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In Vitro Flowering and Fruiting of *Begonia parvula* H. Lév. & Vaniot

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Summary

In this study we investigated the *in vitro* flowering of *Begonia parvula*, a highly ornamental wild species from China. Seeds were sown in a hormone-free defined medium (1/4 MS). Eight weeks after culture, seedlings were transferred to 1/2 MS medium. A period of 5.5 months elapsed from the seed state to the stage when male and female flowers were fully open. A comparison was made between both the *in vivo* and *in vitro* male and female flowers. We noted that a number of the flowers' reproductive organs *in vitro* were smaller than *in vivo*. Flowers from both investigations had identical reproductive organs. Pollen viability was 63 % and 53 % respectively from the *in vitro* and *in vivo* male flowers after incubation in a liquid pollen germination medium for 24 h. *In vitro* fruit ripening was observed and viable seeds were obtained six to seven weeks after manual pollination. The seed size *in vitro* were 323.40 μm long and 205.00 μm wide, which was not significantly different statistically from that of the seeds *in vivo*. Our study showed that the life cycle of *B. parvula* can be completely controlled *in vitro*. As a result, we can eliminate the limitation from unsynchronized bloom in the future breeding attempts using *B. parvula* as a parent.

Key words. *Begonia parvula* – *in vitro* flowering – *in vitro* fruiting

Introduction

Begonia L. is a genus exceeding 1.500 wild species throughout the tropical and subtropical regions of Asia, Africa and America. This genus is one of the largest among the flowering plants and has a huge amount of morphological variation (Kiew 2005; Tebbit 2005). In recent years, many field surveys on begonias have been carried out in the world and new species continued to be discovered and described (Ku et al. 2008; Peng and Ku 2009; Peng et al. 2010).

Begonia parvula H. Lév. & Vaniot is a perennial, deciduous herb that is native to Guizhou and Yunnan provinces in China. It grows on rocky slopes at altitudes ranging from 200 to 1300 m (Gu et al. 2007). It is monoecious, having separate male and female flowers on the same inflorescence (cyme). Each cyme contains 2 or 3 flowers. *Begonia parvula* is a small herb with much horticultural potential: it displays a wide array of leaf shapes, hairiness and maculation patterns. The species forms tubers at base and has a dormancy property that enables it to be bred and stored for the winter season under frost-free conditions.

Many articles have reported on the subject of shoot regeneration system and/or somatic embryogenesis from a leaf or petiole explant in begonias cultivars (Nakano et al. 1999; Espino et al. 2004; Awal et al. 2009). To date, there is only one known case, *Begonia teuscheri* × *coccinea*, of *in vitro* flowering in this highly ornamental genus (Ringe and Nitsch 1968). In our preliminary studies, more than 100 wild species of Asiatic *Begonia* were cultivated *in vitro*, only *B. parvula* was able to produce flowers *in vitro*.

In vitro flowering has been reported for many plants (Zhang and Leung 2000, Wang et al. 2001; Dewir et al. 2006; Zhang 2007; Than et al. 2009; Chang et al. 2010), but *in vitro* fruiting and seed set are not readily encountered (Boodhipadma and Leung 2003; Hee et al. 2007; Saritha and Naidu 2007). Flowering is a complex process regulated by both internal plant factors and environmental signals. *In vitro* flowering can reduce the influence of environmental factors and allow for precise control of environmental factors and the application of plant growth regulars. It may also offer a means of studying the mechanism of flowering (Zhang 2007). The *in vitro* fruiting also offer novel material for research on the physiology of the fruit and seeds developed in an *in vitro* environment. This study examined the morphology and function of *in vitro* flowering and fruiting of *B. parvula* to facilitate *Begonia* breeding involving that species.

