

Plant regeneration via direct and callus-mediated organogenesis from leaf explants of *Chirita swinglei* (Merr.) W. T. Wang

Yulu Chen^{1,2} · Yueya Zhang^{1,2} · Qingwei Cheng^{1,3} · Meiyun Niu^{1,2} · Hanzhi Liang^{1,3} ·
Haifeng Yan^{1,2} · Xinhua Zhang¹ · Jaime A. Teixeira da Silva⁴ · Guohua Ma^{1,2}

Received: 19 November 2015 / Accepted: 12 May 2016 / Published online: 10 June 2016 / Editor: Ewen Mullins
© The Society for In Vitro Biology 2016

Abstract Plant regeneration via direct shoot organogenesis and callus-mediated organogenesis from leaf explants of *Chirita swinglei* (Merr.) W. T. Wang was studied. Cytokinins played a crucial role in the direct formation of adventitious shoots, which originally appeared as small nodular protuberances and developed into adventitious shoots as culture period increased. These protuberances were similar to somatic embryos in external morphology, but their anatomical structure confirmed that these were shoots and not somatic embryos. The highest frequency of shoot buds was induced by 2.0 μM thidiazuron (TDZ) and 2.5 μM 6-benzyladenine (BA). Leaf explants produced the highest frequency of shoot buds (100%) on MS medium supplemented with both 2.0 μM TDZ and 2.5 μM BA. In the presence of 2.0 μM TDZ, leaf explants became swollen after culture for 15 d. Some shoot buds were observed after 20 d of culture. Shoot buds were clearly visible as culture period was extended from 35 to 45 d. Histological analysis revealed the presence of meristematic tissues coincident with shoot tips. Callus could also be

induced from leaves when α -naphthaleneacetic acid (NAA) was used alone or in combination with TDZ and BA. Three types of callus, pink and friable, white and compact, and green-yellow and compact, formed, but only the latter two could differentiate into plantlets. Over 90% of plantlets survived after transplanting into sand or a mixture of sand, loessal clay, and vermiculite (1:1:1, v/v). This protocol provides an efficient method via two organogenic pathways, to mass produce and conserve *C. swinglei*, an attractive ornamental plant and important medicinal herb.

Keywords *Chirita swinglei* · Adventitious shoots · Shoot buds · Callus differentiation

Introduction

The genus *Chirita* (Gesneriaceae) is made up of about 140 species, mainly distributed throughout Southeast Asia (Wood 1974; Wang *et al.* 1990; Wang *et al.* 2011). Many *Chirita* species have ornamental value and are also used as medicinal plants (Harborne 1966; Damtoft and Jensen 1994; Zhou *et al.* 2001; Chen *et al.* 2011). *Chirita swinglei* (Merr.) W. T. Wang, mainly found growing in Guangxi and Guangdong provinces in China, is an evergreen perennial herb with glossy green foliage and baby blue or purple corollas. It is used as a potted ornamental plant and is also an important medicinal herb in South China, and the whole plant is used to treat ulcers and poisoning (Wang *et al.* 1990). *C. swinglei* generally reproduces by seed or is propagated through stem cuttings. However, these two methods are slow and rely on collecting or cutting mother plants. To strengthen the conservation, development, and utilization of this plant resource, it is important to establish an efficient *in vitro* micropropagation and plant regeneration protocol. Only a single tissue culture

Yulu Chen and Yueya Zhang contributed equally to this work.

✉ Jaime A. Teixeira da Silva
jaimetex@yahoo.com

✉ Guohua Ma
magh@scib.ac.cn

¹ Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, the Chinese Academy of Sciences, Guangzhou 510650, China

² University of Chinese Academy of Sciences, Beijing 100039, China

³ Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China

⁴ P. O. Box 7, Miki-cho Post Office, Ikenobe 3011-2, Kagawa-ken 761-0799, Japan

and plant regeneration protocol via adventitious shoot regeneration exists for *C. swinglei* (Yu *et al.* 2012). Callus-mediated induction of shoots has never been reported for this plant. In the *Gesneriaceae*, a callus-mediated regeneration protocol could be useful in genetic improvement programs through transgenic engineering, for example for the genus *Saintpaulia* (Kushikawa *et al.* 2001).

Studies on the *in vitro* propagation of members of the *Gesneriaceae* have mainly focused on the induction of adventitious shoots or somatic embryos (Dolendro *et al.* 2003; Ma *et al.* 2010; Yang *et al.* 2012, 2014). In this study, we established efficient protocols for high-frequency regeneration via two pathways, direct and indirect (i.e., callus-mediated) shoot organogenesis, from *C. swinglei* leaf explants. To be able to conserve plants with great value and low reproductive ability, it is useful to have alternative pathways that can lead to high-frequency and rapid regeneration. In addition, callus-mediated shoot formation is not only a useful method for plant propagation but also a powerful tool for plant genetic improvement, germplasm conservation, and production of useful compounds. We also investigated the effects of different types and concentrations of plant growth regulators (PGRs) on the induction of each of these pathways and studied the histology underlying shoot organogenesis.

