Micropropagation Shortens the Time to Blooming of Begonia montaniformis × Begonia ningmingensis var. bella F1 Progeny

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Abstract. Begonia montaniformis × Begonia ningmingensis var. bella hybrids have high ornamental potential. Hence, the aim of this study was to determine the optimal conditions for the micropropagation of a Begonia montaniformis × Begonia ningmingensis var. bella F₁ progeny by using various concentrations of plant growth regulators (PGRs) and varying light spectra in half-strength Murashige and Skoog (1/2 MS) medium. The results showed that the explant regeneration was optimal when the lamina was incubated in a medium supplemented with 2.0 μM 6-benzylaminopurine and 0.8 μM α-naphthaleneacetic acid (NAAs). Under such conditions, 98% of the explants regenerated adventitious shoots after 8 weeks, and 41 buds were produced per explant on average. The mean shoot length was 9.6 mm, and on average, 4.5 shoots per explant were more than 2 cm long. Subsequently, the induced adventitious shoots were transferred into rooting medium consisting of 1/2 MS and various NAAs concentrations. After 4 weeks, the shoots subcultured in this medium showed ~93% root induction and an average of 3.5 adventitious roots per explant. Furthermore, the applied light spectrum significantly influenced shoot regeneration, and optimal results were achieved under an equal distribution of blue, red, and infrared light. The histological sections of shoots regenerated from direct organogenesis were observed through scanning electron microscopy (SEM). Afterward, the rooting adventitious shoots were subcultured in PGR-free medium for 8 weeks. The seedlings were successfully acclimated 4 weeks after being transferred to soil and bloomed after 11 months in a greenhouse. Thus, the PGR composition in micropropagation efficiently shortened the time to blooming from 25 to 16 months.

Materials and Methods

Plant materials. Individual plants of B. montaniformis and B. ningmingensis var. bella were collected from their natural habitats and cultivated in the experimental greenhouse of the National Museum of Natural Science in Taiwan. The greenhouse was determined to have a natural photoperiod per day, 85% to 95% relative humidity, and 25 °C/20 °C day/night temperature. The aforementioned plants were artificially hybridized, and the seeds of their F₁ hybrids were collected and sterilized with 0.8% sodium hypochlorite. Subsequently, the seeds were germinated in a dark cabinet at 25 ± 1 °C with basal medium containing one-fourth-strength Murashige and Skoog (1962, 1/4 MS) salts and fissures) of the Sino-Vietnamese karst region (Peng et al., 2015; Qin et al., 2017), with most species identified from a single or a several localities and differing from one another in leaf shape, pubescence, texture, and variegation (Gu et al., 2007).

Both Begonia montaniformis and B. ningmingensis var. bella belong to section Coelocentrum and have special leaf variegation. B. montaniformis is an endemic lithophytic species found on limestone hills in North Vietnam. On the leaf surface of this plant dense conic bullae with a silvery green zone among the primary and secondary veins present an impressive strobili, shape, giving the plant high ornamental value. Furthermore, the yellowish-green color of its staminate and carpellate flowers is rare in the Begonia genus (Peng et al., 2015). However, B. montaniformis plants are difficult to grow. The germinated seedlings rarely survive and are extremely sensitive to environmental changes. However, if they grow satisfactorily, the leaves last for long periods. B. ningmingensis var. bella is also an endemic and rare species and is only distributed in the karst area of Southwestern Guangxi, China. The upper surface of its leaf is dark green, brown, or dark brown, with white maculation along the major veins, and its lower surface is reddish or red (Fang et al., 2006).