Materials and Methods

Plant materials and culture conditions

Materials of *Begonia parvula* were collected from about 1050 m altitude on shaded rocky slope of Gejiu city in

Yunnan Province, China. These plants were cultivated in a growth chamber of Biodiversity Research Center of Academia Sinica, Taipei under cool white fluorescent light at 40 μmol m^{-2} s^{-1} (GE, Taiwan), with a 14 h photoperiod per day, 85 % relative humidity, and a 25/20 °C day/night temperature. Seeds collected from mature capsules of manually pollinated plants were sterilized with 0.8 % sodium hypochlorite (NaOCl) supplemented by 1 drop of Tween 20 for 10 min and rinsed three times in sterile distilled water at lamina flow. Aseptic seeds were germinated in 13 × 100 mm test tubes (Pyrex No. 9820) with 7.5 ml of 1/4 strength MS salts (1/4 MS) (Murashige and Skoog 1962) supplemented with (mg l^{-1}) niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (200), *myo*-inositol (100), 2 % (w/v) sucrose and 0.8 % agar (w/v) (Sigma Co., St. Louis, MO). The pH of all media were adjusted to 5.8 with 0.1 M NaOH before autoclaving at 121 °C for 15 min. Seeds were maintained in a dark cabinet at 25 ± 1 °C. Most seeds regular germinated in 8 weeks. Seedling were transfered and incubated in 617-ml Erlenmeyer flasks each containing 100 ml of the 1/2 MS medium (1/2 MS) supplemented with 3 % (w/v) sucrose and 0.1 % peptone (w/v). The other conditions were the same for the seeds sowing medium above. The culture was incubated at 25 ± 1 °C under the 16/8 h light/dark cycle with the light intensity of 40 μmol m^{-2} s^{-1} (daylight fluorescent tubes FL-30D/29, 40 w, China Electric Co, Taipei, Taiwan).

Measurement of morphological traits of sexual reproductive organ

Ten each of the *in vitro* inflorescences and those of the *in vivo* plants grown in a growth chamber were compared. Ten each of fully opened, *in vivo* and *in vitro* flowers were examined for morphological traits of sexual reproductive organ including length of peduncles, pedicel and size of tepals in male and female flowers, anther number and ovary size.

Pollen viability test

Three or four androecia were detached from fully open male flower from both *in vivo* and *in vitro* plants. Two anthers random detached from different androecia were transferred in an aseptic laminar flow hood onto 3 drops of liquid pollen germination medium each in 75 × 25 mm double concavities slide. The liquid pollen germination medium contained (mg l^{-1}) H_3BO_3 (100), Ca $(NO_3)_2 \cdot 4H_2O$ (200), $MgSO_4 \cdot 7H_2O$ (100), KNO_3 (100) and sucrose (10 %, w/v). Detection for pollen grain germination was achieved after 24 h dark incubation at 25 ± 1 °C. Zeiss Axioskop 2 microscope with digital camera ProgRes C3 (Jenoptik, Germany) was used to observe and photograph pollen grain germination through the green light filter. For each concavity of slide three independent fields of vision have been chosen. 80–120 pollen grains per vision were observed for germination. Experiments were repeated three times.

Pollination, fruiting and seed production

Twenty attempts of manual pollinations for both *in vivo* and *in vitro* flowers were made for seed production experiment. In the *in vitro* flowers, pollen grains were transferred from anther to stigma in the laminar flow using a pair of aseptic forceps. At six to seven weeks after manual pollination, mature capsules were harvested. Seeds from *in vitro* fruit were germinated on 1/4 MS medium.

Scanning electron microscope (SEM)

SEM examinations were conducted for pollen and seed trait (size). Materials from both *in vivo* and *in vitro* flowers of *B. parvula* were mounted on aluminum pedestal for coating. Hitachi Ion Sputter Coater E-1010 and Hitachi S-3000N scanning electron microscope were used to coat the materials with gold and to scan the sample. All statistical analyses were carried out using one way ANOVA t-test at 95 % confidence level.

