Rapid In vitro Multiplication of *Chirita longgangensis* W.T. Wang: An Endemic and Endangered Gesneriaceae Species in China

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Abstract. Experiments were conducted to establish an efficient protocol for micro-propagation of *Chirita longgangensis* W.T. Wang. Somatic embryos formed directly at the cut edges of *C. longgangensis* leaf explants on Murashige and Skoog (MS) medium supplemented with benzylaminopurine (BA) and α -naphthalene acetic acid (NAA). During the somatic embryo induction stage, leaf explants and basal leaf explants were used. Leaves were more appropriate explants than the basal leaf explants. The best medium was modified MS macronutrients and micronutrients supplemented with 0.5 mg·L⁻¹ BA and 0.1 mg·L⁻¹ NAA (the best mean number of somatic embryos per explants was 24.10 $\pm$ 1.63). The second stage was root induction and elongation. In vitro regenerated plantlets rooted best on MS medium containing 0.1 mg·L⁻¹ indole-3-acetic acid (IAA) and 30 g·L⁻¹ sucrose. Rooted plantlets, following acclimatization in a greenhouse, were successfully transferred to field conditions, and 95% of the plants survived. Application of this protocol has the ability for mass multiplication, in a limited time, of the endangered species *C. longgangensis*.

Chirita longgangensis W.T. Wang (Wang, 1990) belongs to the family Gesneriaceae, *Chirita* Buch.-Ham. ex D. Don, and is distributed in Guangxi Province (Tiandeng and Longzhou counties). *C. longgangensis* is an endemic and endangered plant of China. It is listed in the China Red Book of endangered plant species (Song et al., 1998). *C. longgangensis* is an herbaceous evergreen with a very beautiful flower, so it is a very good ornamental house plant. It is also an important medicinal plant in southern China, where it is used in herbal medicine to control injuries from falls and to enrich the blood as a hematinic. *C. longgangensis* usually grows in the shade of the valley or in the crack of limestone cliffs; it has a restricted distribution range and small population size (Li and Wang, 2004). When the central distribution area of a species is destroyed by human activities, remaining portions of the popula-

tion along the marginal areas of the distribution range may not have habitat suitable to maintain the survival of the species. Rare and endangered plants usually have limited reproductive capacities and very slow growth rates; the use of in vitro culture can overcome these difficulties (Malda et al., 1999).

There are some reports of in vitro regeneration of Gesneriaceae using various tissues as explant sources, such as *Santipaulia ionantha* Wendl (Bilkey et al., 1978; Kukulczanka and Suszynska, 1972; Mithila et al., 2003; Start and Cumming, 1976), *Cape primrose* (Peck and Cumming, 1984), and *Streptocarpus nobilis* (Handre, 1983). In these reports, adventitious shoots had been induced on modified MS medium (Murashige and Skoog, 1962) from leaf (Mithila et al., 2003; Start and Cumming, 1976) or cross-sections of petiole (Bilkey et al., 1978; Mithila et al., 2003) and even corolla (Peck and Cumming, 1984). This article describes an efficient protocol for rapid multiplication of *C. longgangensis* through high-frequency somatic embryo induction from leaf and basal leaf explants, followed by successful greenhouse establishment of regenerated plants, and discusses the conditions required for efficient shoot elongation and root induction.

Materials and Methods

Plant material was provided by the In Vitro Plant Germplasm Collection, Type

Culture Conservation Committee, Chinese Academy of Sciences. Plants were maintained by vegetative propagation in the greenhouse at 25 °C. Leaves were excised under the dissecting microscope to avoid taking stem tissues surrounding the petiole (Pascual and Marin, 2005). Each leaf used for tissue culture was washed with detergent and water for 20 min, surface-sterilized in 70% (v/v) ethanol for 15 s, washed 5 min with 0.01% HgCl₂, and then rinsed five times with sterilized distilled water. Leaf explants were separated into basal, medium leaf explants, and then medium leaf explants were sliced into $\approx$ 5 mm $\times$ 5 mm explants. The culture medium used for somatic embryo induction consisted of MS medium, 30 g·L⁻¹ sucrose, and 7 g·L⁻¹ agar. The pH of all media was adjusted to 5.8 before autoclaving at 121 °C for 20 min. Unless mentioned otherwise, all cultures were incubated at 25 °C under 14 h illumination (150 μ m·m⁻²·s⁻¹) with fluorescent lamps.