Materials and Methods

Culture establishment *C. swinglei* explant material was obtained from South China Botanical Garden in Guangzhou, China. About 20 immature leaves from one-yr-old mother plants were collected as explants and were washed under running tap water for 15 min. Excised leaves were surface sterilized with 70% (v/v) ethanol for 15 s, rinsed in sterile distilled water, and dipped in 0.1% (w/v) mercuric chloride solution for 10 min followed by five or six rinses with sterile distilled water. After that, they were placed on sterile filter paper and allowed to dry briefly in air. The leaves were cut into 0.5 cm² explants and inoculated adaxial-side down on MS basal medium (Murashige and Skoog 1962). The pH of MS basal medium, which contained 30 g L⁻¹ sucrose, was adjusted to 5.9 before being solidified with 0.7% (w/v) agar (Sigma-Aldrich, St. Louis, MO) and autoclaved at 121°C for 15 min. Culture jars (10 cm high and 6 cm in diameter) were placed in an air-conditioned culture room at 26 ± 2°C with a 12-h photoperiod under 100 μM m⁻² s⁻¹ fluorescent light (Philips, Tianjing, China). These culture conditions were identical for all experiments.

Effect of single PGRs on induction of shoot buds, adventitious shoots and callus from leaf explants Leaf segments were inoculated adaxial-side down on MS medium supplemented with 0.5 or 2.0 μM of a single PGR: 6-benzyladenine (BA), α-naphthaleneacetic acid (NAA), thidiazuron (TDZ), or 2,4-dichlorophenoxyacetic acid (2,4-

D) (Sigma-Aldrich). Induction of shoot buds, shoots, and callus was investigated after culture for a total of 45 d (Table 1). Among the PGRs tested, TDZ was filter-sterilized through a 0.24-μm filter and added after autoclaved medium had cooled while 2,4-D, BA, indole-3-butyric acid (IBA), and NAA were added prior to autoclaving at 121°C for 15 min.

Effect of NAA, BA and TDZ concentrations on induction of shoot buds, adventitious shoots and callus from leaf explants We studied the effects of different PGRs (NAA, BA, and TDZ) concentrations and their combinations (selected by an orthogonal test; Fang and Ma 2001) on the induction of shoot buds, adventitious shoots, and callus from leaf explants. Leaf segments were inoculated adaxial-side down on MS medium supplemented with different concentrations of PGRs. Induction of shoot buds, adventitious shoots, and callus was investigated after 45 d of culture (Table 2).

Shoot proliferation Adventitious shoots induced during the single PGR experiment were transferred to MS medium containing different concentrations and combinations of BA, NAA, and TDZ for shoot proliferation, which was investigated after culture for 45 d (Table 3). The shoot proliferation coefficient (SPC) was calculated as the ratio of the number of adventitious shoots after inoculation/number of adventitious shoots before inoculation.

Morphogenesis from different callus types Type I (pink and friable), II (white and compact), and III (green-yellow and compact) callus were separated from cultured leaf blades and cut into 8–9 mm diameter callus clumps that were transferred to MS medium supplemented with 1.0 μM TDZ to observe callus differentiation (i.e., their ability to differentiate into shoots). All culture jars were placed in the dark for 10 d then transferred to a culture room with a 12-h photoperiod under 100 μM m⁻² s⁻¹ fluorescent light. After culture for 60 days, callus differentiation was investigated.

Root formation and acclimatization Shoots with 2–3 leaves that had reached 2–3 cm in height on MS medium supplemented with 2.0 μM BA and 0.5 μM NAA were isolated from multiple shoot clusters, cut off at the base, and transferred to half-strength MS medium supplemented with 0.5 μM IBA, 0.5 μM NAA, 0.5 μM IBA, and 0.5 μM NAA or no PGRs. After culture for a total of 30 days on these media, root formation was investigated (Table 4). A total of 270 rooted plantlets with 4–5 leaves and 3–4 cm tall were removed from jars representing the above four treatments. The agar was gently washed off roots in tap water. Plantlets were transplanted to square plastic trays (50 cm × 40 cm × 10 cm) containing three substrates: sand, loessal clay, and sand: loessal clay: vermiculite (1:1:1, v/v). A total of 90 plantlets were planted per

Table 1. Effect of different PGRs on induction of shoot buds, adventitious shoots, and callus from leaf explants of *Chirita swinglei*

PGRs (μM)	Explants forming shoot buds (%)	Number of shoot buds/explant	Explants forming shoots (%)	Number of shoots/explant	Explants forming callus (%)
BA 0.5	0.0 d	0.0 d	84.4 \pm 5.1 a	12.4 \pm 0.6 a	0.0 c
BA 2.0	70.0 \pm 5.8 c	13.1 \pm 0.9 c	72.2 \pm 5.1 b	9.6 \pm 0.5 b	0.0 c
NAA 0.5	0.0 d	0.0 d	66.7 \pm 3.3 b	9.3 \pm 0.4 b	84.4 \pm 5.1 b
NAA 2.0	0.0 d	0.0 d	53.3 \pm 3.3 c	5.0 \pm 0.4 c	100.0 a
TDZ 0.5	81.1 \pm 1.9 b	14.1 \pm 0.9 b	0.0 e	0.0 e	0.0 c
TDZ 2.0	93.3 \pm 3.3 a	17.1 \pm 0.8 a	10.0 \pm 0.0 d	1.5 \pm 0.1 d	0.0 c
2,4-D 0.5	0.0 d	0.0 d	0.0 e	0.0 e	0.0 c
2,4-D 2.0	0.0 d	0.0 d	0.0 e	0.0 e	0.0 c

Type I callus (pink and friable) was produced on medium supplemented with 0.5 or 2.0 μM NAA. Values represent means \pm SD. Different *letters* within a *column* indicate significant differences according to Duncan's multiple range test ($P \leq 0.05$). Percentage values were arcsine transformed prior to analysis. $n = 90$ per treatment

substrate type. The trays were placed in a makeshift greenhouse covered with a 90% black net and watered every 2 d with half-strength MS. The temperature in the greenhouse was $27 \pm 2^\circ\text{C}$. Plant survival (%) was assessed after 30 d (Table 5).