Results

Comparison of morphological features of in vivo *and* in vitro *sexual reproduction organs*

Eight weeks after culture, seeds of *B. parvula* germinated in 1/4 MS medium. Seedlings were transferred to 1/2 MS medium. Four weeks after subculture, shoots and tubers formed. Ten weeks after subculture, inflorescences formed from the second and third nodes of stem. Each flowering plantlet continued to produce inflorescences at leaf axils. Male and female flowers were fully open 5.5 months after seeds were sown under *in vitro* condition (Fig. 1a). When plantlets were subsequently subcultured on the 1/2 MS medium, they produced flowers continually. The flowers developed *in vitro* were identical to those *in vivo*, comprising 4 tepals on the male flower and 5 tepals on the female flower, all of which appeared pinkish in both conditions (Fig. 1a, b, c).

Each *in vivo* and *in vitro* inflorescence of *B. parvula* comprises one male and one female flower. Comparisons were made between the *in vivo* flowers and the *in vitro* flowers in relation to the length of peduncle and pedicel, size of tepals and color of male and female flowers, number of anthers, and size of ovaries (Fig. 1). The length of the peduncle of the *in vitro* plantlets and *in vivo* plants were 5.39 and 9.85 cm respectively. The *in vitro* plantlets produced a shorter peduncle than the *in vivo* plants (Table 1). The pedicel length of male and female flowers formed under *in vivo* and *in vitro* conditions did not differ significantly (Table 1). The mean number of anthers derived from the *in vitro* developed flowers and the flowers of *in vivo* were 30.80 and 26.30, which did not differ significantly either (Table 2). The color of androecium and stigma derived from both *in vivo* and *in vitro* flowers was a brilliant yellow (Fig. 1d, e). A notable difference between the *in vivo* and *in vitro* subjects however is that the length of tepal 2 and tepal 4 on the male flowers and the width of tepal 1 and length of tepal 3 and tepal 4 (Fig. 1b, c) on the female flowers and the length of ovary was significantly smaller *in vitro* than in vivo (Tables 2 and 3).

Pollen morphology and viability

Pollen grains from the *in vitro* male flowers were approximately 24.95 μm long and 8.99 μm wide, which were significantly smaller than those of the *in vivo* male flowers (Fig. 2a and Table 4). Pollen viability tests demon-

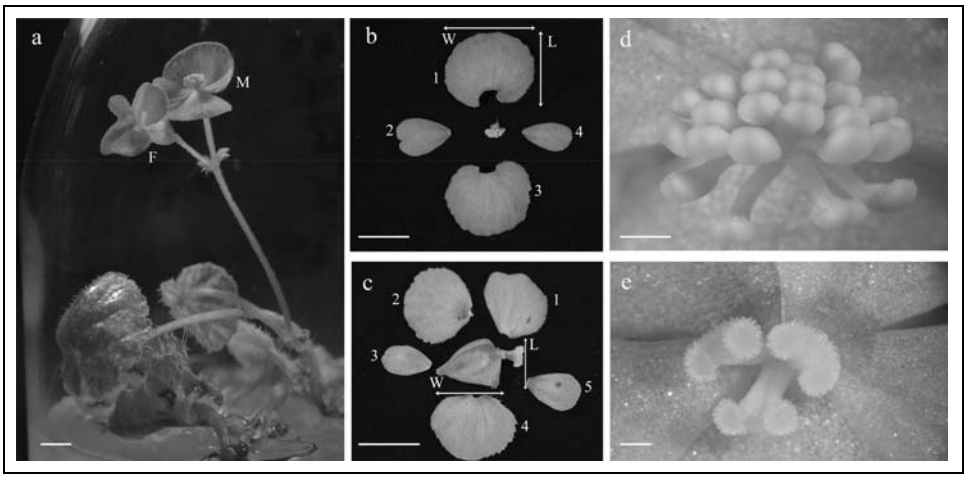


Fig. 1. *In vitro* flowering morphology of *B. parvula*: (a) Fully developed inflorescence with a male and a female flower; (b) Illustration of male flower; (c) Illustration of female flower; (d) Androecium; (e) Style branches and stigma. M: male flower; F: female flower; L: length; W: width; Serial numbers present tepals on male and female flower (b, c). Scale bars equals 1 cm (a, b, c), 1 mm (d, e).

