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Shoot organogenesis and plant regeneration in *Begonia coptidifolia*

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Abstract: An efficient regeneration protocol was established for a rare plant species, *Begonia coptidifolia*. Using leaf explants, adventitious shoots were induced on Murashige and Skoog (MS) basal medium using various concentrations (1.0–10.0 mM) of three cytokinins, 6-benzyladenine (BA), thidiazuron (TDZ) and kinetin (KIN). However, adventitious shoots could not be induced on MS medium supplemented with auxins [α-naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D)] tested in the 1.0–10.0 mM range. The highest number of shoots per explant (31.2) was induced on MS medium with 5.0 mM BA and 1.0 mM NAA. Although roots were induced in ½MS medium free of plant growth regulators, the time to form roots was shorter in the 0.5 mM IBA, 0.5 mM IAA and 0.5 mM NAA treatments. Following acclimatization, over 95% of plantlets survived in a substrate of sand and humus (1:1, v/v). Over 200 plantlets were reintroduced to their natural habit, and over 50% of plants survived after one year.

Key words: *Begonia coptidifolia*, shoot organogenesis, leaf, plant regeneration, transplantation

1. Introduction

The genus *Begonia* L. has more than 1000 species that are widely distributed in tropical and subtropical zones, especially in Central and South America (Doorenbos et al., 1998). About 150 species of *Begonia* are indigenous to China (Shui et al., 2002). *Begonia coptidifolia* is a rare species that is found exclusively in the Ehuangzhang Nature Reserve, Yangchun City, Guangdong Province, in South China. *B. coptidifolia* is usually found near several streams at an altitude of about 600 m, in growth habitats that are particularly hostile. Its leaf margins are deeply serrated, it has high ornamental value, and it occupies an important position in system science, and thus has important scientific research value (Ye et al., 2004). The Ehuangzhang Nature Reserve is located at the northern edge of the tropics with an area of about 15,000 ha of mountain rainforest, with a recorded annual mean rainfall of 3428 mm, well-preserved vegetation, and is the only mountain rainforest recorded in mainland Guangdong. It is the second highest peak in western Guangdong, China, with the highest altitude of 1337.6 m above sea level and the lowest point is only 50 m above sea level (Wang et al., 2003, 2004). Wild populations of *B. coptidifolia* are rare and the species faces extinction. To preserve and propagate *B. coptidifolia*, we established an in vitro propagation and regeneration system by successfully inducing adventitious shoots from leaf explants using two methods.

2. Materials and methods

2.1. Plant material, surface disinfection, and culture conditions

A single plant was removed from the Ehuangzhang Nature Reserve, Yangchun City, Guangdong Province in May of 2014. It was planted in a 10 cm high pot filled with sand and humus (1:1, v/v). Two months later, two young leaves were used as explants. Leaves were washed in running tap water for 10 min, surfaces were cleaned, using cotton wool, with 70% alcohol for 20 s, immersed and stirred in 0.1% mercuric chloride for 10 min, then rinsed three times with sterile distilled water. Leaves were dried in airon paper towels then cut into 0.4 cm^2 explants (usually about 0.6–0.7 cm long squares)that were inoculated onto Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal medium with 0.54 μM α-naphthaleneacetic acid (NAA) and 4.4 mM 6-benzyladenine (BA), based on ideal levels indicated for other *Begonia* species (Burritt and Leung, 1996; Espino et al., 2004). Culture jars were 10 cm high and 6 cm in diameter. Each culture jar contained 30 mL of medium. All media contained 30 g L^{-1} sucrose and were solidified with 0.7% agar (Sigma-Aldrich, St. Louis, MO, USA). pH

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was adjusted to 5.8 with 1.0 N NaOH or 1.0 N HCl prior to autoclaving culture jars at 121 °C for 16 min. Culture jars, each containing five leaf explants, were placed in the dark for 2 weeks to minimize leaf tissue browning, then placed under a 10-h photoperiod with a photosynthetic photon flux density of 100 μmol $m^{-2} s^{-1}$ (2× white fluorescent lamps, 40 W each; Philips, Tianjing, China) in a culture room at 25 ± 1 °C to induce adventitious shoots. In June of 2016, we revisited the Ehuangzhang Nature Reserve and took two *B. coptidifolia* plants back to South China Botanical Garden. We also used the above methods to propagate adventitious shoots via leaf explants. When induced adventitious shoots were about 2 cm long, leaves were excised and subcultured every 80 days onto the same fresh medium. When 50 jars of in vitro shoots had been cloned, adventitious shoot clusters were divided into clusters with 3–4 shoots each that were transferred to plant growth regulator (PGR)-free MS medium for shoot growth. The experiments described next were then initiated.