Histology of shoot organogenesis from in vitro leaves To study the ontogeny and development of *C. swinglei* shoots, leaf explants were incubated on shoot induction medium (SIM) supplemented with 2.0 μM TDZ, which was the optimal concentration shown to induce shoot buds in the single PGR experiment. After culture for 15, 25, and 35 days, the leaves were fixed in FAA (37% (*w/v*) formaldehyde:glacial acetic acid:glycerol 1:1:18 (*v/v/v*) in 50% (*v/v*) alcohol), solution at room temperature for more than 48 h, then transferred to 70% (*v/v*) alcohol for storage until analysis. The samples were dehydrated through an alcohol series and embedded in paraffin. Longitudinal sections 10 μm thick were made with a paraffin-compatible microtome (Fourth Shanghai Medicine Manufacturing Co., Shanghai, China). The sections were observed under a microscope (Leica DM1000 LED, Wetzlar, Germany).

Statistical analyses All experiments were repeated three times within a 2-wk interval. Each treatment contained six explants per culture jar and five jars per treatment. The data were reported as mean \pm SD (standard deviation). Percentage values were arcsine transformed prior to analysis. Means were statistically analyzed by one-way analysis of variance (ANOVA), and treatment means were considered to be significantly different from controls by Duncan's multiple range test at $P \leq 0.05$ using SPSS v. 19.0 (IBM, New York, NY).

Results

Effect of single PGRs on induction of shoot buds, adventitious shoots and callus from leaf explants Significant differences were observed in adventitious shoot formation among the different PGRs or PGR concentrations (Table 1). On SIM supplemented with 0.5–2.0 μM BA, many adventitious shoot buds developed directly on the leaf surface and leaf blade (Fig. 1A). On SIM containing 2.0 μM TDZ, leaf explants became swollen after culture for 15 d. Globular-shaped shoot

Table 2. Effect of different combinations of NAA, BA, and TDZ on induction of shoot buds, adventitious shoots, and callus from leaf explants of *Chirita swinglei*

PGRs (μM)	Explants forming shoot buds (%)	Number of shoot buds/explant	Explants forming shoots (%)	Number of shoots/explant	Explants forming callus (%)	Type of callus
BA 2.0+TDZ 1.0	91.1 \pm 5.1 b	16.8 \pm 0.4 b	35.6 \pm 1.9 b	3.1 \pm 0.3 b	0 d	—
BA 2.5+TDZ 2.0	100 a	23.1 \pm 1.2 a	15.6 \pm 1.9 c	1.2 \pm 0.0 c	0 d	—
NAA 0.5+BA 1.5+TDZ 1.0	40.0 \pm 3.3 d	8.1 \pm 0.4 d	92.2 \pm 3.9 a	14.9 \pm 0.5 a	34.4 \pm 1.9 c	II
NAA 0.5+BA 2.0+TDZ 2.0	51.1 \pm 3.8 c	9.6 \pm 0.4 c	0 d	0 d	38.9 \pm 1.9 c	II, III
NAA 1.0+BA 2.0	35.6 \pm 1.9 d	7.9 \pm 0.5 d	0 d	0 d	58.9 \pm 1.9 b	II
NAA 1.0+BA 2.5+TDZ 1.0	22.2 \pm 1.9 e	3.7 \pm 0.2 e	0 d	0 d	72.2 \pm 5.1 a	III

Type II callus: white and compact. Type III callus: green-yellow and compact. Values represent means \pm SD. Different *letters* within a *column* indicate significant differences according to Duncan's multiple range test ($P \leq 0.05$). Percentage values were arcsine transformed prior to analysis. $n = 90$ per treatment

Table 3. Effect of different of PGRs on shoot proliferation of *Chirita swinglei*

PGRs (μM)	SPC
0	5.1 \pm 0.1 c
BA 2.5	15.5 \pm 0.5 a
TDZ 2.5	12.7 \pm 0.3 b
BA 2.5+NAA 0.5	16.1 \pm 0.3 a
TDZ 2.5+NAA 0.5	12.1 \pm 0.4 b

Values represent means \pm SD. Different *letters* within a *column* indicate significant differences according to the Duncan's multiple range test ($P\leq 0.05$). $n=90$ per treatment. SPC, shoot proliferation coefficient=number of adventitious shoots after inoculation/number of adventitious shoots before inoculation

buds were observed after 20 (Fig. 1B) and 25 d of culture (Fig. 1C). These enlarged by 35 d (Fig. 1D) and formed distinct leaf initials after culture for 45 d (Fig. 1E). The shoot buds developed into complete shoots that could be harvested when culture period was prolonged to 60 d (Fig. 1F). The response of shoot buds reached 93.3% and an average of 17.1 shoot buds per explant on medium containing 2.0 μM TDZ. A lower concentration of TDZ and BA (0.5 μM) could only induce fewer shoot buds but many adventitious shoots whereas a higher concentration of TDZ or BA (2.0 μM) induced both shoot buds and adventitious shoots.

On medium supplemented with 0.5 or 2.0 μM NAA, callus was observed on the abaxial surface of leaf blades and at the same time, many adventitious shoot buds developed directly on the adaxial leaf surface (Fig. 1G). This callus was defined as type I callus, which was pink, friable, and slow growing. As the culture period was prolonged, adventitious roots developed from callus. Leaf explants could not induce shoot buds, adventitious shoots, or callus on SIM supplemented with 0.5–2.0 μM 2,4-D, and generally became brown and necrosed.