Table 1. Comparison of *in vivo* and *in vitro* of inflorescence of *B. parvula*[1].

	Peduncle (cm)	Male flowers pedicel (cm)	Female flowers pedicel (cm)
In vivo	9.85 ± 3.31 a	1.92 ± 0.51 a	2.08 ± 0.38 a
In vitro	5.39 ± 0.93 b	2.36 ± 0.44 a	2.07 ± 0.62 a

[1] Values are means of 10 replicates ± SE in which the measurement unit was centimeter (cm). Data marked by the same letter in a column are not significantly different (t-test, P<0.05).

strated that about 63 % of the pollen grains from the male flowers that formed *in vitro* were viable, which did not differ significantly from the 53 % of viable pollen grain of the *in vivo* male flowers (Fig. 2b, 3).

Seed morphology and seed production

The capsules developed *in vivo* and *in vitro* (Fig. 2c) were harvested six to seven weeks after manual pollination when the pedicel began to deteriorate. The seeds from the *in vitro* fruit samples were 323.40 μm long and 205.00 μm wide, which were similar to the seeds obtained from capsules developed *in vivo* (316.40 μm long, 199.50 μm wide) (Table 4). The seeds produced from the *in vitro* plants were sown on 1/4 MS medium. They germinated five to six weeks after incubation. SEM observation (Fig. 2d) and *in vitro* germination (Fig. 2e) demonstrated that seeds formed in the *in vitro* samples were viable. They exhibited similar morphology and level of viability to seeds of plants grown *in vivo* (data not shown).

Discussion

We have successfully raised plants of *B. parvula* from seed state to flower formation *in vitro* in 5.5 months under hormone-free conditions. We observed that the peduncles and some tepals of *in vitro* flowers were smaller than those of the *in vivo* flowers (Table 1, 2, 3). The smaller flowers produced *in vitro* may have been resulted, at least in part, from competition and/or nutritional deficiencies (Sankhla et al. 1994). We conjecture that growth of *in vitro* plantlets may have been limited by the culture container. In our study, the morphology of both male and female reproductive organs of *B. parvula* obtained *in vitro* was normal. Previous studies have shown that *in vitro* plantlets produced malformed flowers that are also smaller than those under *in vivo* conditions (Zhang and Leung 2000; Chang and Chang 2003; Hee et al. 2007; Than et al. 2009). The *in vitro* flowering by exogenous plant growth regulators has been reported (Chang and Chang 2003; Than et al. 2009). The results of numerous

Table 2. Comparison of *in vivo* and *in vitro* male flower organs size of *B. parvula*[1].

Organ source	Tepal 1L[2]	Tepal 1W[3]	Tepal 2L	Tepal 2W	Tepal 3L	Tepal 3W	Tepal 4L	Tepal 4W	Anther number
In vivo	1.28 ± 0.28 a	2.04 ± 0.32 a	0.77 ± 0.08 a	1.10 ± 0.16 a	1.42 ± 0.23 a	2.10 ± 0.33 a	0.83 ± 0.15 a	1.10 ± 0.18 a	30.80 ± 2.20 a
In vitro	1.32 ± 0.19 a	1.81 ± 0.23 a	0.63 ± 0.13 b	1.13 ± 0.21 a	1.45 ± 0.18 a	1.71 ± 0.29 a	0.55 ± 0.13 b	1.10 ± 0.18 a	26.30 ± 4.81 a

[1] Values are means of 10 replicates ± SE except of anther number in which the measurement unit was centimeter (cm). Data marked by the same letter in a column are not significantly different (t-test, P<0.05).
[2] L: Length.
[3] W: Width.

Table 3. Comparison of *in vivo* and *in vitro* female flower organs size of *B. parvula*[1].