Conserving the superior F₁ hybrid is potentially valuable for horticultural purposes, and in vitro regeneration techniques may be advantageous in coping with market demand for this hybrid. Therefore, in vitro hybrid regeneration techniques should be investigated and the corresponding control mechanism determined before this hybrid is promoted for commercial use. Furthermore, light quality influences adventitious shoot regeneration (Burritt and Leung 2003; Zhou et al., 2016). A novel technology has been used to examine the effect of the light spectrum on in vitro micropropagation (Fang et al., 2011; Lee et al., 2011). This study attempted to develop a micropropagation protocol for rapid in vitro propagation of the F₁ hybridized progeny of B. montaniformis × B. ningmingensis var. bella (Novel F₁), especially in terms of plant growth regulators (PGRs) and light quality.
Supplemented with niacin (0.5 mg/L), pyridoxine HCl (0.5 mg/L), thiamine HCl (0.1 mg/L), myoinositol (100 mg/L), glycine (200 mg/L), and sucrose (2%, w/v) and solidified with 0.75% (w/v) agar. Most seeds germinated within 4 to 6 weeks.

Subculture and culture medium. After 8 weeks, the germinated F1 seedlings were incubated in 617-ml erlenmeyers flasks containing 100 mL of half-strength (1/2 MS) medium supplemented with 3% (w/v) sucrose and 0.1% peptone (w/v). The other conditions were identical to those used for the aforementioned seed medium.

After 3 months, there were 3–4 leaves present on the plants. The length of leaf was 4–5 cm. We randomly selected the same leaves regardless of leaf position or age. The laminas were cut randomly from whole leaf presents with stereoscopic and white spotted leaf laminae were cut randomly from whole leaf laminae regardless of leaf position or age. The explants were then placed individually with their adaxial sides upward in a scalpel. The explants were then placed in medium supplemented with 3% (w/v) sucrose and 0.1% peptone (w/v).

Table 1. Effect of different concentrations of plant growth regulators on Begonia montaniformis var. bella shoot organogenesis after 8 weeks of culture.

| Type of PGR (µM) | Adventitious shoots (%) | Mean shoot length (mm) | No. shoots/explant | Mean no. of elongated shoots (>2 mm) | Explant necrosis (%) |
|-----------------|-------------------------|------------------------|--------------------|--------------------------------------|----------------------|
| BA Zeatin mT NAA |                         |                        |                    |                                      |                      |
| 0 0 0 0          | 51 ± 51 a               | 0.0 ± 0.0 e            | 1.6 ± 2.2 f        | 0.0 ± 0.0 e                          | 51 ± 50 a            |
| 2.0             | 86 ± 35 abcd            | 3.0 ± 4.9 d            | 19.5 ± 13.9 c      | 1.3 ± 2.3 cd                         | 10 ± 37 def          |
| 4.0             | 95 ± 22 ab              | 2.9 ± 4.2 c            | 25.6 ± 15.4 b      | 1.4 ± 2.1 c                          | 10 ± 30 def          |
| 8.0             | 87 ± 34 abc             | 1.7 ± 3.2 d            | 23.4 ± 20.5 c      | 1.2 ± 1.9 cde                        | 18 ± 39 cde          |
| 2.0 0.8         | 98 ± 14 a               | 9.6 ± 9.6 a            | 41.2 ± 26.3 b      | 4.5 ± 4.7 a                          | 4 ± 59 f             |
| 4.0             | 98 ± 14 a               | 6.1 ± 8.8 b            | 50.1 ± 23.7 a      | 3.1 ± 4.4 b                          | 0 ± 0 f              |
| 8.0 0.8         | 98 ± 14 a               | 5.0 ± 6.5 b            | 56.4 ± 30.9 a      | 2.4 ± 3.2 b                          | 0 ± 0 f              |
| 2.0             | 66 ± 48 cef             | 0.8 ± 2.2 d            | 6.0 ± 7.5 def      | 0.4 ± 1.1 cde                        | 25 ± 44 bcd          |
| 4.0             | 90 ± 31 ab              | 0.9 ± 2.3 d            | 5.5 ± 7.1 def      | 0.4 ± 1.1 cde                        | 24 ± 43 bcd          |
| 8.0             | 84 ± 37 abcd            | 1.5 ± 3.2 d            | 10.3 ± 10.0 de     | 0.7 ± 1.4 cde                        | 26 ± 44 bcd          |
| 2.0 0.8         | 67 ± 47 dgef            | 0.5 ± 2.1 d            | 3.8 ± 4.8 ef       | 0.2 ± 0.9 d                          | 4 ± 20 ef            |
| 4.0             | 69 ± 47 dgef            | 1.8 ± 4.0 d            | 5.0 ± 5.8 d        | 0.8 ± 1.9 c                          | 2 ± 19 ef            |
| 8.0 0.8         | 85 ± 36 abcd            | 1.6 ± 3.7 d            | 11.7 ± 9.9 d       | 0.7 ± 1.6 d                          | 6 ± 24 ef            |
| 2.0             | 76 ± 44 bcdf            | 2.5 ± 4.8 cde          | 3.5 ± 4.2 ef       | 1.0 ± 1.9 c                          | 52 ± 50 a            |
| 4.0             | 50 ± 58 fg              | 2.9 ± 1.2 cd           | 1.8 ± 2.8 f        | 0.2 ± 0.5 cd                         | 54 ± 50 a            |
| 8.0             | 58 ± 50 fg              | 0.9 ± 2.2 d            | 3.1 ± 4.1 ef       | 0.5 ± 1.0 c                          | 37 ± 49 b            |
| 2.0 0.8         | 17 ± 38 h               | 0.0 ± 0.0 e            | 0.8 ± 2.2 f        | 0.0 ± 0.0 e                          | 33 ± 47 bc           |
| 4.0 0.8         | 12 ± 33 h               | 0.0 ± 0.0 e            | 0.8 ± 2.2 f        | 0.0 ± 0.0 e                          | 33 ± 47 bc           |
| 8.0 0.8         | 28 ± 46 h               | 0.0 ± 0.0 e            | 1.6 ± 3.5 f        | 0.0 ± 0.0 e                          | 10 ± 30 def          |

