

CALLUS INDUCTION AND ADVENTITIOUS BUD DIFFERENTIATION OF *CYCLOCODON LANCIFOLIUS* (ROXB.) KURZ

INDUCCIÓN DE CALLOS Y DIFERENCIACIÓN ADVENTICIA DE YEMAS DE *CYCLOCODON LANCIFOLIUS* (ROXB.) KURZ

YIN-KAI XI^{1,2}, YE WANG^{1,3}, BIAO ZENG⁴, HENG-YU HUANG^{1,5*}, WU-DE YANG²

¹Yunnan Breeding and Cultivation Research and Development Center of Endangered and Daodi Chinese Medicinal Materials, Yunnan University of Chinese Medicine, Kunming, China

²Chemistry department, Guizhou University of Traditional Chinese Medicine, Guiyang, China

³Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China

⁴Yunnan Academy of Agricultural Sciences, Kunming, China

⁵Qiucheng Breeding Company Limited, Lijiang city, China

* Author for correspondence: hyhy96@163.com

Abstract

Background: *Cyclocodon lancifolius* (Roxb.) Kurz is a perennial medicinal and edible plant with a huge potential economic value. The wild resources of this plant are gradually scarce by the serious destruction of the habitat and the limitations of sexual reproduction. This is the first attempt to establish an *in vitro* reproductive system for the species.

Hypothesis: The suitable plant regulator types and its mass concentration range, combined with the explants, can induce the development of plants at various stages. We expected to establish an *in vitro* regeneration system of *C. lancifolius* based on these factors.

Species studied: *Cyclocodon lancifolius*

Study site and years of study: Yunnan Breeding and Cultivation Research and Development Center of Endangered and Daodi Chinese Medicinal Materials, Yunnan University of Chinese Medicine, from 2017 to 2019.

Methods: The plant regeneration of *C. lancifolius* was established by single factor, L_9 (3^4) orthogonal and complete combination experiments.

Results: Stem segments were the best explants for callus induction on MS medium containing $0.05 \text{ mg} \cdot \text{L}^{-1}$ KT, $0.5 \text{ mg} \cdot \text{L}^{-1}$ 6-BA and $0.5 \text{ mg} \cdot \text{L}^{-1}$ NAA. MS medium with $0.2 \text{ mg} \cdot \text{L}^{-1}$ TDZ and MS medium were used alternately as the culture method to conduct differentiation and proliferation of adventitious shoot. The optimal protocol for the rooting was MS medium combined with $0.1 \text{ mg} \cdot \text{L}^{-1}$ 6-BA and $1.0 \text{ mg} \cdot \text{L}^{-1}$ NAA.

Conclusions: A rapid propagation system of *C. lancifolius* was established which provided a possible solution for the protection of wild resources and artificial cultivation.

Key words: Adventitious buds, callus, orthogonal experiments, tissue culture, stem segments with nodes.

Resumen

Antecedentes: *Cyclocodon lancifolius* es una planta perenne medicinal y comestible. Los recursos silvestres de esta planta escasean gradualmente debido a la grave destrucción del hábitat y las limitaciones de la reproducción. Este es el primer intento de establecer un sistema reproductivo *in vitro* para esta especie.

Hipótesis: El tipo de regulador de crecimiento adecuado y su rango de concentración másica, combinado con los explantes, pueden inducir el crecimiento de plantas en varias etapas. Se espera establecer un sistema de regeneración *in vitro* de *C. lancifolius* basado en estos factores.

Especie estudiadas: *Cyclocodon lancifolius*.

Sitio y años del estudio: Universidad de Medicina China de Yunnan, De 2017 a 2019.

Métodos: La regeneración de plantas de *C. lancifolius* se estableció mediante los experimentos de un solo factor, ortogonales de tipo L_9 (3^4) y los de combinación completa.

Resultados: Los segmentos del tallo fueron los mejores explantes para la inducción de callos en un medio MS que contenía $0.05 \text{ mg} \cdot \text{L}^{-1}$ KT, $0.5 \text{ mg} \cdot \text{L}^{-1}$ 6-BA y $0.5 \text{ mg} \cdot \text{L}^{-1}$ NAA. El medio MS + $0.2 \text{ mg} \cdot \text{L}^{-1}$ TDZ se usó de manera alternativa con el medio MS como el método de cultivo para realizar la diferenciación y proliferación de brotes adventicios. El protocolo óptimo para el enraizamiento fue el medio MS combinado con $0.1 \text{ mg} \cdot \text{L}^{-1}$ 6-BA y $1.0 \text{ mg} \cdot \text{L}^{-1}$ NAA.