Table 4. Effect of PGRs in rooting media (half-strength MS) on the rooting of *Chirita swinglei*

PGRs (μM) in rooting media	Rooting (%)	Number of roots/plantlet	Rooting observations
0.0	91.1 \pm 3.8 b	9.2 \pm 0.3d	Thin short roots; few side roots; no callus at shoot base
IBA 0.5	100.0 a	15.6 \pm 1.1 a	Many strong roots, and many well-developed side roots; callus at shoot base
NAA 0.5	100.0 a	11.9 \pm 0.8 c	Many thin roots; few side roots; callus at shoot base
IBA 0.5+NAA 0.5	100.0 a	13.5 \pm 0.5 b	Many strong roots; few side roots; callus at shoot base

For number of roots, values represent means \pm SD. Different *letters* within a *column* indicate significant differences according to the Duncan's multiple range test ($P\leq 0.05$). Percentage values were arcsine transformed prior to analysis. $n=90$ per treatment

Table 5. Effect of different culture media on plantlet survival of *Chirita swinglei*.

Culture media	Survival of transplanted plants (%)
Sand	94.4 \pm 6.9 a
Loessal clay	57.8 \pm 1.9 b
Sand: loessal clay: vermiculite (1:1:1)	90.0 \pm 3.3 a

Values represent means \pm SD. Different *letters* within a *column* indicate significant differences according to the Duncan's multiple range test ($P\leq 0.05$). Percentage values were arcsine transformed prior to analysis. $n=90$ per treatment

Effect of NAA, BA and TDZ concentrations on the induction of shoot buds, adventitious shoots and callus from leaf explants

Culture on MS medium, supplemented with 2.5 μM BA and 2.0 μM TDZ, produced globular shoot buds, which were initially white, turned light green, and emerged in large numbers. Some shoot buds were observed after 40 d of culture. Thereafter, many shoot buds gradually developed into shoots: 100% of explants formed an average of 23.1 shoot buds per explant on this SIM (Table 2).

MS medium supplemented with 1.5 μM BA combined with a low concentration of NAA (0.5 μM) and TDZ (1.0 μM) produced 92.2% of responding leaf explants, and 14.9 adventitious shoots formed per explant. Callus was only induced on MS medium when NAA was included, forming two kinds of callus, which were defined as type II and type III callus.

Type II callus was compact, white, and slow growing (Fig. 2B). The highest callus response was 58.9% on medium with 1.0 μM NAA and 2.0 μM BA. Only 34.4% callusing response was observed on medium supplemented with 0.5 μM NAA, 1.5 μM BA, and 1.0 μM TDZ (Table 2).

Type III callus was green-yellow, compact, and grew the slowest among the three types of callus. In some cases, type II and type III callus were induced on the same medium and the amount of type III callus was higher than type II callus.

Shoot proliferation Adventitious shoots grew slowly and SPC was 5.1 after culture for 45 d on PGR-free MS medium. After 45 days of culture, SPC reached 15.5 on medium containing 2.5 μM BA and was 12.7 on medium containing 2.5 μM TDZ (Fig. 1H). On medium supplemented with 2.5 μM BA alone or in combination with 0.5 μM NAA, there was no significant difference in SPC. Similarly, there was also no significant difference in SPC when the medium was supplemented with 2.5 μM TDZ alone or combined with 0.5 μM NAA (Table 3).

Morphogenesis from different types of callus MS medium supplemented with 1.0 μM TDZ, produced type I callus which gradually became brown and necrotic, and never differentiated. Type II callus showed no response within the first 15 d of culture, but after culture for 25 d, it began to differentiate and green granular structures became visible. As

culture period was extended, 46.7% of callus produced adventitious shoots. Moreover, type II callus could convert into type III callus as culture period progressed. Type III callus began to proliferate rapidly then differentiated into adventitious shoots (shoot response was 55.6%) and green shoot buds (globular shoot bud response was 34.4%) by the 60th day of culture (Fig. 2D).

Root formation and acclimatization Plantlets could induce adventitious roots on all rooting media. However, root development was slow and the rooting response only reached 91.1% on half-strength PGR-free MS medium. 100% of plantlets could develop roots when medium contained 0.5 μM IBA, 0.5 μM NAA, or 0.5 μM IBA and 0.5 μM NAA (Table 4). On rooting medium with any auxin, callus was visible at the base of shoots, but this callus had no effect on plant survival during acclimatization. When plantlets were