2.2. Effect of plant growth regulators on shoot organogenesis from leaf explants in vitro

In vitro leaf explants were cut into 0.5 cm^2 squares and inoculated onto medium containing different concentrations (1.0, 2.5, 5.0, and 10 mM) of single PGRs [thidiazuron (TDZ), kinetin (KIN), BA, NAA and 2,4-dichlorophenoxyacetic acid (2,4-D); Sigma-Aldrich]. We used PGR-free medium as the control (Table 1). Cultures were kept in the dark for 2 weeks, then transferred to light. Each treatment contained 40 explants (8 × 5 = 40) that were divided among eight jars and five leaf explants per jar. After 8 weeks in culture, shoot organogenesis (i.e. the number of induced adventitious shoots) was investigated.

2.3. Shoot induction and proliferation

Two methods to induce shoots were assessed. The first method (method 1) was conventional shoot propagation in which clusters of axillary shoots (20–40 shoots) 2 cm high were divided into smaller clusters (3–4 shoots) that were inoculated onto medium with different PGRs to propagate them (Table 2). Each treatment included five clusters per culture jar, with a total of 8 jars and 40 clusters (8 × 5 = 40). Propagation efficiency was assessed after 8 weeks. The second method induced adventitious shoot organogenesis from leaf explants (0.5 cm^2 squares) in vitro. In the second method (method 2), leaf explants were inoculated into induction medium with different PGRs (Table 2) in culture jars, and adventitious shoot organogenesis was assessed after 8 weeks.

2.4. In vitro root induction and ex vitro acclimatization

B. coptidifolia is a herbal species that does not form obvious axillary shoots, so 3–4 leaves clumps with invisible shoots were subcultured on shoot proliferation medium, i.e. MS medium with 5.0 mM BA and 1.0 mM NAA, for 8 weeks.

Table 1. Effect of single PGRs on number of adventitious shoots per explant and response of *Begonia coptidifolia* leaf explants after culture for 8 weeks.

PGR in induction medium (μM)	Mean number of shoots per leaf explant	Response of leaf explants
PGR-free	0 ± 0 f	No callus and no shoot
KIN 1.0	10.5 ± 1.2 d*	No callus, normal shoots
KIN5.0	20.3 ± 1.8 b	No callus, normal shoots
KIN10.0	23.6 ± 2.1 b	Little callus, irregular shoots
BA 1.0	15.2 ± 1.3 c	No callus, normal shoots
BA 5.0	22.8 ± 1.9 b	No callus, normal shoots
BA 10.0	29.5 ± 2.3 a	Little callus, irregular shoots
TDZ 1.0	23.2 ± 1.2 b	Few callus, normal shoots
TDZ 5.0	16.7 ± 0.2 c	More callus, adventitious shoots
TDZ 10.0	5.8 ± 0.2 e	Callus, somatic embryo-like shoots
2,4-D 1.0 2,4-D 2.5 2,4-D 10.0	0 ± 0 f 0 ± 0 f 0 ± 0 f	Callus, no shoots
NAA1.0 NAA2.5 NAA10.0	0 ± 0 f 0 ± 0 f 0 ± 0 f	Callus, no shoots

* Different letters within a column indicate significant differences according to Duncan's multiple range test ($p < 0.05$; n = 40 explants per treatment).

Table 2. Comparison of two methods (conventional axillary shoot propagation and adventitious shoot organogenesis from leaf explant) for shoot propagation of *Begonia coptidifolia*.

PGRs in MS medium (μM)	Induced shoot number of the two methods	
	Conventional axillary shoot propagation (method 1)	Shoot organogenesis from leaf explant (method 2)
BA 1.0 + NAA 0.2	4.3 ± 0.2 c B	17.5 ± 1.2 c A**
BA 1.0 + NAA 0.5	4.4 ± 0.2 c B	18.3 ± 1.3 c A
BA 2.5 + NAA 0.5	5.2 ± 0.3 bB	24.8 ± 1.4 b A
BA 2.5 + NAA 1.0	5.3 ± 0.3 bB	25.6 ± 1.4 b A
BA 5.0 + NAA 0.5	6.1 ± 0.4 a B	29.5 ± 1.8 a A
BA 5.0 + NAA 1.0	6.2 ± 0.4 a* B	31.2 ± 1.9 a A

* Different letters within a column indicate significant differences according to Duncan's multiple range test ($p < 0.05$; n = 40 explants per treatment) and ** different capital letters indicate significant differences (different propagation methods) by the LSD test ($p \leq 0.05$; n = 40 explants per treatment).