Selecting the best combination of plant growth regulators. Medium leaf laminae were cut into 5 mm $\times$ 5 mm squares and placed with the abaxial side down on MS medium. Basal leaf explants were placed with the abaxial side down also. Different combinations of cytokinins, such as benzylaminopurine (BA), kinetin (KT), and auxins indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α -naphthalene acetic acid (NAA) were added to the induction medium (for exact combination, see Table 1).

Determining the best concentrations of NAA and BA. Medium leaf laminae and basal leaf explants were placed on modified MS medium as described above. NAA (0, 0.02, 0.1, or 0.5 mg·L⁻¹) and BA (0, 0.02, 0.1, or 0.5 mg·L⁻¹) were added in combination to the medium (for exact concentrations, see Table 2).

Determining the best concentrations of activated charcoal (AC) and IAA. For rooting, shoots $\approx$ 1.5–2 cm long with at least four fully expanded leaves were excised and transferred to 100-mL glasses containing 40 mL of MS or half-strength MS, supplied with AC and IAA. Those treatments were as follows: a) 1 $\times$ MS, 30 g·L⁻¹ sucrose, 0.1 mg·L⁻¹ IAA, 5 g·L⁻¹ AC; b) 0.5 $\times$ MS, 30 g·L⁻¹ sucrose, 0.5 mg·L⁻¹ IAA, 5 g·L⁻¹ AC; c) 1 $\times$ MS, 30 g·L⁻¹ sucrose, 0.1 mg·L⁻¹ IAA; d) 1 $\times$ MS, 30 g·L⁻¹ sucrose, 0.5 mg·L⁻¹ IAA; e) 0.5 $\times$ MS, 30 g·L⁻¹ sucrose, 0.1 mg·L⁻¹ IAA; and f) 0.5 $\times$ MS, 30 g·L⁻¹ sucrose, 0.5 mg·L⁻¹ IAA.

For the above experiments, six explants were cultured in a glass vessel (6.5 cm diameter $\times$ 11 cm high) containing 40 mL of medium. The vessels were sealed with a polypropylene plastic film-sandwiched Millipore membrane.

Measurements. For the somatic embryo induction experiments, the percentage of explants giving rise to somatic embryos and the somatic embryo number per explant were recorded after 6 weeks of the first bud induction. For rooting experiment analysis, parameters were mean number of roots per

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Table 1. Somatic embryo (SE) induction frequency and number of plantlets per explant from medium leaf and basal leaf explants of *C. longgangensis* cultured on MS medium containing different growth regulator combinations.^z

Growth regulators (mg·L ⁻¹)					Medium leaf explants		Basal leaf explants	
BA	KT	NAA	IAA	IBA	SE-forming explants (%)	No. of plantlets per explant	SE-forming explants (%)	No. of plantlets per explant
0.2		0.2			90.33 ± 2.67 a	19.23 ± 2.31 a	88.83 ± 4.17 a	7.77 ± 0.18 a
0.2			0.2		72.12 ± 4.73 ab	6.25 ± 1.72 b	85.71 ± 8.25 a	6.25 ± 0.96 a
0.2				0.2	0.00 ± 0.00 c	0.00 ± 0.00 c	49.01 ± 8.97 b	2.09 ± 0.20 bc
	0.2	0.2			67.59 ± 9.35 b	6.04 ± 0.8 b	76.03 ± 7.12 ab	8.27 ± 0.54 a
		0.2	0.2		51.94 ± 9.33 b	3.68 ± 0.45 bc	94.44 ± 5.56 a	6.13 ± 0.13 a
	0.2			0.2	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c	0.00 ± 0.00 c

^zValues are means ± SE. Different letters indicate statistically different values at the 0.05 level, the bold type signifies the optimal medium.

plantlet, mean length of root, and mean length of the plantlet, which were measured 8 weeks after transfer to the rooting medium.