Conclusiones: Se estableció un sistema de propagación rápida de *C. lancifolius* que proporcionó una posible solución para la protección de los recursos silvestres, así como para el cultivo artificial.

Palabras clave: Callo, cultivo de tejidos, experimentos ortogonales, segmentos de tallo con nudos, yemas adventicias.



In recent years, medicinal and edible plants are increasingly used for the industrial production of pharmaceutics and nutraceuticals in the healthcare sector. The viewpoint of “medicine food homology” conforms to today’s food requirements of returning to a natural and green healthy life (Hou & Jiang 2013). In addition to their abundant nutritional value, these plants also have other health care functions such as preventing and treating diseases. *Cyclocodon lancifolius* (Roxb.) Kurz is a medicinal and edible plant with potential development prospective. The plant is a perennial plant belonging to *Cyclocodon* genus in Campanulaceae family, and it is known as Zhi Zhu Guo, Rou Suan Pan or Ye Ling Guo in Chinese phonetic writing (Editorial Board of State Administration of Traditional Chinese Medicine 1999). It grows in grassy slopes, gullies or forests regions ascending up to an altitude of 300~1,800 m, mainly distributed in some countries of Southeast Asia, especially the southern region of the Yangtze River in China (Institute of Chinese Academy of Sciences 2016).

The root of *C. lancifolius* is used as a traditional Chinese medicine with the function of treating traumatism, fatigue, intestinal colic, etc. Its young shoot tip is a delicious vegetable in daily life. Modern researches have validated that leaves were rich in flavonoids (Chen et al. 2014a), which have the effects of preventing and treating neuropathic diseases, delaying aging, strengthening the protection of DNA, treating coronary heart disease, anti-cancer and anti-virus (Dean 2003). The fruit of this plant is also popular and is sold in some supermarkets in various forms, such as fresh fruit, dried fruit or drinks made from it. It was reported that the fruit contained polysaccharide, nature fiber, crude protein, crude fat, mineral element and beneficial component, especially polysaccharide, whose content is up to 45.80 %, which is beneficial for eliminating hydroxyl radical and superoxide free radical. Meanwhile, the polysaccharide can suppress oxidation of grease obviously (Chen et al. 2013, Chen et al. 2014b).

However, there are many problems in the resources and propagation of *C. lancifolius*. First, the wild resources are gradually scarce due to the serious destruction of the habitat (Yi et al. 2016). Second, this species can be harvested after planted in the same year, and the local government encourages the farmer to plant the species in order to develop the economy and increase the income of local people. Thus, a large number of seedlings are needed. Furthermore, *C. lancifolius* is propagated mainly via seeds and cuttings. The field sowing has some problems such as large segregation of character, poor resistance and high mortality. Similarly, cutting also has many problems of growth diseases, long cycle and low reproduction coefficient. Tissue culture is not restricted by land, seasonal and climatic factors, with a fast growth speed, which is beneficial for facilitating intensive management and factory

production. Therefore, there needs an urgent protocol to resolve the problem mentioned above by an optimized method of tissue culture to protect wild resources and improve reproduction efficiency of *C. lancifolius*.

Until now, the literature about *C. lancifolius* mainly concentrated on chemical components, extraction technology and nutrition research (Chen et al. 2013, Chen et al. 2014a, b). Studies on propagation and tissue culture have not been reported. In this study, the stem segments with nodes and leaves were used as explants for rapid propagation *in vitro*. The well-established regeneration system provided a possible way for protecting the wild resources and seedling propagation of *C. lancifolius*. The protocol also laid a foundation for the genetic transformation of the plant.

Materials and methods

Plant materials. Twenty *C. lancifolius* samples were collected from the Jishou City of Xiangxi Tujia and Miao Autonomous Prefecture in Hunan Province of China. After collection, fifteen plants were transplanted in the greenhouse of the Yunnan University of Chinese Medicine where these plants were cultivated a year. Then, stems with one or two nodes and leaves from healthy individuals were chosen as explants for propagation *in vitro*. The stems with one or two nodes and leaves were immersed into 5 % (w/v) mild detergent for 5 min, washed under running tap water for 30 min before shifted on a sterile operating platform. Furthermore, these materials were immersed into 75 % (v/v) ethyl alcohol for 10 s and surface sterilized using 0.1 % $HgCl_2$ (w/v) for 8 min (stem segments with nodes) and 3 min (leaves) successively and followed by treatment with sterile distilled water eight times that was more than 3 min every time.