La: Lamina explants were cultured in half-strength MS medium supplemented with various cytokinins and NAA; PGRs: plant growth regulators.

aMeans ± SE followed by the same letters indicate that the values are not significantly different according to Duncan’s multiple test at P ≤ 0.05.

Spectral quality affects morphogenesis of explant plantlets during in vitro culture. A novel system equipped with light-emitting diodes (LEDs) as light sources for tissue culture (TC) plantlets (Nano Bio Light Technology Co., Ltd., Taiwan; Chen et al., 2016; Fang et al., 2011) was used to test the impact of spectral quality on the micropropagation of novel F1 hybrids. Eight sets of LED chips were configured through a combination of blue, green, red, and infrared (IR) (B/G/R/IR) LEDs and mounted on the tips of the TC vessels to produce the same light intensity. The LED peaks for B/G/R/IR were 450 ± 3, 525 ± 3, 660 ± 5, and 730 ± 5 nm, respectively. Two combinations produced cool white light and warm white light with a spectrum similar to that of daylight. The B/G/R/IR ratios in these two combinations were 26:26:2:6 and 10:45:51:4. Four sets had chip ratios of 3B:3R:3IR, 1B:7R:1IR, 1B:1G:7R, and 1B:8R. Two sets had only one band each, namely 9B and 9R. Five laminae of the F1 hybrid were transferred to one TC vessel holding 1/2 MS medium with 2 µM BA and 0.8 µM NAA. Three laminae were placed in each light quality set. The experiments were repeated thrice. The photosynthetic photon flux density was adjusted to 42 µmol·m⁻²·s⁻¹ in each light quality set. The other culture conditions were kept the same as in the aforementioned experiments. The regeneration percentage of adventitious shoots, number of shoots, and shoot length per explant were recorded after 8 weeks.