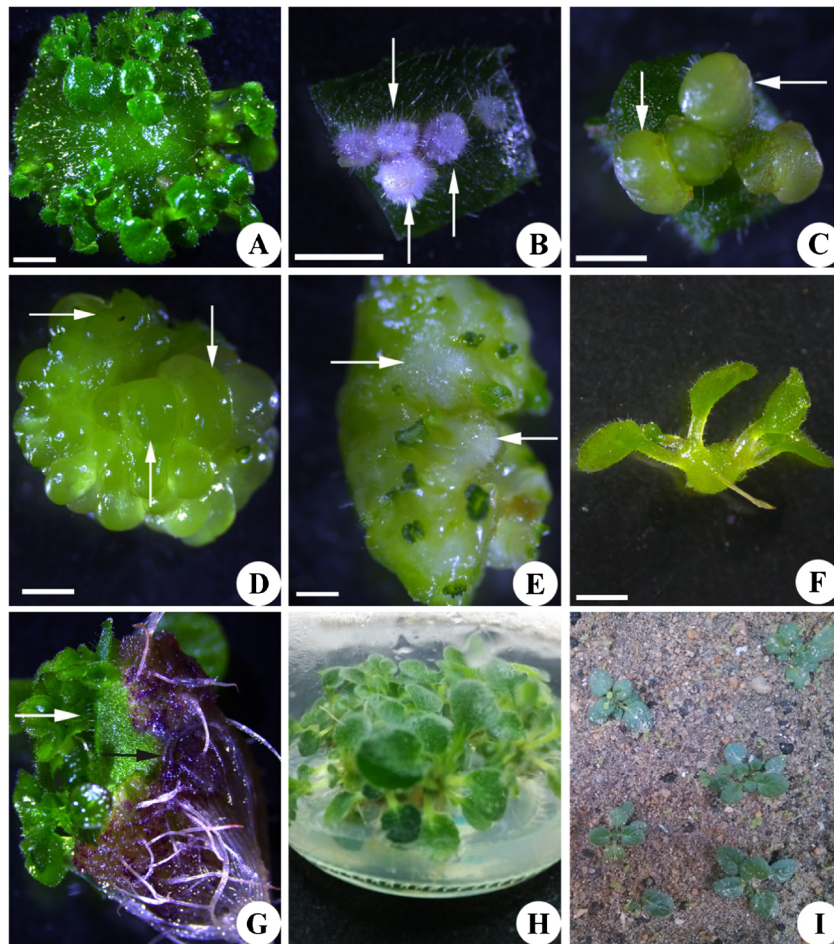
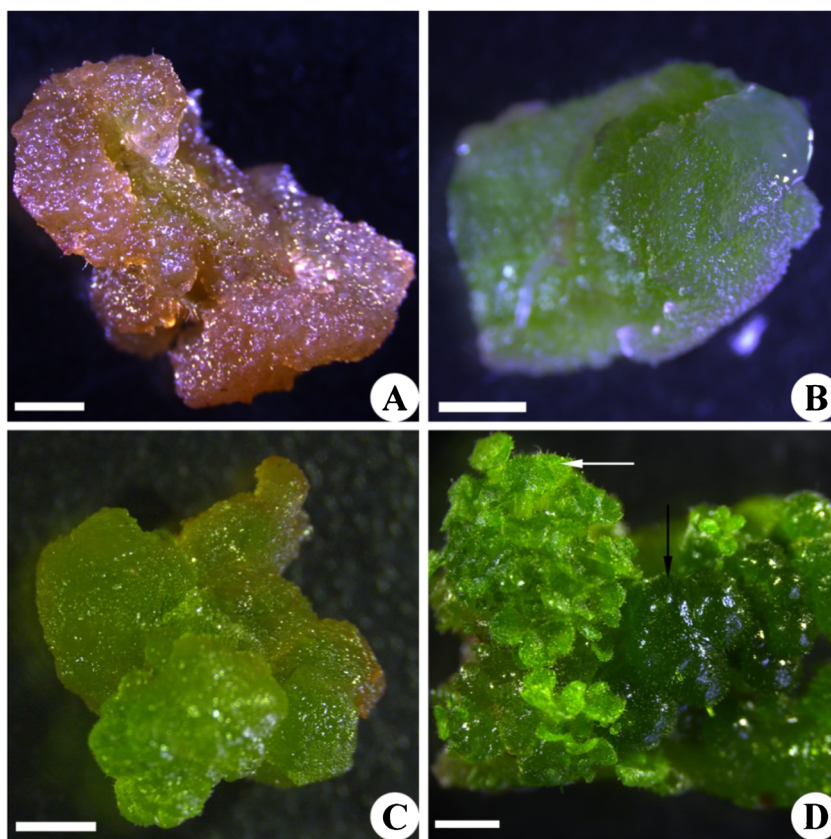


Figure 1. Plant regeneration via shoot organogenesis from leaf explants of *Chirita swinglei* (bars, 2 mm). (A) Adventitious shoots were induced directly from the leaf surface on MS medium containing 0.5 μM BA after culture for 30 d. (B and C) Globular shoot buds (white arrows) were induced on MS medium with 2.0 μM TDZ for 20 and 25 d, respectively. (D) Shoot buds with different shapes were induced on MS medium containing 2.0 μM TDZ after 35 d of culture. (E) Larger shoot buds (white arrows) were induced on MS medium supplemented with

2.0 μM TDZ for 45 d. (F) A shoot developed from a shoot bud on MS containing 2.0 μM TDZ after culture for 60 d. (G) Adventitious shoots (white arrow) were induced on the leaf surface and callus (black arrow) was induced on the lamina's abaxial side on MS medium with 2.0 μM NAA when cultured for 30 d. (H) Shoot clusters proliferated on MS medium with 2.5 μM BA after 45 d. (I) Plantlets in a plastic tray containing sand, 30 d after transplanting from *in vitro* conditions.

Figure 2. Callus induction and morphogenesis from different types of callus induced from leaf explants of *Chirita swinglei*. (A) Type I callus (pink and friable) was induced on medium supplemented with 2.0 μM NAA after 40 d. (B) Type II callus (white and compact) was induced on medium with 1.0 μM NAA and 2.0 μM BA after culture for 40 d. (C) Type III callus (green-yellow and compact) was induced on medium containing 1.0 μM NAA, 2.0 μM BA, and 2.0 μM TDZ after culture for 40 d. (D) Type III callus differentiated into adventitious shoots (white arrow) or shoot buds (black arrow) on medium supplemented with 1.0 μM TDZ after 55 d. Bars, 2 mm.



transplanted to sand 30 days later, 94.4% of plantlets survived after 30 d. Only 57.8% of plantlets could survive in loessal clay (Fig. 1I, Table 5).

Histology of shoot organogenesis from *in vitro* leaves

Histological analysis revealed that meristematic tissues and some darkly stained primordial cell clumps were visible after 15 d of culture (Fig. 3A, B). As culture period approached 25 days, some globular shoot buds were visible on both the abaxial and adaxial leaf surfaces (Fig. 3C, D, respectively). After leaf explants were cultured for 35 days, some adventitious shoots formed (Fig. 3E). As culture period was prolonged, shoot buds formed leaves (Fig. 3F).