The rooting criterion was the presence of at least one visible whitish, polar, cylindrical structure ≈2 mm long (Corrêa et al., 2005).

Histological examination. Histological analysis was made with light microscopy to study the origin of somatic embryos. Tissue samples were collected at 5, 7, 9, 13, and 15 d after placing the medium leaf explants on the medium. Explants were fixed in 70% FAA solution (formalin/glacial acetic acid/70% ethyl alcohol, 5:5:90, v/v) for 24 h to confirm them. The samples were dehydrated in a graded ethanol series (70%, 85%, 95%, and 100%) and embedded in paraffin. Sections, 8 µm thick, were cut with a rotary microtome, mounted onto glass slides which were then double-stained with 1% safranin and 0.1% fast green, and observed under a light microscope.

Statistical analysis. Six glass vessels (36 medium leaf explants and 36 basal leaf explants) per treatment were used for somatic embryo induction experiments. Twelve replicates per treatment were used for shoot elongation and root induction experiments. All experiments were repeated at least three times with the same clone. Data were analyzed using SPSS 11.5 software. The mean difference is significant at 0.05.

Results and Discussion

Influence of different plant growth regulators. Six different auxin and cytokinin combinations were tried for regeneration derived from medium leaf and basal leaf

explants. Among the various combinations used, MS medium with NAA and BA proved to be the most effective, but regeneration was dependent on the type of explant. BA was the optimum cytokinin for in vitro propagation of many plants, which has been reported in many experiments (Arunyanart and Chaitrayagun, 2005; Cid and Cruz, 2004; Pascual and Marín, 2005; Prem et al., 2005; Shu et al., 2005; Song and Sink, 2005; Yuan et al., 2005), but many kinds of auxin, such as 2,4-dichlorophenoxyacetic acid (2,4-D) (Arunyanart and Chaitrayagun, 2005; Elhag et al., 2004), IBA (Kim et al., 2004), KT (Stefanello et al., 2005), and NAA (Pascual and Marín, 2005), were used.

According to medium leaf explant experiments, somatic embryo regeneration frequency varied from 51.94% to 90.33%, and the number of embryos per explant varied between 3.68 and 19.23 (Table 1). No somatic embryos formed on MS medium with IBA. According to basal leaf experiments, the number of somatic embryos per explant was not more than 7.77. Clearly, NAA showed a synergistic effect with BA for enhanced induction of somatic embryogenesis from explants compared with other cytokinin–auxin combinations. Similar synergistic triggering effects of NAA and BA have also been recently shown for *Vigna radiata* (Amutha et al., 2003) and *Cyamopsis teragonoloba* (Prem et al., 2005). The influence of the auxin and cytokinin used and their interaction were statistically significant ($P = 0.05$).

MS basal medium supplemented with different concentrations of NAA and BA stimulated somatic embryo induction and development in 6 weeks. Rates of embryo-

genic induction in medium leaf and basal leaf explants were significantly affected by plant growth regulators. The formation of embryos in clusters and their asynchronous development made it difficult to accurately estimate the frequency of embryogenesis. Therefore, the counting of embryos with developed green cotyledonary leaves was done separately (Elhag et al., 2004).

For medium leaf explant induction, tissues of explants on MS media were not obviously enlarged and thickened. First initiation of somatic embryo was visible within ≈13–15 d both at wounded edges and around leaf veins (Fig. 1A). Somatic embryos were green, small, and appeared in clusters. These embryos were loosely attached to the tissue of explants and could develop into plantlets without transfer into new medium (Fig. 1C). The tested concentration of NAA and BA significantly influenced the somatic embryo induction rate, which varied between 18.33% and 96.97%. The number of plantlets per explant varied from 0.71 up to 24.10 after being cultured for 6 weeks. Very few somatic embryos formed on MS medium with NAA or BA only.