Historesin sections and SEM. The adventitious shoots of a lamina placed in medium supplemented with 2 µM BA and 0.8 µM NAA were periodically removed for anatomic analysis. The shoots were cut into small sections and fixed at 4 °C for 6–8 h. The other conditions were then washed with 0.1 M sodium phosphate buffer (pH 6.8). These sections were then dehydrated and embedded with increasing concentrations of Technovit 7100 resin (Kulzer, Hanau, Germany) under vacuum. After the final polymerization of the sample resin, serial transversal sections were cut using a rotary microtome (RM2245; Leica, Bensheim, Germany) and stained with periodic acid–Schiff (PAS) reaction for insoluble carbohydrate and amido black 10B for protein. These preparations were photographed and examined using a light microscope (AxioCam ERC 5s; Zeiss, Jena, Germany; Lee et al., 2006; Yeung, 1999). Other parts of the tissues were dehydrated in a critical point dryer. Subsequently, they were coated with gold using an ion sputter coater (E-1010; Hitachi Ltd., Tokyo, Japan) and observed through SEM (S-3000N; Hitachi Ltd.).

Statistical analysis. In adventitious shoot induction experiments, 15 explants were used per treatment. In the rooting and light spectrum experiments, five explants were used per treatment. All experiments were repeated thrice. One-way ANOVA was performed using the Statistical Package for the Social Sciences, version 12.01 (IBM, Armonk, NY), and significant differences between the means were evaluated using Duncan’s multiple range test at P < 0.05.
Results

Adventitious shoot induction. Among the three cytokinins examined, BA was found to induce greater shoot regeneration than did zeatin and mT (Table 1). In the medium containing BA and 0.8 μM NAA, 98% of the explants regenerated adventitious shoots after 8 weeks, and more than 41 shoots were produced per explant. Although the addition of either 4.0 or 8.0 μM BA along with 0.8 μM NAA to the medium induced more shoots than those observed before the addition, supplementation with 2.0 μM BA and 0.8 μM NAA produced significantly more elongated shoots (>2 mm) than those observed before supplementation, and the mean shoot length achieved after 8 weeks was 9.6 mm (Table 1; Fig. 1A and B). Thus, the PGR composition was determined for further rooting and light spectrum experiments. The addition of 0.8 μM NAA efficiently reduced mortality irrespective of the cytokinin combination. However, compared with a low concentration of zeatin and the control set, a high concentration of zeatin induced slightly more shoots, but the results were much worse than those obtained using BA. Compared with the control set, mT exhibited no significant effect on shoot regeneration and produced fewer shoots when 0.8 μM NAA was added.

Root induction, plantlet elongation, and acclimatization. To improve root induction, the adventitious shoots from the explants incubated in the medium supplemented with 2.0 μM BA and 0.8 μM NAA were carefully separated and transferred to the rooting medium with 1/2 MS supplemented with three concentrations of NAA. The root induction had begun in all rooting media after 2 weeks. After 4 weeks, more than 66% root induction was observed in the adventitious shoot cultured in media without and with NAA (Table 2; Fig. 1C). Plantlets rooted in the 1/2 MS media containing different concentrations of NAA did not differ significantly in rooting frequency. When the NAA concentration was increased to 5.38 μM, the mean number of roots per plantlet decreased to 2.27. Plantlets in auxin-free medium had significantly more leaves (average 4.53) than those in medium containing 2.68–5.38 μM NAA.

The wounded lamina slightly thickened after 3 weeks of culture (Fig. 1A) and showed expanded areas at the excised edges. The optimum PGR combination for adventitious shoot regeneration was determined in this study to be 1/2 MS medium supplemented with 2 μM BA and 0.8 μM NAA. Adventitious shoot initiation occurred on the surface and cut margins of the lamina explants within 8 weeks of inoculation in culture medium (Fig. 1B). Root induction in media containing different concentrations of NAA was observed. After 4 weeks, the roots were well developed (Fig. 1C) and fit for subculturing in PGR-free medium for future plantlet production. Plantlets grown from rooting explants required up to 8 weeks in PGR-free