Organ source	Tepal 1L[2]	Tepal 1W[3]	Tepal 2L	Tepal 2W	Tepal 3L	Tepal 3W	Tepal 4L	Tepal 4W	Tepal 5L	Tepal 5W	Ovary L	Ovary W
In vivo	0.98 ± 0.17 a	1.42 ± 0.27 a	0.66 ± 0.28 a	0.91 ± 0.15 a	1.09 ± 0.20 a	1.20 ± 0.28 a	1.08 ± 0.20 a	1.18 ± 0.26 a	0.56 ± 0.12 a	0.80 ± 0.21 a	1.06 ± 0.10 a	0.98 ± 0.25 a
In vitro	0.88 ± 0.22 a	1.00 ± 0.16 b	0.60 ± 0.30 a	0.86 ± 0.22 a	0.73 ± 0.25 b	0.88 ± 0.25 a	0.81 ± 0.25 b	0.97 ± 0.18 a	0.51 ± 0.27 a	0.78 ± 0.25 a	0.92 ± 0.11 b	0.90 ± 0.24 a

[1] Values are means of 10 replicates ± SE, the measurement unit was centimeter (cm). Data marked by the same letter in a column are not significantly different (t-test, $P<0.05$).
[2] L: Length.
[3] W: Width.

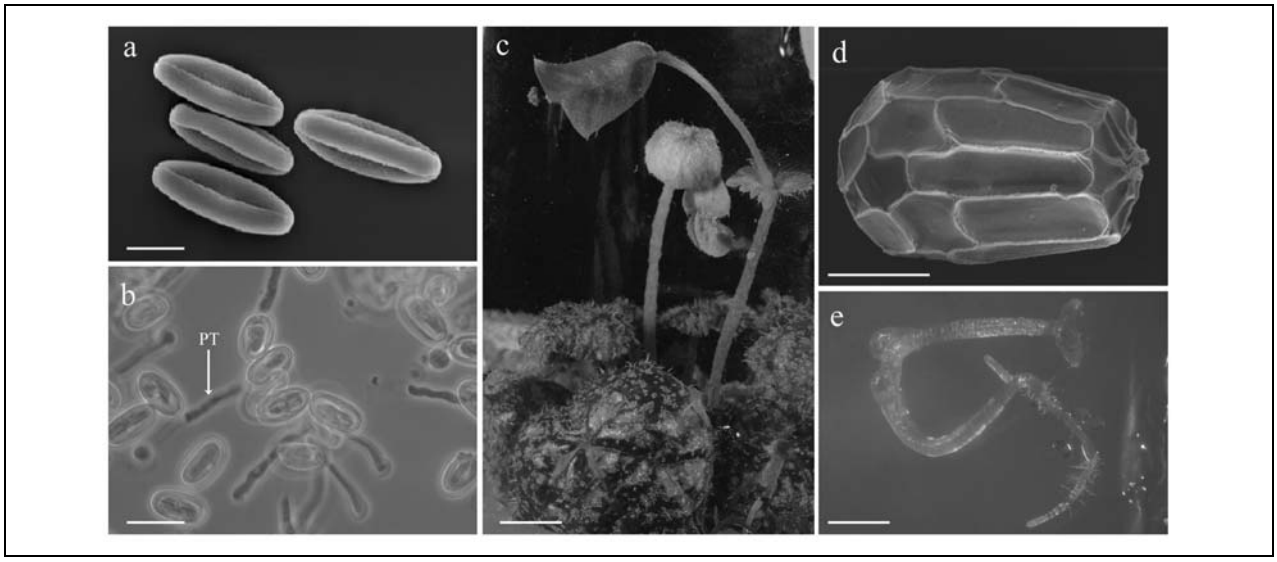


Fig. 2. (a) SEM observation of pollen grains from *in vitro* male flower of *B. parvula*; (b) Germination of pollen grain derived from *in vitro* male flower on a liquid pollen germination medium contain (mg l^{-1}) H_3BO_3 (100), Ca $(NO_3)_2 \cdot 4H_2O$ (200), $MgSO_4 \cdot 7H_2O$ (100), KNO_3 (100) and sucrose (10 %, w/v) (c) Capsule formed after three weeks of aseptic manual pollination (d) SEM observation of a seed from *in vitro* fruiting (e) Germination of seed derived from *in vitro* ripe fruit 5 weeks after sowing in 1/4 MS. PT: pollen tube. Scale bar equals 10 μm (a), 25 μm (b), 5 mm (c), 100 μm (d), 1 mm (e).