For basal leaf segment induction, results were similar to those for medium leaf explants (Fig. 1B). Basal leaf segment showed direct somatic embryogenesis (DSE) without passing through the callus stage. Although the somatic embryo formation frequency was very high, the number of somatic embryos was not more than 16.00. The first somatic embryo induced within 15 d; then more and more somatic embryos induced and developed into plantlets cultured on MS medium containing 0.1 mg·L⁻¹ NAA and 0.5 mg·L⁻¹ BA (Table 2).

Table 2. Somatic embryos (SE) induction frequency and number of plantlet per explant from medium leaf and basal leaf explants of *C. longgangensis* cultured on MS medium containing different concentrations of NAA and BA.^z

Growth regulators (mg·L ⁻¹)		Medium leaf explants		Basal leaf explants	
NAA	BA	SE-forming explants (%)	No. of plantlet per explant	SE-forming explants (%)	No. of plantlet per explant
0	0.02	21.67 ± 1.67 e	3.67 ± 1.20 d	21.67 ± 1.67 fg	2.17 ± 0.17 cdefg
0	0.1	43.33 ± 3.33 bcde	4.67 ± 0.33 dh	35.33 ± 2.33 bcdeg	2.17 ± 0.17 cdefg
0	0.5	24.33 ± 4.33 e	2.00 ± 0.57 efgh	18.33 ± 4.41 fg	1.33 ± 0.33 defg
0.02	0	21.00 ± 6.66 e	2.33 ± 0.33 d	21.67 ± 6.01 fg	2.33 ± 0.33 cdefg
0.1	0	33.33 ± 3.33 e	4.33 ± 0.33 d	40.00 ± 5.78 bcdef	4.50 ± 0.29 b
0.5	0	18.33 ± 4.41 e	3.33 ± 0.33 d	18.33 ± 4.41 fg	1.67 ± 0.33 cdefg
0.02	0.02	70.91 ± 0.90 ab	6.65 ± 0.17 df	74.44 ± 12.62 ab	7.60 ± 1.70 bd
0.02	0.1	68.52 ± 9.26 ac	3.31 ± 0.53 d	63.33 ± 8.81 ad	2.10 ± 0.26 cdefg
0.02	0.5	21.11 ± 5.48 e	0.71 ± 0.24 efgh	73.33 ± 6.67 ac	6.60 ± 0.12 be
0.1	0.02	89.32 ± 5.36 a	17.81 ± 1.98 ab	88.89 ± 5.56 a	11.22 ± 1.53 ab
0.1	0.1	93.94 ± 6.06 a	14.88 ± 4.02 bc	88.89 ± 11.11 a	16.00 ± 3.71 a
0.1	0.5	96.97 ± 3.03 a	24.10 ± 1.63 a	100.00 ± 0.00 a	15.50 ± 2.16 a
0.5	0.02	87.41 ± 2.06 a	7.27 ± 0.54 de	100.00 ± 0.00 a	8.61 ± 0.41 bc
0.5	0.1	80.47 ± 10.58 a	5.77 ± 1.01 dg	61.11 ± 20.03 ae	3.67 ± 1.69 cdefg
0.5	0.5	56.09 ± 4.68 ad	9.57 ± 1.98 cd	100.00 ± 0.00 a	4.60 ± 0.83 bf

^zValues are means ± SE. Different letters indicate statistically different values at the 0.05 level, the bold type signifies the optimal medium.

Analysis of the data for embryogenic frequency in the second experiment revealed significant differences among the treatments. The highest embryogenic frequency was obtained with medium leaf explant cultivar on MS basal medium containing $0.5 \text{ mg}\cdot\text{L}^{-1}$ BA with $0.1 \text{ mg}\cdot\text{L}^{-1}$ NAA, as 96.97% of medium leaf explant with somatic embryogenic induction and 24.10 plantlets per explants were obtained on the medium (Table 2).

Root induction and elongation. Rooting is generally a very slow process in *C. longgangensis*, and after 4 weeks the roots were still rarely longer, $\approx 5 \text{ mm}$. Auxin was observed to influence rooting, and shoot elongation was reported (Gulen et al., 2004; Kadota and Niimi, 2004; Nas and Read, 2004). Our preliminary studies showed that IAA was better than NAA or IBA.