![Fig. 1. Adventitious shoot regeneration using laminae of in vitro plantlets of *B. montaniformis* × *B. ningmengensis* var. *bella.* (A) Growth from the excised edges of the lamina explants after 3 weeks in half-strength MS medium supplemented with 2 μM BA and 0.8 μM NAA. (B) Adventitious shoot induction from the lamina explants after 8 weeks in the regeneration medium. (C) Rooting of in vitro regenerated shoots after 4 weeks in PGR-free half-strength MS medium. (D) Explants at 11 months after transferring to soil in a greenhouse. (E) Well-developed plantlets after 8 weeks in PGR-free half-strength MS medium. (F) Explants at 4 weeks after transferring to soil in a greenhouse. Scale bars: 2 mm (A), 2 mm (B), 1 cm (C), 4 cm (D), 3 cm (E), and 3.5 cm (F).](http://example.com/fig1)

Table 2. Effects of half-strength MS medium, with or without various NAA concentrations, on the in vitro rooting of regenerated shoots after 4 weeks of culture.

| NAA (μM) | Rooted shoots (%) | No. roots | No. leaves | Explants necrosis (%) |
|----------|-------------------|-----------|------------|----------------------|
| 0        | 93.33 ± 6.67 a    | 3.47 ± 0.84 ab | 4.53 ± 0.84 a | 0.00 ± 0.00 a |
| 2.69     | 86.67 ± 9.09 a    | 6.33 ± 1.77 a  | 1.87 ± 0.38 b  | 20.00 ± 10.69 a |
| 5.38     | 66.67 ± 12.60 a   | 2.27 ± 0.94 b  | 2.13 ± 0.51 b  | 26.67 ± 11.82 a |

*Means ±SE followed by the same letters indicate that the values are not significantly different according to Duncan’s multiple test at P ≤ 0.05.*
medium for suitable shoot elongation and root development (Fig. 1E). The seedling successfully acclimated 4 weeks after transfer to soil (Fig. 1F) and produced slightly yellowish flowers after 11 months (Fig. 1D). The period from adventitious shoot induction to flowering was ≈16 to 17 months.

Effect of light quality on micropropagation. The lamina of the explants incubated in ½ MS medium supplemented with 2.0 μM BA and 0.8 μM NAA grew optimally under an equal distribution of blue, red, and IR light, such as in 3B3R3IR (Table 3). The induction frequency of adventitious shoots, shoot length, and number of shoots were significantly greater under 3B3R3IR light than under other light sources.

Under such conditions, the explant produced on average 30 shoots, an average shoot length of 2.4 mm, and an adventitious shoots percentage of 95%. The explant under warm white light (WW) and only blue light (9B) produced the second best results in terms of the percentage of adventitious shoots and number of shoots. The results were less significantly different from the explant cultured in 3B3R3IR light. However, the blue light slightly inhibited shoot elongation. The least optimal regeneration was recorded under cold white light (CW) and strong red light (1B8R).

Historesin sections and SEM. Histological studies showed that morphogenesis gradually changed after the initiation of the culture in ½ MS medium containing 2 μM BA and 0.8 μM NAA. After 13 d of culture, both periclinal and anticlinal division occurred in the areas within the outermost one to two epidermal cell layers. Cross-sections revealed cell activation or dedifferentiation, with small and dense cytoplasm in which a series of organized divisions continued (Fig. 2A). Nineteen days after the beginning of induction, the cells of the meristematic zone had rapidly divided and had high cytoplasmic content with small vacuoles and abundant starch grains. The division of cells protruded from the leaf surface and had epidermal layers connected with the original lamina (Fig. 2B). The emerged cells had developed a well-defined meristem and two new leaf primordia after 28 d of initiation. The base of the explant epidermis had differentiated into parenchyma cells, indicating the early stages of shoot bud differentiation (Fig. 2C). The thoroughly developed bud had an obvious apical meristem with lively differentiation of cells after 33 d of culture (Fig. 2D).

