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Callus induction and haploid plant regeneration from baby primrose (*Primula forbesii* Franch.) anther culture

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ABSTRACT

A protocol for successful callus induction and plant regeneration from *Primula forbesii* Franch. anthers is described. We first examined morphological characteristics of flower buds at different microspore developmental stages, then utilized anthers at the appropriate developmental stage for callus induction. Cultures were initiated on Murashige and Skoog (MS) medium supplemented with 1.0–2.0 mg/l 6-benzylaminopurine (BAP) and 0.5–2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). The highest callus induction ratio (2.4%) was produced on MS + 1.0 mg/l BAP + 0.5 mg/l 2,4-D. Nine regeneration media were tested for callus shoot regeneration, with BAP from 0.1 to 2.0 mg/l and α-naphthaleneacetic acid (NAA) from 0.01 to 0.5 mg/l. The highest induction and proliferation of indefinite buds (55.2%) was produced on MS + 0.2 mg/l BAP + 0.01 mg/l NAA. Plant regulator free MS was also found to be the best rooting medium. Among a total of 516 anther-derived plantlets flow cytometry and cytological analysis identified 2% to be haploid, 65% diploid, 9% triploid, 5% tetraploid, 2% hexaploid, and 17% mixoploid. This protocol provides a useful foundation for further research toward the development of homozygous *P. forbesii* or other *Primula* species.

1. Introduction

Primula is an ornamental plant popular for its early spring blooms and diversity of colors. This genus consists of approximately 500 species, which occur in the temperate zones of Europe, South America, North Africa, and Asia (Smith and Forrest, 1929). China is a major center of *Primula* biodiversity with approximately 300 species (Hu, 1990), specimens of which were collected and introduced to the Western world by botanists of England and France, such as Frank Kingdon-Ward and George Forrest, in the early 19th century. After centuries of conventional breeding, many primroses have become indispensable pot flowers and garden plants in European gardens.

Baby primrose (*Primula forbesii* Franch.) is a biennial wild flower abundant in many locales across Sichuan and Yunnan Provinces in China; the species exhibits desirable horticultural traits such as long flowering season, plentiful flowers, and a pleasant fragrance. Baby primrose was introduced into Europe by Vilmorin seed company in 1891 but is rarely seen today (Richards, 1993). During our glasshouse breeding of baby primrose using seeds from wild varieties, we have found that improvement of this valuable species is hampered by its high heterozygosity. Producing homozygous lines by conventional methods is time consuming and difficult, because the species is self-incompatible.

Homozygous genotypes are valuable for plant breeding and genetic studies. Haploid and doubled haploid (DH) lines carrying a gametophytic chromosome constitution are an important resource. To date, over 200 crop and horticultural plant varieties have been developed using various haploid and DH methods (Thomas et al., 2003). Most haploid or DH lines have originated from anther and microspore cultures, or from gynogenesis, and among these anther culture is the most effective and widely used method (Li et al., 2013; Parra-Vega et al., 2013). Anther culture has been successfully applied in many crops, including rice, barley, wheat, maize, tomato, cotton and rapeseed (Dunwell, 2010; Germanà, 2011; Xu et al.,

Abbreviations: MS, Murashige and Skoog medium; PGRs, plant growth regulators; 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; NAA, α-naphthaleneacetic acid; IBA, indole-3-butyric acid; DH, doubled haploid; DAPI, 4,6-diamidino-2-phenylindole.

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Table 1
Effects of various induction media on callus production from cultured *P. forbesii* anthers.

Abbreviated number	PGRs (mg/l)		No. of anthers cultured[1]	Callus induction rate (%)[2]	Callus growth pattern
	BAP	2,4-D			
I_1	1.0	0.5	603	2.4 ± 0.64a	Three types of calli, most green, granular calli
I_2	1.0	1.0	600	1.0 ± 0.42b	Most green, compact calli and few reddish-yellow, soft calli
I_3	1.0	1.5	596	1.1 ± 0.76b	Most green, compact calli and few soft, watery calli
I_4	1.0	2.0	598	1.2 ± 1.09b	Three types of calli
I_5	2.0	0.5	600	1.0 ± 1.21b	Three types of calli, most soft and watery calli
I_6	2.0	1.0	595	0.7 ± 0.55c	Most yellowish-white, friable calli
I_7	2.0	1.5	605	0.5 ± 0.21c	Yellowish-white, watery calli
I_8	2.0	2.0	602	1.0 ± 1.02b	Most friable calli and few green, compact calli

[1] Approximately 20 anthers were inoculated in one Petri dish; 30 replicates of each medium were performed.
[2] Mean separation in columns by Duncan's multiple-range test at $P < 0.05$.

2011; Zhang et al., 2002; Doi et al., 2010), but its use is still limited in ornamentals. The production of haploid plants from anther culture provides *Primula* breeders with a means of accelerating cultivar development.