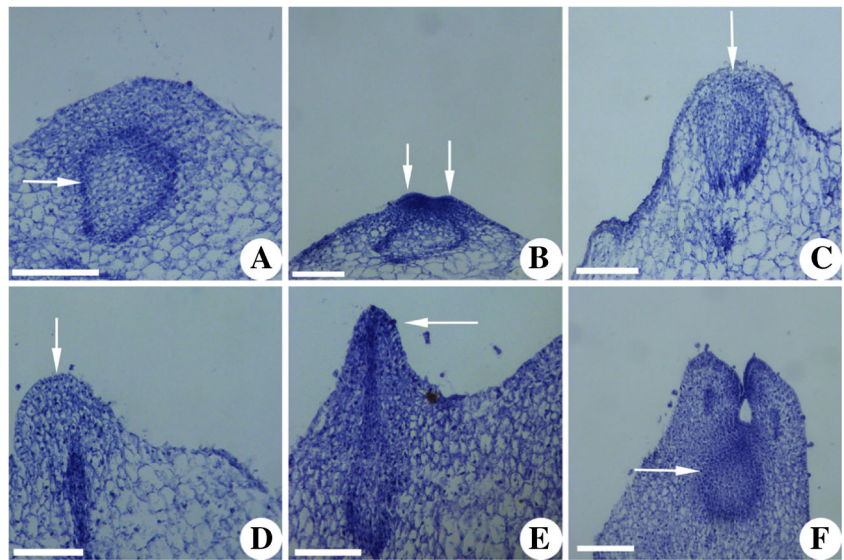
Discussion

In several species of the *Gesneriaceae*, shoot organogenesis has been reported. For example, the use of BA and kinetin is a popular means to propagate African violet (*Saintpaulia ionantha*) from leaf explants (Dewir *et al.* 2015). In *Metabriggsia ovalifolia*, MS medium with 5.0 μM TDZ and 0.5 μM BA could induce adventitious shoots from *in vitro* leaf explants and MS medium with 5.0 μM BA and 0.5 μM NAA could induce adventitious shoots from petiole explants (Ma *et al.* 2011a). In *Titanotrichum oldhamii*, adventitious shoots

formed on medium with 0.1 μM BA from *in vitro* leaf explants (Takagi *et al.* 2011). *In vitro* leaf explants of *Dayaoshania cotinifolia* also induced adventitious shoots on MS medium with 1, 3, or 5 μM TDZ, BA or NAA alone (Yang *et al.* 2014). *In vitro* leaf explants of *Chirita flavimaculata*, *Chirita eburnea*, and *Chirita speciosa* formed adventitious shoots on MS medium containing 0.5 μM NAA and 0.4 μM BA (Nakano *et al.* 2009). MS medium supplemented with 0.5–2.0 μM NAA produced adventitious shoots formed from *in vitro* leaf explants of *Lysionotus pauciflorus* (Godo *et al.* 2010). Yu *et al.* (2012), using *in vitro* leaves as explants, noted that optimal SIM was MS medium supplemented with 0.5 μM BA and 0.1 μM NAA. The optimal differentiation medium was MS medium supplemented with 0.3 μM BA and 0.1 μM NAA. The optimal rooting medium was half-strength MS medium supplemented with 0.1 μM NAA and 1.0 g L^{-1} activated charcoal. In our experiments, BA, TDZ, and NAA could induce adventitious shoots from leaf explants (Fig. 4).

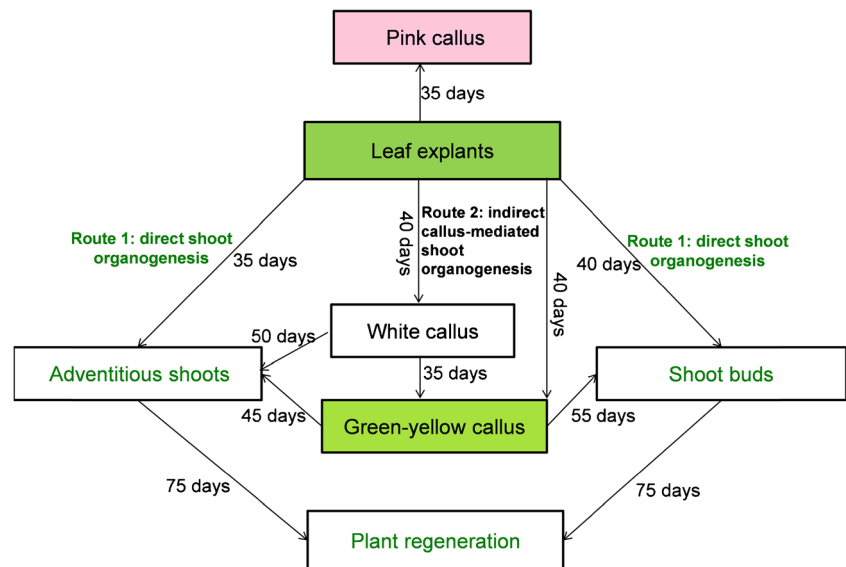
In other species of many plant families, previous reports have shown that TDZ can induce shoot organogenesis, including in *Albizia julibrissin* (Hosseini-Nasr and Rashid 2002), *Curcuma alismatifolia* (Mahadatanapuk *et al.* 2006), *Primulina tamiana* (Padmanabhan *et al.* 2014), and *Primulina dryas* (Padmanabhan *et al.* 2015). In other cases, TDZ induced somatic embryos such as in *Arachis hypogaea* (Murch and

Figure 3. Histology of shoot organogenesis on MS medium supplemented with 2.0 μM TDZ from leaves of *Chirita swinglei*. (A and B) Meristematic tissue was visible after culture for 15 d (white arrows). (C and D) Globular shoot buds (white arrows) were visible protruding from the leaf surface after culture for 25 d. (E) Adventitious shoots were visible after 35 d (white arrow). (F) Shoot buds (white arrow) after culture for 35 d. Bars, 0.2 mm.