The leaf epidermis of novel F1 explants at the beginning of culture (0 d) exhibited flat orderly rows of cells and distinct raised trichomes in SEM observations (Fig. 3A). Well-developed buds with numerous leaf

Table 3. Effects of various light spectra on shoot regeneration. Medium containing half-strength MS supplemented with 2 μM BA and 0.8 μM NAA after 8 weeks of culture.

| Treatment | Adventitious shoot (%) | Shoot length (mm) | No. shoots |
|-----------|------------------------|-------------------|------------|
| CW        | 76 ± 43 bc             | 1.4 ± 1.0 d       | 18.4 ± 21.9 bc |
| WW        | 88 ± 33 abc            | 2.0 ± 1.0 ab      | 26.8 ± 22.4 ab |
| 9B        | 90 ± 30 ab             | 1.8 ± 1.0 bcd     | 26.5 ± 34.7 ab |
| 3B3R3IR   | 95 ± 22 a              | 2.4 ± 0.8 a       | 30.4 ± 24.3 a |
| 1B7R1IR   | 79 ± 42 abc            | 1.6 ± 1.1 bcd     | 21.2 ± 29.0 abc |
| 1B1G7R    | 81 ± 40 abc            | 1.7 ± 1.1 bcd     | 18.5 ± 24.3 bc |
| 1B8R      | 71 ± 46 c              | 1.4 ± 1.1 cd      | 15.7 ± 21.6 bc |
| 9R        | 83 ± 38 abc            | 1.9 ± 1.0 bc      | 12.2 ± 12.3 c |

*Means ±SE followed by the same letters indicate that the values are not significantly different according to Duncan’s multiple test at P ≤ 0.05.
primordia were observed after 33 d in the induction medium (Fig. 3B). Different developmental stages of primordia that involved early swelling to completely formed buds with apical meristems and leaf primordia were observed after 50 d of induction (Fig. 3C and D).

**Discussion**

**Effect of plant regulators on induction of adventitious shoots.** The regeneration of adventitious shoots of novel F1 hybrids from lamina explants was dependent on the presence of both auxin and cytokinin in the medium. Our study findings suggest that BA produces more favorable results than zeatin and mT. Shoot multiplication of the novel F1 hybrid was most efficient when the medium was supplemented with 2.0 μM BA and 0.8 μM NAA. BA is one of the most effective and low-cost cytokinins and has thus been used in numerous plant micropropagation studies (Werbrouck et al., 1996). BA has also been commonly used in TC media to stimulate adventitious shoot induction in *Begonia* leaf explants (Espino et al., 2004; Kaviani et al., 2015; Kumari et al., 2017; Nakano et al., 1999). In this study, using BA alone resulted relatively unsatisfactory mean shoot elongation and high explant necrosis (Table 1), whereas combining BA with NAA improved the number of shoots and shoot length. A similar observation has also been reported in other *Begonia* species (Godo et al., 2008; Kumari et al., 2017; Mendi et al., 2009; Nada et al., 2011; Nakano et al., 1999), indicating that combining cytokinins at a high concentration with auxin is more effective for shoot multiplication compared with using cytokinins alone. The same situation was observed in a *Begonia* petiole transverse thin-cell-layer culture study (Nhut et al., 2005). Combining BA and a low concentration of auxin (NAA) has also promoted direct shoot regeneration and the formation of multiple shoots from leaf explants in various plants such as *Lysimachia* (Zheng et al., 2009) and *Solanum* (Ghimire et al., 2012). 

*Meta-topolin* has been used in adventitious shoot induction of *B. semipartetals* (which belongs to section Coelocentrum) (Chung et al., 2016). *Begonia* sect. *Coelocentrum* is a highly diverse group and generally found in cave-like microhabitats of karst limestone (Chung et al., 2014; Hughes and Hollingsworth, 2008). Most sect. *Coelocentrum* species show isolated distribution patterns and are single-site endemic (Chung et al., 2014; Qin et al., 2017). Therefore, their genetic background variety may lead to different regeneration patterns.