When leaf clusters were 1-3 cm high, they were cut into smaller clusters and transferred to half-strength (macro- and micronutrients) MS medium (½MS) with 0.5 mM of an auxin, NAA, indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA), to induce roots (Table 3). Auxin-free ½MS medium served as the control. After culture for 45 days in this medium, plantlets with 4–6 leaves and 3–5 cm high were removed from jars. Agar was gently removed from roots by rinsing in tap water, and 100 plantlets from each treatment were transplanted to a substrate of sand and humus (1:1, v/v) in rectangular plastic plates (30 cm × 40 cm) under a shaded shed. Plantlets were watered once daily with tap water and with ½MS solution (only microelements) once a week. Root formation in acclimatized plants was not assessed. Plant survival was assessed after 4 weeks.

2.5. Reintroduction of acclimatized plants to their natural habitat

After growing plantlets on the above substrate for 3 months, more than 200 plantlets were transported to and transplanted to their habitat (Ehuangzhang Nature Reserve) in 2017. We searched for some small gaps among rocks near streams, and roots of plantlets were covered with moss and gently inserted into the gaps. Survival was assessed twice a year.

2.6. Statistical analysis

Experiments were repeated in triplicate. Means were separated by one-way analysis of variance (ANOVA) and significant differences between PGR treatments were assessed with Duncan's multiple range test and between the two propagation methods by the least significant difference (LSD) test at $p \leq 0.05$ using SPSS v. 18.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Effect of plant growth regulators on morphogenesis from leaf explants in vitro

Leaf explants did not respond to 1.0–10.0 mM BA and KIN in the first two weeks of culture (no data). However, 4 weeks later, leaf explants began to swell, but no callus was induced. Some protuberances developed directly on the surfaces of leaf lamina within 6 weeks. After 8 weeks, some adventitious shoots were visible (Figure 1a). When BA or KIN concentrations were increased, more adventitious shoots were induced (Table 1). Secondary shoot organogenesis was induced when culture period was prolonged. In the latter case, new adventitious shoots formed on the leaves of induced adventitious shoots, forming adventitious shoot clusters (Figure 1b). On MS medium with 1.0 mM TDZ, leaf explants initially showed no differences relative to explants exposed to BA and KIN within 4 weeks. However, leaf explants became swollen and some callus was visible on cut surfaces and on the leaf lamina within 6 weeks. After culture for 8 weeks, adventitious shoots became visible (Figure 1c). When TDZ was added at 5.0 mM, more adventitious shoots formed from callus after 8 weeks (Figure 1d; Table 1). When TDZ was applied at 10.0 mM, leaf explants swelled within 4 weeks, callus was induced, and more globular somatic embryo-like adventitious shoots formed (Figure 1e; Table 1). On MS medium with 1.0, 2.5 and 10.0 mM NAA or 2,4-D, leaf explants swelled within 3 weeks, inducing some callus, but this callus became necrotic and no adventitious shoots formed (Table 1).

3.2. Shoot proliferation

After 8 weeks of culture, the two methods to induce shoots revealed differences. A mean of 17.5–31.2 adventitious shoots formed in any medium with different PGRs from leaf explants (Table 2). Among the MS media supplemented with 5.0 mM BA and 1.0 mM NAA, a single leaf explant induced 31.2 adventitious shoots on average. However, the adventitious shoots were very small, less than 1.0 cm. As culture time was prolonged, more adventitious shoots elongated, forming longer shoots. In method 1, 4.3–6.2 axillary shoots were induced within 8 weeks (Figure 1g; Table 2). As culture time was prolonged, more axillary shoots were induced and the shoots usually grew to a height of 1–3 cm. Method 2 formed more shoots than method 1 (Table 2).

3.3. Root induction and acclimatization

When shoot clusters were transferred to root induction medium with different auxins (Table 3), roots developed within 2 weeks in ½MS medium containing 0.5 mM IBA, IAA or NAA (Figure 1g). Roots also developed on auxin-free ½MS medium, but within 3 weeks. After plantlets were transferred to a substrate of sand and humus for one month, more than 95% of plantlets survived. Plants 5 cm high showed the highest survival (97.5%) (Figure 1h) relative to 3 cm high plants (91.5% survival) indicating larger plantlets has a higher survival percentage.