Saxena 1999), *Capsicum annuum* (Khan *et al.* 2006), and *Ricinus communis* (Kumari *et al.* 2008). In *Cajanus cajan*, a low concentration of TDZ (0.05–1.0 μM) induced multiple shoots while a higher concentration (10 or 20 μM) induced somatic embryos (Dolendro *et al.* 2003). In *Ochna integerrima*, a higher concentration of TDZ (10.0–15.0 μM) could induce both somatic embryos and adventitious shoots whereas a low concentration of TDZ (5.0 μM) could only induce adventitious shoots (Ma *et al.* 2011b). Therefore, TDZ not only functions as a cytokinin but also as an auxin and can substitute for auxins or combinations of auxins and cytokinins (Murthy *et al.* 1998; Singh *et al.* 2003). Our results also showed that a higher level of TDZ or BA (2.0 μM) could induce both shoot buds and adventitious shoots, whereas a low level of TDZ and BA (0.5 μM) could only induce fewer shoot buds and many adventitious shoots (Table 1).

Figure 4. Simplified flow diagram of two regeneration pathways (direct and indirect) from *Chirita swinglei* leaf explants. Even though shoot buds and adventitious shoots are most likely the same organs (adventitious shoots), they form as distinct pathways with distinct periods of formation. They are both represented in the figure as *Route 1* (i.e., direct shoot organogenesis).



There are many reports that used 2,4-D to induce callus and plant regeneration. When MS medium was supplemented with 2,4-D combined with TDZ and BA, callus was induced and plant regeneration of *Curcuma kwangsiensis* took place (Zhang *et al.* 2011). MS medium containing 2.0 μM 2,4-D induced callus from the leaves of *Lysionotus pauciflorus* and showed fourfold proliferation in liquid medium after culture for 4 wk (Lu *et al.* 2006). In *Curcuma attenuata*, 4.5 μM 2,4-D could induce callus then differentiate into shoots (Kou *et al.* 2013). However, in this study, all explants generally become brown and necrotic on SIM with 2,4-D, which indicates that 2,4-D is not suitable for *C. swinglei* plant regeneration. However, NAA combined with cytokinins (BA and TDZ) could induce callus and be used for plant regeneration.

For plants with great ornamental or medicinal value but low reproductive ability, such as *C. swinglei*, a single tissue culture and plant regeneration protocol via adventitious shoot

regeneration is sub-optimal for conservation purposes. The mass propagation and conservation of this medicinal ornamental herb would benefit from the establishment of multiple plant regeneration pathways. In addition, callus-mediated shoot regeneration is a powerful tool for plant genetic improvement when used in combination with traditional agricultural techniques and is also an important technique to understand plant growth and development and mechanisms of cell differentiation. Our study expands on previous research on plant regeneration system of this species, which was limited to the simple induction of adventitious buds. This is the first efficient *in vitro* shoot induction and plant regeneration system for *C. swinglei* from young *in vitro*-derived leaf explants through two distinct pathways, namely indirect callus-mediated shoot organogenesis and direct shoot organogenesis (Fig. 4). This protocol provides a foundation for future transgenic and other biotechnological applications and also serves as a very practical and useful technique to mass propagate this ornamental and medicinal plant.