Single factor experiment. The sterilized leaves and stems with one or two nodes were cultured in the MS medium containing different type and concentration plant growth regulators (PGRs) which included 2,4-dichlorophenoxy-acetic acid (2, 4-D) (0.05, 0.1, 0.5 and 1.0 $mg \cdot L^{-1}$), 6-benzylaminopurine (6-BA) (0.1, 0.5, 1.0 and 2.0 $mg \cdot L^{-1}$), kinetin (KT) (0.1, 0.5, 1.0 and 2.0 $mg \cdot L^{-1}$) and α -naphthaleneacetic acid (NAA) (0.1, 0.5, 1.0 and 2.0 $mg \cdot L^{-1}$). Every group of single factor experiment had 5 culture bottles with 2 leaves or stems. The growth conditions were recorded and the types and concentration ranges of PGRs suitable for the growth of *C. lancifolius* were selected.

Callus induction and regeneration. Based on the results of chapter “Single factor experiment”, $L_9 (3^4)$ orthogonal experiment was design via KT (A: 0.01, 0.05 and

0.1 mg·L⁻¹), 6-BA (B: 0.5, 1.0 and 1.5 mg·L⁻¹) and NAA (C: 0.1, 0.5 and 1.0 mg·L⁻¹) as factors. Some representative groups were selected from the comprehensive test and the results equivalent to the comprehensive tests were achieved with the minimum number tests (Table 1). Every group of callus induction and regeneration contained 5 culture bottles with 2 explants (aseptic leaves or stems obtained from single factor experiment). The rate of callus induction was calculated after 30 days, and the callus was evaluated by its color and texture.

Embryonic callus induction and bud differentiation. Although both explants (leaves and stems with one or two nodes) could induce callus in the L₉ (3⁴) orthogonal experiment, the proliferation coefficient of cluster buds failed to obtain satisfactory results in the subsequent experiment. Therefore, the MS medium containing different concentration TDZ (0.05, 0.1, 0.2 and 0.3 mg·L⁻¹) were used for inducting the embryonic callus. Every group of embryonic callus induction and bud differentiation contained 6 culture bottles with 5 explants (1×1 cm callus induced from stems with one or two nodes). The incidence of embryonic callus was calculated after 30 days.

Rooting and transplantation of the plantlets. In light of the results of PGRs selection, 3-4 cm individual bud excised from adventitious buds was cultured on the MS medium with the complete combination of NAA (0.5, 1.0 and 1.5 mg·L⁻¹) and 6-BA (0.05, 0.1 and 0.5 mg·L⁻¹). Every group of the rooting culture contained 6 culture bottles with 5 explants (3-4 cm individual bud excised from adventitious buds). The rooting rate was determined after 30 days.

The rooting plantlets of 5-6 cm long were removed from the culture room to adapt to the environment for 3 days and the agar on the roots was gently cleaned with water. Then, they were transplanted into peat matrix and controlled the temperature at 20-25 °C and 75-95 % humidity. The survival rate was determined after 30 days.

Growth condition. All the media in the experiment contained 3 % sucrose and 0.47 % agar, and all the experiments were repeated three times.

The cultivation temperature was maintained between 21 and 23 °C and 12 h photoperiod with 40-60 μmol m⁻² s⁻¹ irradiance provided by cool-white fluorescence tubes.

Table 1. Results of callus induction from stem segments with nodes of *Cyclocodon lancifolius* (Roxb.) Kurz by L₉ (3⁴) orthogonal test.

NO.	A	B	C	D Rate of callus induction *	
	KT (mg·L ⁻¹)	6-BA (mg·L ⁻¹)	NAA (mg·L ⁻¹)	(Blank)	(%)
C01	0.01	0.5	0.1	1	86.67
C02	0.01	1.0	0.5	2	76.67
C03	0.01	1.5	1.0	3	63.33
C04	0.05	0.5	0.5	3	100.00
C05	0.05	1.0	1.0	1	86.67
C06	0.05	1.5	0.1	2	70.00
C07	0.1	0.5	1.0	2	90.00
C08	0.1	1.0	0.1	3	76.67
C09	0.1	1.5	0.5	1	70.00
K₁	75.56	92.22	77.78	81.11	
K₂	85.56	80.00	82.22	78.89	
K₃	78.89	67.78	80.00	80.00	
R	10.00	24.44	4.44	2.22	

Note: NO.: The group number of the experiment; *: all callus with tight texture as showed in Figure 2A; K: the mean value; R: range.

Statistical analysis. The calculation method of the data in each stage was as follows:

Rate of callus induction (%) = (the number of explants that produced callus / the number of explants) $\times 100\%$. Occurrence rate of adventitious shoot (%) = (the number of callus that produced adventitious buds / the number of callus inoculations) $\times 100\%$. Callus proliferation coefficient = the number of callus in subculture / the number of initial inoculations.