Table 3. Root formation and root formation time of *Begonia coptidifolia* grown on different PGR concentrations.

PGR (μM) in rooting medium	Root formation (%)	Root formation time (weeks)
PGR-free	100 ± 0 a*	3.3 ± 0.3 a
IAA 0.5	100 ± 0 a	2.2 ± 0.2 b
IBA 0.5	100 ± 0 a	2.1 ± 0.2 b
NAA 0.5	100 ± 0a	2.3 ± 0.2 b

*Different letters within a column indicate significant differences according to Duncan's multiple range test ($p < 0.05$; $n = 40$ explants per treatment).

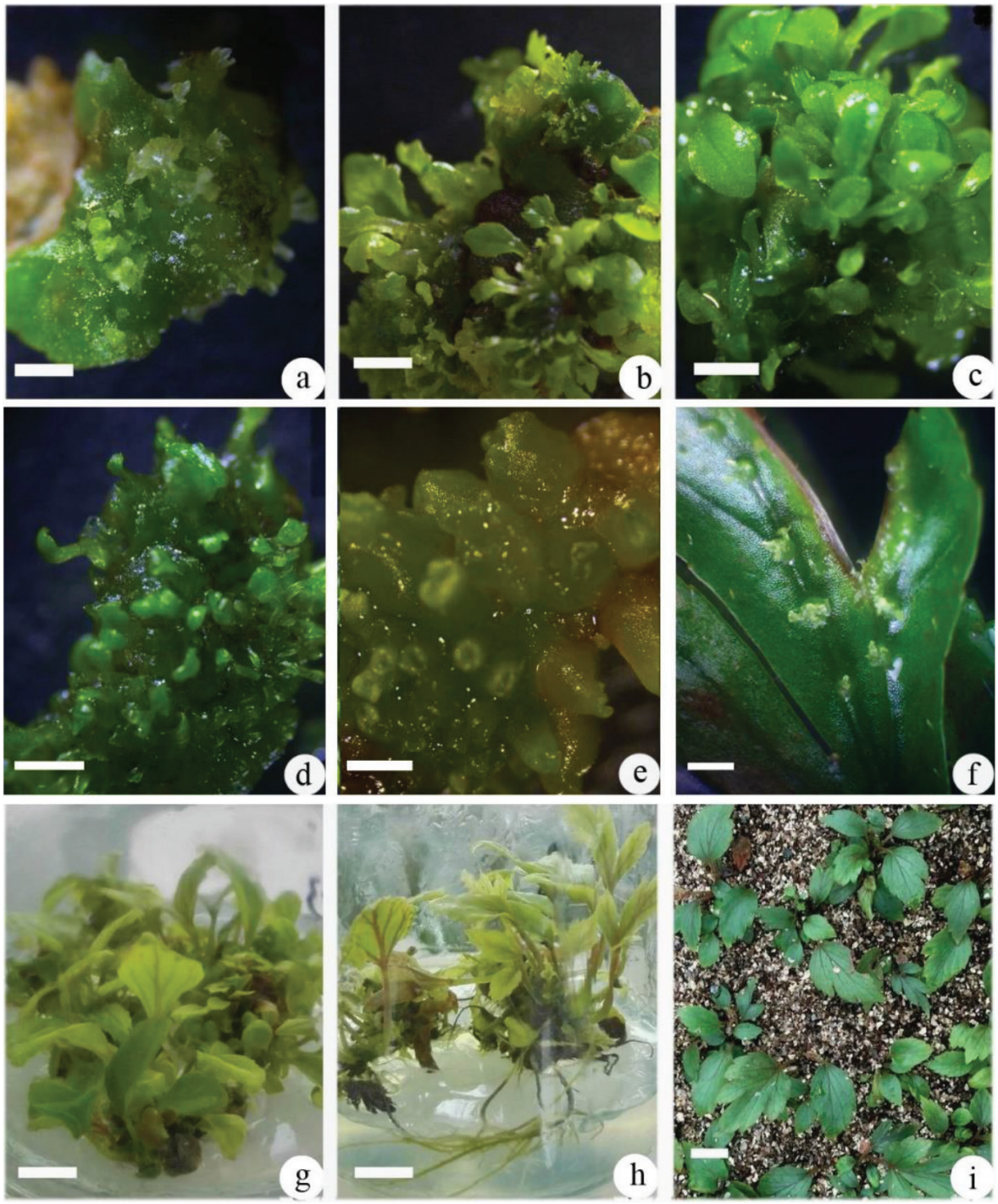

Figure 1. Shoot organogenesis and plant regeneration in *Begonia coptidifolia* on MS medium (a–f) with PGR treatments. (a) Adventitious shoots were induced from leaf explants cultured for 8 weeks with 5.0 µM BA. (b) Secondary shoot organogenesis on induced adventitious shoots after culture for 12 weeks with 10.0 µM BA. (c) Adventitious shoot formation with 1.0 µM TDZ after culture for 9 weeks. (d) Adventitious shoots and some callus were induced with 5.0 µM TDZ after culture for 9 weeks. (e) Somatic embryo-like adventitious shoots and some callus were induced with 10.0 µM TDZ after culture for 14 weeks. (f) Shoot organogenesis from leaf explant on the MS medium supplemented with 5.0 µM BA and 0.5 µM NAA within 8 weeks. (g) Axillary shoot propagation on the MS medium supplemented with 5.0 µM BA and 0.5 µM NAA. (h) Root formation after culture for 4 weeks on ½MS medium with 0.5 µM NAA. (i) Plantlets transplanted to a tray containing a substrate consisting of a mixture of sand and humus (1:1, v/v) for one month. Bars = 0.5 mm (a–f), 1 cm (g–i).

3.4. Reintroduction of tissue-cultured plants to their natural habitat

We investigated plant survival and collected plants during the first verification stage in June of 2018, finding that 63.4% of plants were still alive (Figure 2a). These plants had grown to about 15 cm in height (Figure 2b). Some plants had begun to flower (Figure 2c). After one year (December, 2018), during the second verification stage, most of the plants had grown to more than 20 cm in height and > 50% of the plants survived, most having senesced flowers and set fruit (Figure 2d). No visually obvious morphological aberrations were observed in reintroduced plants after one year.