In vitro propagation of several *Primula* species has been successfully achieved *via* somatic embryogenesis or direct and indirect organogenesis using explants from floral buds, leaves, hypocotyls, and shoot tips (Coumans et al., 1979; Shimada et al., 1997; Yamamoto et al., 1999; Mizuhiro et al., 2001a, 2001b; Schween and Schwenkel, 2003; Morozowska and Wesołowska, 2004; Okršlar et al., 2007; Takihira et al., 2007; Sharaf et al., 2011). However, the only existing report of successful anther culture in this genus was reported 32 years ago, in *Primula obconica*, utilizing anthers that had been frozen in liquid nitrogen (Bajaj, 1981).

In this study, we describe a comprehensive culture method for callus induction and plant regeneration from anthers of *P. forbesii*. Special attention was given to several factors influencing androgenesis, such as the correlation of bud morphology and size, microspore developmental stages, plant growth regulator (PGR) concentrations. This is the first report of the production of haploid plants in *Primula* and contributes to the development of primrose breeding.

2. Materials and methods

2.1. Plant material

Baby primrose (*P. forbesii*) seeds were collected in the glasshouse of Beijing Forestry University. The seeds were sown in September and emerging plants were grown in the glasshouse under standard cultivation conditions. Flower buds appeared three months after sowing. About 200 plants were used as anther donor plants.

2.2. Determination of microspore developmental stages

The relationship between the flower bud morphology, bud size, and microspore developmental stage was determined firstly. Different sizes of flower buds were collected and measured the lengths. Anthers were then isolated from the buds and placed on glass slides, stained with Carbol fuchsin solution, mashed, and observed under a microscope (Olympus BX51, Japan). Cell size and position of the nucleus were recorded, and one hundred pollen grains per anther were counted in randomly chosen fields of vision using 400× magnification to determine viability. Microspores that were not stained by fuchsin were considered non-viable.

2.3. Preparation of induction media and induction of callus

Using MS as the basic culture medium (Murashige and Skoog, 1962), eight induction media labeled I_1–I_8 were used to determine optimal conditions supporting *in vitro* callus formation of *P. forbesii* (Table 1). Among the eight different media, two levels of BAP (1.0 and 2.0 mg/l) and four levels of 2,4-D (0.5, 1.0, 1.5, 2.0 mg/l) were tested. All media were adjusted to pH 5.9–6.0 and contained 30 g/l sucrose and 6.5 g/l agar. Following 20 min of autoclaving at 121 °C and 1.1 kg/cm^2 pressure, 30 ml aliquots of media were poured into 90-mm-diameter Petri dishes. The dishes were sealed with parafilm while cooling.

Flower buds were collected as donor material when most microspores were at the late-uninucleate to early-binucleate stage of development. Buds were placed under running tap water for 1 h, then surface-sterilized by immersion in 70% (v/v) ethanol for 30 s followed by 2% (v/v) sodium hypochlorite (NaClO) for 8–10 min. The buds were then rinsed three times in sterile distilled water. Under aseptic conditions, anthers were isolated from buds by excision from the filaments, then 20 anthers were placed immediately onto a single plate of induction medium. Different media were tested using a completely randomized design, with 30 replicates for each type of induction medium. Anther cultures were incubated for 20 d at 25 ± 1 °C in darkness, then incubated under fluorescent 20 W daylight lamps (30–40 μmol/m^2/s) for 14/10 h (light/dark) conditions at 25 ± 1 °C until callus formed. Callus induction rates were determined on day 60 after anthers were placed into culture. Data were analyzed using the Statistical Package for Social Science (SPSS 17.0; Chicago, IL, USA). Analysis of variance (one-way ANOVA) was used to determine which variables were significant ($P = 0.05$ level). Duncan's multiple range test was used to determine significant differences between treatment means.

2.4. Preparation of differentiation media and plant regeneration

Nine different types of differentiation media were tested and labeled as D_1–D_9 (Table 2). For all nine media, MS salts were combined with 30 g/l sucrose and 6.5 g/l agar and pH was adjusted to 5.9–6.0. Five levels of BAP were tested (0.1, 0.2, 0.5, 1.0, 2.0 mg/l) along with four levels of NAA (0.01, 0.1, 0.2, 0.5 mg/l). After autoclaving at 121 °C and 1.1 kg/cm^2 pressure for 20 min, 40 ml aliquots of media were poured into 100 ml Erlenmeyer flasks.

In order to easily identify the origin of calli in this phase of the experiment, only calli produced in induction medium supplemented with 0.5 mg/l 2,4-D and 1.0 mg/l BAP (I_1) were used for differentiation. Calli were subcultured 2–3 times for one month each round on the same medium used for callus multiplication, until calli numbers were sufficient for the regeneration experiment. The calli were divided into small pieces (2 cm × 2 cm), then one piece was transferred to each flask of differentiation media mentioned. Flasks were incubated under a 12 h photoperiod at 30–40 μmol/m^2/s provided by fluorescent 20 W daylight lamps at 25 ± 1 °C for 30 d. A completely randomized design was used, with