studies asserted that without cytokinins – or in the presence of low cytokinin concentration – flowering did not occur (Dielen et al. 2001, Wang et al. 2001). Cytokinins have also been reported to affect the morphogenesis of flowers (Chen et al. 1997). Ringe and Nitsch (1968) have reported flower formation on excised *Begonia teuscheri* × *coccinea* fragments cultured *in vitro*. In their experiments only *in vivo* inflorescence explants were able to produce male flowers *in vitro*. The male flowers thus formed, however, had rudimentary tepals and reduced stamens.

Pollen viability is correlated with breeding and hybridization success rate (Hee et al. 2007). Our results showed a high germination percentage (63 %) of pollen grains from the *in vitro* male flowers.

Commercially cultivated begonias are mostly hybrids between very limited wild species. The blooming times of wild *Begonia* vary greatly (Guan et al. 2008). Our study of the *in vitro* flowering of *B. parvula* has a practical value in wide hybridization studies, because *in vitro* conditions can eliminate environmental interference and we have demonstrated that *B. parvula* was able to flower *in vitro* all year round.

Begonia parvula is a deciduous herb with a natural blooming season in September (Gu et al. 2007). Our experiments have demonstrated that its life cycle can be controlled completely – they were not dormant and they flowered all year round *in vitro*. Our study offers the potential of *B. parvula* for horticultural breeding without being limited by unsynchronized blooming and seasonal dormancy in the field.

To sum up, the present work represents the first report of *in vitro* flower and fruit development from seeds of *B. parvula*. The *in vitro* flowers assumed normal morphology and the reproductive organs were fully functional. In addition, they were successfully matured into ripe fruits with viable seeds after manual pollination. Our results promise the prospect of using the handsome *B. parvula* for effective breeding in the future.

Table 4. Comparison of *in vivo* and *in vitro* pollen and seed sizes of *B. parvula*[1)].

	Pollen (μm)		Seed (μm)	
	Length	Width	Length	Width
In vivo	27.16 ± 0.98 a	10.13 ± 0.36 a	316.40 ± 19.25 a	199.50 ± 12.48 a
In vitro	24.95 ± 1.10 b	8.99 ± 0.53 b	323.40 ± 19.35 a	205.00 ± 6.50 a

[1)] Values are means of 10 replicates ± SE, the measurement unit was micrometer (μm). Data marked by the same letter in a column are not significantly different (t-test, P<0.05).

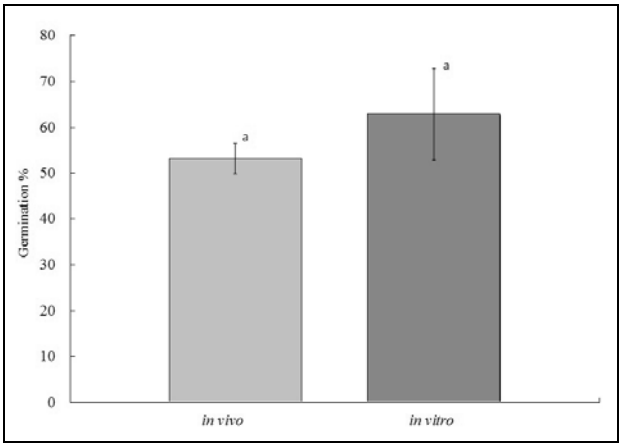


Fig. 3. Comparison of *in vivo* and *in vitro* pollen germination rate of *B. parvula*. Values are means of three replicates ± SE. Data marked by the same letter are not significantly different (t-test, P<0.05).

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