantlets |
|----------------|--------------------------|---------------------------------|---------------------------------------------|-----------------------------------|
| Cold pretreatment | 920                      | 285 (31.0)a                     | 22 (2.4)a                                   | 38 (4.1)a                         |
| Control        | 940                      | 57 (6.1)b                       | 5 (0.5)b                                    | 32 (3.4)b                         |

Anthers were cultured on MS medium supplemented with 0.075 mg L⁻¹ 2,4-D or 0.075 mg L⁻¹ picloram or 0.75 mg L⁻¹ 2,4-D. Numbers in parentheses indicate percentages to the total numbers of cultured anther. Values within a column followed by the same letter are not significantly different at the 5% level by DMRT. Pretreatment was 10°C for 5 days, and plantlet production was examined 120 days after the start of culture. Each value represents the average value of three cultivars.

Fig. 3. Effect of cold pretreatment on the production of haploid plantlets through anther culture.

3. Cytological analysis

Root tips were excised from regenerated plantlets, and immersed in a solution of 0.1% colchicine for 2 hours at 20°C. After fixation in a mixed solution of ethanol and acetic acid (3 : 1 v/v) for 30 min. at 5°C, they were immersed in 1N HCl for 5 min. and stained with 1% aceto-orcein solution. The stained root tips were squashed in 45% acetic acid under a cover slip and chromosome number was counted. Chromosomes of plants grown from seed were also observed using the root tips.
Table 1 shows the percentage of callus formation in the anthers of three genotypes of *B. falcatum* in both the light and dark conditions. The average percentage of callus formation in the dark and light was 30.0 and 3.7%, respectively, and the percentage varied with the cultivar in the dark condition, but not in the light.
condition. The percentage of callus formation in the cultivar of 2n=16 and n=20 was lower than that in the cultivar of 2n=26 in the dark.

2. The efficiency of low temperature pretreatment

Table 2 shows the effect of the cold pretreatment on callus and embryo formation in anther culture of B. falcatus. In the anthers given cold pretreatment the callus induction rate (31.0%) and embryo induction rate (2.4%) were higher than in the anthers of the control (6.1 and 0.5%, respectively). The rate of regeneration of plantlets from the calli formed on the anthers pretreated with and without low temperature was 4.1 and 3.4%, respectively, showing no significant difference. Thus, callus and embryo induction was clearly promoted by cold pretreatment of anthers.

The cold pretreatment also promoted the frequency of haploid plantlet production (Fig. 3). Root tips of the plantlets regenerated from the anthers with or without cold pretreatment were investigated for chromosome number. In the plantlets developed from the anthers with cold pretreatment, 17 out of 120 roots (14.2%) were haploid, but in the plantlets developed from the anthers without cold pretreatment only 3 out of 120 root tips (2.5%) were haploid (Fig. 3).

The percentage of callus formation was 5.1-fold higher (Table 2) and that of haploid production was 5.7-fold higher (Fig. 3) in the anthers with cold pretreatment than in the control anthers. Although the calli were formed more rapidly in the control anthers than in the anthers with cold pretreatment, the cold pretreatment is highly efficient for callus formation and also for haploid production.

3. Haploid plantlet production through anther culture

After 6 weeks of culture on MS medium supplemented with 0.075 mg L^{-1} 2,4-D + 0.075 mg L^{-1} picloram or 0.75 mg L^{-1} 2,4-D, calli with somatic embryos, about 5 mm in size, were transplanted onto the MS medium without phytohormones. After a 3-month culture, haploid plantlets were selected from the regenerated haploid plantlets by examination of chromosome number in root tips and transplanted to Jiffy pots filled with vermiculite. They were maintained in 90% humidity for about one month for acclimatization in the laboratory, and then transplanted to pots filled with upland soil (Fig. 4). Thus haploid plants of B. falcatus were successfully produced.

Discussion

The composition and concentration of phytohormones in the medium are important factors not only for the callus and embryoid formation from anthers, but also for the subsequent regeneration of the plantlets. For instance, eggplant, pepper and carrot anthers require both auxins and cytokinins, but optimal concentrations of the 2,4-D are different among these species (Hu et al., 1993; Matsubara et al., 1992). B. falcatus, however, application of auxin (0.75mg of 2,4-D per liter) alone was effective. The dark condition was more favorable than light condition for callus induction in B. falcatus. The rate of callus induction was similar in all cultivars examined.