Cluster bud proliferation coefficient = the number of cluster buds in subculture / the number of initial inoculations. The rooting rate (%) = (the number of rooted seedlings / the number of seedlings inoculations) $\times 100\%$. The survival rate (%) = (the number of survival seedlings / the number of transplantations) $\times 100\%$

The results obtained from every section were subjected to analysis of variance (ANOVA) using SPSS software (IBM Corp, Armonk, USA). The minor difference among every treatment method was determined using Least Significant Differences Test at 5 % probability ($P \leq 0.05$), the mean values were further separated using analysis of range.

Results

Single factor experiment. In the MS medium containing 6-BA or 2, 4-D, all explants (stem segments with nodes and leaves) could induce callus, but most of the texture was loose and fragile. Among them, the most excellent callus was observed in MS medium with 0.5 mg·L⁻¹ 2, 4-D

(Figure 1A-B), whereas in concentration of 2, 4-D higher than 0.5 mg·L⁻¹, all the explants died. Besides, in the 6-BA groups, axillary buds began to germinate after 12 days (Figure 1C), and then some of them were continuously elongated (Figure 1D). Adventitious buds from the leaves occasionally differentiated and the probability of recurrence was very low (Figure 1E-F). Regarding to the KT groups, no callus was found on the stem segments with nodes, while the germinated axillary buds from the nodes showed vitrification (Figure 1G). The redifferentiation time of callus from leaves was long. Two kinds of explants in NAA treatment group had no callus, among which adventitious roots were formed in the nodes. Especially, in the MS medium with 1.0 mg·L⁻¹ NAA, the rooting rate reached 36.67 %, but the roots were thin and easily broken (Figure 1H).

Callus induction. According to the results of a single factor experiment, 6-BA, NAA and KT were selected as the factors of optimization experiments of callus induction to design L_9 (3^4) orthogonal experiment. Two types of explants were cultured after 30 days, the callus induction results and Range (R) analysis were shown in Table 1, and the Variance analysis was shown in Table 2 and 3.

As could be seen from Table 1, R of different PGRs sorted in descending order was 6-BA (24.44) > KT (10.00) > NAA (4.44) > Blank (2.22). It was indicated that 3 kinds of hormone contributed to callus induction equally

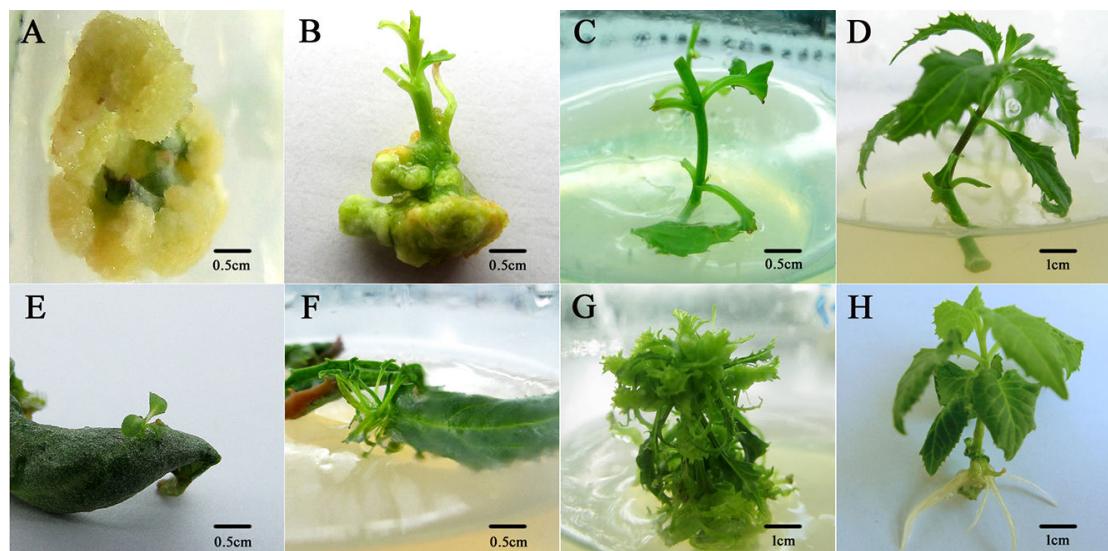


Figure 1. Results of single factor experiment of *Cyclocodon lancifolius*. A. The induced callus from leaves in the MS medium containing 0.5 mg·L⁻¹ 2, 4-D. B. The induced callus from stem segments with nodes in the MS medium with 0.5 mg·L⁻¹ 2, 4-D. C. The germinated axillary buds in 6-BA group. D. The elongated axillary buds in the MS medium with 6-BA. E-F. The differentiated adventitious buds from leaves in 6-BA group. G. The vitrified seedlings from stem segments with nodes by KT treatments. H. Adventitious roots from stem segments with nodes by NAA treatments.