Effects of AC and IAA on rooting of *C. longgangensis* cuttings are summarized in Table 3. As basal medium, MS medium was better than $\frac{1}{2}$ MS medium. IAA induced root regeneration. A brown-colored, stringent exudate also influenced root induction; this exudate was tested, and phenolic compounds were identified. The browning phenomenon of cultured tissue was attributed to oxidized phenolic compounds (Das and Pal, 2005; Huang et al., 2002). With the IAA concentration increased, the brown-colored exudate was increased. AC could adsorb some phenolic compounds (Fig. 1E), but AC could adsorb IAA at the same time. For this experiment, the optimal rooting medium was MS basal medium, $0.1 \text{ mg}\cdot\text{L}^{-1}$ IAA, and $30 \text{ g}\cdot\text{L}^{-1}$ sucrose (Table 3; Fig. 1D).

Histological observation. Somatic embryogenesis is the development from the somatic cell, through an orderly series of characteristic morphological stages, structures that resemble zygotic embryos (Emons, 1994). This in vitro morphogenetic pattern is defined as a process in which a bipolar structure resembling a zygotic embryo develops from a nonzygotic cell without vascular connection with the original tissue (Arnold et al., 2002).

We could observe the successive stages of somatic embryo development from leaf laminae explants. Somatic embryos were observed in explants after being cultured on medium containing $0.1 \text{ mg}\cdot\text{L}^{-1}$ NAA and $0.5 \text{ mg}\cdot\text{L}^{-1}$ BA. Usually, embryos formed on the surface of the leaves and near cutting margins.

Longitudinal sections of the tissue revealed its component with epidermis, sub-epidermis, and a large number of parenchyma cells. Leaf explants seldom enlarged and thickened at the cutting margin (Fig. 2B). At the subepidermis, some cells displayed the typical traits of a spongy parenchymatous callus after 5 d of culturing on the MS medium with $0.1 \text{ mg}\cdot\text{L}^{-1}$ IAA and $0.5 \text{ mg}\cdot\text{L}^{-1}$ BA (Fig. 2A). Proembryos were observed after 7 d of culturing (Fig. 2B) and further developed into globular somatic embryos after 9–13 d of culturing (Fig. 2C). Globular somatic embryos developed into heart-shaped somatic embryos and then underwent further development to form mature, cotyledon-

Fig. 1. Induction of somatic embryos, development, and plantlet establishment from medium leaf and basal leaf explants. (A) Plantlets developed from medium leaf explants on MS medium containing $0.5 \text{ mg}\cdot\text{L}^{-1}$ BA with $0.1 \text{ mg}\cdot\text{L}^{-1}$ NAA after 6 weeks of culture (bar = 2.1 mm). (B) Plantlets developed from basal leaf explants on MS medium containing $0.5 \text{ mg}\cdot\text{L}^{-1}$ BA with $0.1 \text{ mg}\cdot\text{L}^{-1}$ NAA after 6 weeks of culture (bar = 2.3 mm). (C) Plantlets clustered after 8 weeks of culture (bar = 11.8 mm). (D) Rooting of in vitro regenerated plantlets cultured on MS basal medium containing $0.1 \text{ mg}\cdot\text{L}^{-1}$ IAA and $30 \text{ g}\cdot\text{L}^{-1}$ sucrose after 8 weeks (bar = 2.4 cm). (E) Root induction and elongation on MS basal medium containing $0.1 \text{ mg}\cdot\text{L}^{-1}$ IAA, $30 \text{ g}\cdot\text{L}^{-1}$ sucrose, and $5 \text{ g}\cdot\text{L}^{-1}$ AC after 8 weeks of culture (bar = 1.3 cm). (F) Plantlets transferred to pots containing sterile sand (bar = 1.5 cm). (G) Plants acclimatized in greenhouse (bar = 4 cm).