4. Discussion

Some *Begonia* species can induce adventitious shoots and plants can be regenerated with ease (Kereša et al., 2011). Highest shoot bud induction of *Begonia tuberosus* was possible on medium with 0.9 µM TDZ and 1.0 µM NAA, giving rise to 100% shoot formation after 8 weeks using transverse thin cell layers (TCLs) from petioles (Nhut et al., 2005). In *B. tuberosus*, as many as 210 ± 9.7 shoots could be induced from TCLs after 8 weeks (Nhut et al., 2010). In *B. tuber-hybrida*, leaves and petioles formed shoots on MS medium with 0.54 µM NAA and 0.44 µM BA (Nakano et al., 1999). Also, in *B. tuber-hybrida*, shoots were induced directly on leaf and petiole segments, but the highest frequency (90% of explants) and maximum number of shoots (132/leaf explant) was on MS medium containing 5.5 µM NAA and 9.0 µM TDZ (Nada et al., 2011). In petioles, the highest frequency of shoot induction was 82% while a maximum of 33 shoots per explant were induced with 2.2 µM NAA and 9.0 µM TDZ (Nada et al., 2011). In *Begonia erythrophylla*, petioles induced adventitious shoots on MS medium with 0.54 µM NAA and 0.44 µM BA (Burritt and Leung, 1996, 2003). Espino et al. (2004) induced shoots from leaves and petioles in *Begonia semperflorens*, *Begonia rex*, *Begonia* × *elatior*, and a *Begonia* hybrid, three of which responded to different concentrations of NAA and BA in MS medium. In *Begonia malabarica*, MS medium with 4.4 mg/L BA and 1.4 mg/L IAA induced a maximum of 10 shoots from nodes while leaf-derived callus differentiated into more than 28 shoots on medium with 150 mg/L adenine sulphate (Nisha et al., 2009). In *Begonia homonym*, a maximum of 85 ± 4.3 adventitious shoots were induced from leaves on MS medium with 15.0

Figure 2. Transplanting and flowering of *Begonia coptidifolia.* (a) mother plant (bar = 20 cm); (b) transplanted plants on a mossy rock in their natural habitat (bar = 20 cm); (c) flowering plant in June of 2018 (bar = 10 cm); (d) seed setting in December of 2018 (bar = 10 cm).