References

- Chen YY, Chen WJ, Li DP, Huang YL, Wen YX (2011) Preparative isolation and purification of five phenylethanoid glycosides from *Chirita eburnea*. *Chem Nat Compd* 47:615–618
- Damtoft S, Jensen SR (1994) Three phenylethanoid glucosides of unusual structure from *Chirita sinensis* (Gesneriaceae). *Phytochemistry* 37:441–443
- Dewir YH, El-Mahrouk ME-S, Hafez YM, Teixeira da Silva JA, Naidoo Y (2015) Hyperhydricity in African violet (*Saintpaulia ionantha* H. Wendl)—biochemical aspects of normal versus hyperhydric shoots regenerated via direct adventitious shoots formation. *Propag Ornament Plants* 15:53–62
- Dolendro SN, Sahoo L, Sarini NB, Jaiwal PK (2003) The effect of TDZ on organogenesis and somatic embryogenesis in pigeonpea (*Cajanus cajan* L. Mill. sp.). *Plant Sci* 164:341–347
- Fang KT, Ma CX (2001) Orthogonal and uniform experimental design. Science Press, Beijing, pp 35–43 (In Chinese)
- Godo T, Lu YX, Mii M (2010) Micropropagation of *Lysionotus pauciflorus* Maxim. (Gesneriaceae). In: Jain SM, Ochatt SJ (eds) *Methods in molecular biology*, vol 589. Berlin, Springer Science and Business Media, pp 127–139
- Harborne JB (1966) Comparative biochemistry of flavonoids—I distribution of chalcone and aurone pigments in plants. *Phytochemistry* 5: 111–115
- Hosseini-Nasr M, Rashid A (2002) Thidiazuron-induced shoot-bud formation on root segments of *Albizia julibrissin* is an apex-controlled, light-independent and calcium-mediated response. *Plant Growth Regul* 36:81–85
- Khan H, Siddique I, Anis M (2006) Thidiazuron induced somatic embryogenesis and plant regeneration in *Capsicum annum*. *Biol Plant* 50:789–792
- Kou YP, Ma GH, Teixeira da Silva JA, Liu N (2013) Callus induction and shoot organogenesis from anther cultures of *Curcuma attenuata* wall. *Plant Cell Tissue Organ Cult* 112:1–7
- Kumari GK, Ganesan M, Jayabalan N (2008) Somatic organogenesis and plant regeneration in *Ricinus communis*. *Biol Plant* 52:17–25
- Kushikawa S, Hoshino Y, Mii M (2001) *Agrobacterium*-mediated transformation of *Saintpaulia ionantha* Wendl. *Plant Sci* 161:953–960
- Lu YX, Godo T, Chin DP, Mii M, Guan KY (2006) Establishment of callus culture with high plant regeneration ability from leaf segments of *Lysionotus pauciflorus* Maxim. *Propag Ornament Plants* 6:180–186
- Ma GH, He CX, Ren H, Zhang QM, Li SJ, Zhang XH, Bunn E (2010) Direct somatic embryogenesis and shoot organogenesis from leaf explants of *Primulina tabacum* Hance. *Biol Plant* 54:361–365
- Ma GH, Lü JF, Teixeira da Silva JA, Zhang XH, Zhao JT (2011a) Somatic embryogenesis and shoot organogenesis from leaf and shoot explants of *Ochna integerrima* (Lour). *Plant Cell Tissue Organ Cult* 104:157–162
- Ma GH, Teixeira da Silva JA, Lü JF, Zhang XH, Zhao JT (2011b) Shoot organogenesis and plant regeneration in *Metabriggsia ovalifolia*. *Plant Cell Tissue Organ Cult* 105:355–361
- Mahadatanapuk S, Topoonyanont N, Handa T, Sanguanserm Sri M, Anuntalabhochai S (2006) Genetic transformation of *Curcuma alismatifolia* Gagnep. using retarded shoots. *Plant Biotechnol* 23: 233–237
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15: 473–497
- Murch SJ, Saxena PK (1999) The role of proline in thidiazuron induced somatic embryogenesis of peanut. *In Vitro Cell Dev Biol Plant* 5: 102–105
- Murthy BNS, Murch SJ, Saxena PK (1998) Thidiazuron: a potent regulator of *in vitro* plant morphogenesis. *In Vitro Cell Dev Biol Plant* 34:267–275
- Nakano TH, Sugawara S, Saito T, Watanabe Y, Lu YX, Guan KY, Godo T (2009) Adventitious shoot regeneration and micropropagation of *Chirita flavimaculata* W. T. Wang, *C. eburnea* Hance, and *C. speciosa* Kurz. *Propag Ornament Plants* 9:216–222
- Padmanabhan P, Murch SJ, Sullivan JA, Saxena PK (2014) Development of an efficient protocol for high frequency *in vitro* regeneration of a horticultural plant *Primulina tamiana* (B. L. Burtt) Mich. Möller & A. Webber. *Can J Plant Sci* 94: 1281–1287
- Padmanabhan P, Murch SJ, Sullivan JA, Saxena PK (2015) Micropropagation of *Primulina dryas* (Dunn) Mich. Möller & A. Webber: high frequency regeneration from leaf explants. *Sci Hortic* 192:250–255
- Singh ND, Sahoo L, Sarin NB, Jaiwal PK (2003) The effect of TDZ on organogenesis and somatic embryogenesis in pigeonpea (*Cajanus cajan* L. Mill.). *Plant Sci* 164:341–347
- Takagi H, Sugawara S, Saito T (2011) Plant regeneration via direct and indirect adventitious shoot formation and chromosome-doubled somaclonal variation in *Titanotrichum oldhamii* (Hemsl.). *Solerefer. Plant Biotechnol Rep* 5:187–195
- Wang WC, Pan KY, Li ZY (1990) *Flora of China: Gesneriaceae* (vol. 69). Science Press, Beijing, pp 333–334 (In Chinese)
- Wang YZ, Mao RB, Liu Y, Li JM, Dong Y, Smith JF (2011) Phylogenetic reconstruction of *Chirita* and allies (Gesneriaceae) with taxonomic treatments. *J Syst Evol* 49:50–64
- Wood D (1974) A revision of *Chirita* (Gesneriaceae). *Notes Roy Bot Gard Edinburgh* 33:123–205
- Yang G, Lü JF, Teixeira da Silva JA, Chen HF, Ma GH (2014) Shoot organogenesis from leaf explants of *Dayaoshania cotinifolia* W. T. Wang. *In Vitro Cell Dev Biol Plant* 50:451–457
- Yang XY, Lü JF, Teixeira da Silva JA, Ma GH (2012) Somatic embryogenesis and shoot organogenesis from leaf explants of *Primulina tabacum*. *Plant Cell Tissue Organ Cult* 109:213–221

- Yu HX, Zhang ZJ, Lu HZ, Wei Y, Huang XY (2012) Study on tissue culture of *Chirita swinglei*. Hubei Agric Sci 51:2606–2608 (In Chinese)
- Zhang SJ, Liu N, Sheng AW, Ma GH, Wu GJ (2011) *In vitro* plant regeneration from organogenic callus of *Curcuma kwangsiensis* Lindl. (Zingiberaceae). Plant Growth Regul 64: 141–145
- Zhou LD, Yu JG, Guo J, Yang SL (2001) Compounds from roots of *Chirita fimbrisepala* Hand–Mazz. China J Chinese Mater Med 26: 114–117