**Rooting and acclimatization.** Rooting is a critical step in plant TC. Adventitious shoots can be induced using high concentrations of cytokinin. However, such shoots inhibit rooting, leading to explants with inadequate development after acclimatization (Werbrouck et al., 1995). The balance of cytokinins and auxins is crucial when considering in vitro organ regeneration (Su et al., 2011). In this study, significant differences in the number of leaves were determined between the control and NAA-treated explants after 4 weeks in the rooting medium (Table 2). Endogenous auxin may be produced when more shoot formation occurs (Song et al., 2011); however, adding exogenous auxins to the culture may inhibit shoot growth. Comparing the effect of different concentrations on root induction and development revealed that PGR-free medium was superior to all the other treatments. This is in agreement with the finding of previous studies on *Begonia* micropropagation, in which the recovery of intact plants was easy even when the regenerated shoots were rooted in PGR-free medium (Chung et al., 2016; Espino et al., 2004). Werbrouck et al.
organogenesis could be accurately examined through histological and electron microscopy techniques. Both techniques have been used for observing organogenesis in various explant species (Burritt and Leung, 1996, 2003; Chlyah and Tran Thanh Van, 1984; Hunter and Burritt, 2004; Pickens et al., 2006; Vatankhah et al., 2014). Organogenesis can occur either/both directly or indirectly through callus formation (Burritt and Leung, 1996; Ma et al., 2011). Our SEM observation indicated that regenerated tissues and primordia formed directly on the leaf epidermis. The ability to regenerate shoots from epidermal or subepidermal layers has gained considerable attention in histological Begonia studies (Burritt and Leung, 1996). A similar differentiation pathway of leaf epidermis was reported by Chlyah and Tran Thanh Van (1984) for Begonia rex. Moreover, histochemical staining results have ascertained that energy and carbon sources are required for cell differentiation. Therefore, starch accumulation (Fig. 2C and D) could play a crucial role in the differentiation and formation of apical meristems during in vitro organogenesis (Yeh et al., 2017). Accordingly, the relationship between starch accumulation and tissues and regeneration potential has been reported for many species (Chen and Ziv, 2005; Fortes and Pais, 2000).

Studies have indicated that higher starch levels are correlated with higher regeneration potentials (Chen and Ziv, 2005; Stoyanova-Koleva et al., 2012).

Both B. montaniformis and B. ningmingsensis var. bella are rare and difficult to regenerate under natural conditions. B. ningmingsensis var. bella leaves fall easily, and B. montaniformis does not flower easily and is difficult to breed; therefore, they were artificially hybridized in this study. The patterns of leaf variegation of the F1 hybrid were closer to those of B. ningmingsensis var. bella. The leaf variegation was rich along the veins, manifesting as white bands, and was sparsely distributed on the leaf surface. It contrasted sharply between the mesophyll and veins. The unique tipped conic bulla of the leaf surface of the B. montaniformis (Peng et al., 2015) was lacking in the hybrid. The summit-shape remained to give the leaf a more stereoscopic shape. The selected F1 hybrids inherited the yellowish flowers and the long-lasting attribute from B. montaniformis (Fig. 1D). Under natural conditions, the period from seed germination to flowering of the F1 hybrid is approximately 25 months. Our micropropagation protocol reduced this period to 16–17 months. Micropropagation not only preserved the unique hybrid characteristics but also shortened the breeding duration. In the presence of suitable amounts of PGRs, BA, and NAA, shoot and root productivity levels higher than 80% could be achieved. Moreover, the leaves of the F1 hybrids grew faster and lasted for 8–9 months. Our study successfully devised a method for massively breeding of stable hybrid begonia; this method therefore increases their potential for commercial application.

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