μM BA and 5.0 μM NAA after 12 weeks, and after a second transfer to MS medium with 2.0 μM BA and 0.5 μM NAA for an additional 6 weeks (Kumari et al., 2017). These studies used leaves or petioles as explants in the presence of cytokinins (BA or TDZ) singly or in combination with auxins (IAA, IBA or NAA). Other explants have also been used to induce shoot organogenesis, such as pedicels of *B. elatior* 'Toran orange', which showed a maximum of 70% shoot induction on MS medium with 2.0 mg/L BA and 1.0 mg/L NAA (Mendi et al., 2009).

In this study, adventitious shoots were successfully induced from leaf explants on MS medium with cytokinins, but not auxins (2,4-D and NAA). A high concentration of cytokinin combined with a low level of auxin (NAA, IBA) was usually more effective than cytokinin alone for shoot regeneration in *Begonia* (Nisha et al., 2009; Kumari et al., 2017). A similar trend was observed from leaf explants in shoot organogenesis of *Euryodendron excelsum* L. (Chen et al., 2020) and *Scaevola sericea* Vahl (Liang et al., 2020).

Different *Begonia* species require different culture times to induce adventitious shoots although quantitative outcome might be influenced by the timing of sampling (Teixeira da Silva and Dobránszki, 2013). For *B. erythrophylla*, adventitious shoots were induced within only 1 week from petioles (Burritt and Leung, 1996). However, *B. homonym* needed 18 weeks to induce adventitious shoots (Kumari et al., 2017). In *B. semiparietalis*, 8 weeks were needed to induce adventitious shoots on MS medium with 4.0 μM meta-topolin (Chung et al., 2016). Progeny of *B. montaniformis* × *B. ningmingensis* var. bella F_1 hybrids needed 8 weeks to induce adventitious shoots (Lai et al., 2018). In four *Begonia* genotypes (*B. semperflorens*, *B. rex*, *B. elatior*, and a *Begonia* hybrid with 'Tiger' parents), adventitious shoots were induced within 6 weeks from leaves and petioles (Espino et al., 2004). In this study, *B. coptidifolia* adventitious shoots were visible on leaf explants after 8 weeks on MS medium with 1.0–10.0 μM TDZ, BA or KIN. This shows that shoot induction in different *Begonia* species, even when different explants are used, need a wide range of time (6–18 weeks in the studies assessed), i.e. a genotype-dependent response, as well as the likely influence of explant (leaf) age and culture conditions.

In this study, we compared two methods of propagation. One method was adventitious shoot organogenesis from leaf explants and another method was simple axillary shoot proliferation by subculturing shoot clusters. Since the culture period for both methods was 8 weeks, evidently, shoot organogenesis (method 1) produced more shoots than simple shoot proliferation (method 2). However, the shoots from method 1 were much smaller and more frail than shoots from method 2. Therefore, at an earlier stage (method 1) was used to induce shoots from leaf explants for large-scale shoot multiplication, and at a later stage (method 2) was used to proliferate and grow shoots. A similar dual tissue culture strategy was employed for *Metabriggsia ovalifolia* (Ma et al., 2011).

Many *Begonia* species are rare and endemic plants (Kumaria et al., 2012; Kumari et al., 2017). *B. coptidifolia* is also a rare species that faces extinction. In this study, we established an efficient mass propagation and regeneration system that would allow *B. coptidifolia* to be rapidly propagated and preserved. After reintroducing >200 plantlets to their natural habitat, in excess of 50% of plants survived, flowered and set fruit after one year, demonstrating that reintroduction is possible, and successful. Future biotechnological studies that focus on micropropagation need to assess the genetic, biochemical and physiological variation in clonal propagules while the survival of tissue culture-derived plantlets versus seed-derived progeny after reintroduction to their natural habitat also merit assessment. Tissue culture medium mineral nutrition studies on the effect of macro- and micronutrients, as well as ions and proportions of ions (Akin et al., 2016; Akin et al., 2017a; Akin et al., 2017b; Kovalchuk et al., 2017; Akin et al., 2020) could also be conducted to further improve the quality and quantity of plantlets and the success of reintroduction into their wild habitat.

Contribution of authors

BYG, YPX, TW, HR, SJZ, JATdS and GHM designed the experiment and provided guidance for the study. YPX and BYG prepared samples for all analyses. BYG and YPX participated in the statistical analysis of physiological changes. BYG provided statistical analyses and cowrote the manuscript with JATdS. JATdS provided advice and interpretation. All authors read and approved the manuscript.

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Conflicts of interest

The authors declare no conflicts of interest.

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