The high frequency of callus formation and plantlet regeneration through somatic embryogenesis suggests the possibility of mass production of haploid or diploid plants of B. falcatus through anther culture. Shon et al. (1997) reported a difference in callus formation among cultivars of different origins. In the cultivar with 2n=20, the highest frequency of callus formation was observed on the medium supplemented with 0.1 mg L^{-1} 2,4-D + 0.1 mg L^{-1} picloram, but in the cultivar with 2n=26, on the medium supplemented with 1 mg L^{-1} 2,4-D. The difference in the frequency of callus formation between 2n=20 and 2n=26 cultivars had been observed in previous studies (Shon et al., 1998). In the present experiment using anthers at the uninucleate stage, however, the best results were obtained on the medium supplemented with 0.75 mg L^{-1} 2,4-D in all cultivars with 2n=16, 2n=20 and 2n=26.

The optimum developmental stage of anther or microspore for anther culture has been reported to be the late uninucleate stage in wheat (Wang et al., 1973), between the late tetrad stage and the early uninucleate stage in the microspore of Digitalis purpurea (Cirdyab and Souxm, 1975), near this stage in Oryza sativa (Oono, 1975) and early binucleate stage in Nicotiana attenuata, N. rainhondii, and N. knightiana (Collines and Sunderland, 1974). In the present study, callus formation was obtained at a high frequency in the anthers at the uninucleate stage in agreement with these results.

Lu et al. (1991) reported that embryoid yield in wheat anther culture significantly varied with the genotype and the environmental conditions. One of the most important environmental factors for callus induction is temperature. A low temperature was effective in rice (Yamaguchi et al., 1990; Genovesi and Magill, 1979). In B. falcatus, pretreatment with 10°C for 5 days (cold pretreatment) was effective for callus formation in anther culture (Table 2) as has been reported previously (Shon et al., 1997). The cold pretreatment might activate cell division at the uninucleate stage. Yamaguchi et al. (1990) reported difference among rice cultivars in callus-formation in response to the temperature, but no difference was found among cultivars of B. falcatus in this experiment.

Cold pretreatment was also efficient for haploid production through anther culture (Fig. 3). We suppose that the anther was separated from its filament more easily under a cold condition thereby increasing...
the frequency of haploid production. One albino plantlet was produced from the anthers with cold pretreatment, but the effect of the low temperature on albino production is uncertain.

The developmental stage of anthers seems to be an important factor for callus formation (Fig. 2). Optimum stage of anthers for anther culture was 9–12 DBF in cultivar of 2n=26, but 12–18 DBF in cultivars of 2n=16 and 2n=20. Can et al. (1998) reported that the growth condition of the mother plant is an important factor for callus induction in anther culture. That the growth condition of the mother plant is an important factor for callus induction in anther culture. For example, the percentage of callus induction in the anthers collected from the plants grown in a greenhouse differed from that in the anthers collected from the plants in the field, in six genotypes of sorghum plants. The anthers grown in the field induced calli at a higher rate (6.4% on the average) than the anthers grown in the greenhouse (3.7% on the average).

* B. falcatum* cultivars from different geographical origins have various chromosome numbers; 2n=16, 20, 26 and 32 although aneuploid variations were also observed (Amano et al. 1989; Chung et al., 1995; Ohta, 1991; Ohta et al., 1986; Shimokawa et al., 1980; Shon et al., 1997). Most of the chromosome numbers were the same as the basic numbers with a few variations (Chung et al., 1995; Ohta et al., 1986; Ohta, 1991). Kohda et al. (1990) reported that *B. falcatum* showed high stability in the chromosome number with less than 1% variation. However, Amano et al. (1989) reported that chromosome number in the plantlets regenerated by tissue culture showed no variation. The plantlets of *B. falcatum* regenerated in this experiment had a stable chromosome number of n=8 or 2n=16.

We reported have the rapid large-scale regeneration of plantlets through somatic embryogenesis in anther-derived callus of *B. falcatum*. This information may offer a tool for efficient breeding programs and genetic studies in *B. falcatum* as well as other plants. Generally, biomass production in medicinal plants varies with the environmental condition and genotype. However, little information is available for the rapid large-scale production of haploid plantlets in *B. falcatum* except for our previous work (Shon et al., 1997). *B. falcatum* shows intraspecific variation in external morphology as well as in internal cytology (Tani et al., 1988; Shimokawa et al., 1980; Kim et al., 1995). Thus, for production of high-yield and good-quality cultivars, plant lines obtained through tissue culture are useful.

* B. falcatum* is genetically heterozygous and shows great variation in cytological and plant characters (Shimokawa et al., 1980; Kim et al., 1995). Therefore, this system can be used to produce genetically homozygous plants and to breed specific lines, with high contents of ingredients and stable high yield.

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