Table 3. Effect of AC and IAA on in vitro root induction and shoot elongation medium, determined after 8 weeks of culture on MS or $\frac{1}{2}$ MS medium.^a

Treatment	No. of roots per cutting	Mean root length (mm)	Mean plantlet length (mm)
MS/5 g Ac/0.1 mg·L ⁻¹ IAA	19.83 ± 1.11 b	8.33 ± 0.43 a	23.58 ± 1.47 a
$\frac{1}{2}$ MS/5 g Ac/0.5 mg·L ⁻¹ IAA	8.42 ± 0.48 c	5.08 ± 0.23 b	16.08 ± 0.51 b
MS/0.1 mg·L⁻¹ IAA	24.75 ± 1.20 a	9.00 ± 2.04 a	25.75 ± 1.68 a
MS/0.5 mg·L ⁻¹ IAA	21.00 ± 0.83 ab	5.75 ± 0.28 b	21.08 ± 1.06 a
$\frac{1}{2}$ MS/0.1 mg·L ⁻¹ AA	19.17 ± 1.55 b	4.50 ± 0.40 bc	24.42 ± 1.20 a
$\frac{1}{2}$ MS/0.5 mg·L ⁻¹ IAA	17.33 ± 1.40 b	3.17 ± 0.17 c	16.00 ± 0.67 b

^aValues are means ± SE. Different letters indicate statistically different values at the 0.05 level, the bold type signifies the optimal medium.

stage somatic embryos that had a well-defined epidermis, distinct growth centers, and a pair of cotyledons (Fig. 2D) after 15 d of culturing. Subsequently, adventitious buds formed, and plantlets were obtained ≈ 4 weeks later (Fig. 2E).

In this study, the bipolar embryogenic structures could be observed, and there were

no vascular connections with the original tissue.

Proliferation procedure. On the basis of this investigation, a tissue culture and rapid propagation procedure for *C. longgangensis* is proposed via direct somatic embryos induction. In this system, ≈ 8 weeks of culture on MS medium containing $0.5 \text{ mg}\cdot\text{L}^{-1}$

Fig. 2. Light micrographs showing evidence for somatic embryogenesis in leaf explants of *C. longgangensis* at different developmental stages when cultured on MS medium containing $0.5 \text{ mg} \cdot \text{L}^{-1}$ BA with $0.1 \text{ mg} \cdot \text{L}^{-1}$ NAA. (A) Cell division to form meristematic zone (arrow) in subepidermis after 1 week of culture (bar = $50 \text{ } \mu\text{m}$). (B) Proembryo consisting of a ball of actively dividing cells; arrows show distinct epidermis and compact cells (bar = 0.33 mm). (C) Globular-stage somatic embryos consisting of a mass of compactly arranged actively dividing cells surrounded by a well-defined epidermis (arrow, bar = $50 \text{ } \mu\text{m}$). (D) Cotyledon-stage somatic embryo; arrows show a pair of cotyledons (bar = 0.1 mm). (E) Well-developed plantlets formed on the margin of leaf sections after 4 weeks culture (bar = 2 mm).

BA with $0.1 \text{ mg} \cdot \text{L}^{-1}$ NAA was needed to induce somatic embryos from leaf lamina of adult plants of *C. longgangensis*. Plantlets with at least two pairs of well-developed leaflet can be separated from each other and placed on MS medium containing $0.1 \text{ mg} \cdot \text{L}^{-1}$ IAA and $30 \text{ g} \cdot \text{L}^{-1}$ sucrose for rooting. This step took ≈ 8 weeks. After rooting, regenerated plantlets were washed carefully and transferred to pots containing sterile sand (Fig. 1F). The potted plants were covered with glasses to ensure high humidity. The glasses were opened after 2 weeks to acclimatize the plants to field conditions. After 4 weeks, surviving plants were transferred to pots containing garden soil and maintained in a greenhouse (Fig. 1G). The overall process takes ≈ 22 weeks.

This is the first report of a successful micropropagation and whole-plant regeneration system for *C. longgangensis* via direct somatic embryo induction. In addition, the protocols reported here provide a basis for Gesneriaceae systematic research and the establishment of a genetic transformation system for *C. longgangensis*.

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