Table 2. Variance analysis of callus induction in *Cyclocodon lancifolius*.

Sources of variance	Type III sum of squares	DOF	Mean square	F value	Significance
A (KT)	155.556	2	77.778	20.979	$P < 0.05$
B (6-BA)	896.456	2	448.230	120.901	$P < 0.01$
C (NAA)	29.615	2	14.807	3.994	$P > 0.05$
D (Blank)	7.415	2	3.707		

Note: DOF: degree of freedom.

with reliable ability. Based on the results of variance analysis in **Table 2**, B (6-BA) had an extremely significant effect to callus induction ($P < 0.01$), A (KT) also had a significant effect ($P < 0.05$), while C (NAA) had no significant effect ($P > 0.05$). According to Duncan test in **Table 3**, the level 1 (0.5 mg·L⁻¹) of 6-BA had the vast influence on callus induction, which was significantly different with the level 3 (1.5 mg·L⁻¹). Moreover, level 2 of KT (0.05 mg·L⁻¹) had obviously effect on callus induction, which was significantly different with level 1 (0.01 mg·L⁻¹). By the mean value (*K*) analysis, the optimal hormone combination for callus induction was A2B1C2 (C04: MS + 0.05 mg·L⁻¹ KT + 0.5 mg·L⁻¹ 6-BA + 0.5 mg·L⁻¹ NAA) with 100 % callus induction rate. The statistical standard of **Table 1** was the tight texture callus (**Figure 2A**).

Stem segments with nodes were cultured in the medium of C04 group, axillary buds showed signs of germination after 8 days and then the base formed green callus with tight texture after 15 days (**Figure 2B**). After 30 days, the expanded callus at the base was removed from stem segments with nodes and divided into 1cm×1cm for transfer to the fresh medium (**Figure 2C**). After 10 days of subculture, although the divided callus surface occasionally had bud differentiation, the growth of buds was not normal (the bud was small, thin and vitrification). Meanwhile, the callus gradually became loose and lost green and finally withered (**Figure 2D**). When the callus were cultured in the subsequent experiment of adventitious bud differentiation, the differentiation rate was very low and the growth was slow even after changing the combination of different hormone types and concentrations. Therefore, we considered the induction of embryonic callus with TDZ of high cytokinin activity. In addition, we also found that in the callus induction process, the induced callus from leaves was looser than that from stem segments with nodes and was not suitable for adventitious bud differentiation.

Differentiation and proliferation of adventitious shoot. Tight callus from stem segments with nodes was transferred into the MS medium containing different concentrations of TDZ. Surprisingly, there was not embryogenic callus and signs of somatic embryogenesis in all experimental groups. On the contrary, a large number of adventitious buds differentiated with callus proliferation (**Table 4**). Proliferation coefficient of callus and adventitious shoot increased with increasing the concentration of exogenous hormone TDZ in a certain range. When the concentration was 0.2 mg·L⁻¹, proliferation coefficient of callus and adventitious shoot reached the highest, which was 4.0 and 12.0 respectively with 100 % the occurrence rate of adventitious shoot. When the TDZ concentration was more than 0.2 mg·L⁻¹, the proliferation coefficient decreased rapidly, which indicated that the lower concentration of TDZ was beneficial for the callus proliferation and adventitious shoot differentiation (**Table 4**, **Figure 3**).

The formed callus in C04 medium were cultured after 30 days and then transferred into the MS medium containing 0.2 mg·L⁻¹ TDZ for callus proliferation and adventitious buds differentiation. After 10 days of culture, as the axillary buds on the nodes grow rapidly, callus was also continuously proliferating and differentiating a large number of conical buds on the surface (**Figure 4A**). Next, these conical buds gradually formed adventitious bud clusters after 20 days (**Figure 4B**). After 25 days, the growth of the main buds was accompanied by the differentiation and growth of cluster buds at the base (**Figure 4C**). Finally, the proliferation coefficient of adventitious cluster buds reached more than 12.0 after 30 days (**Figure 4D**). However, in the subsequent subculture, it was found that the callus proliferation was slow, the base gradually turned yellow and started to lose the redifferentiation ability (**Figure 4E**), accompanied by a sharp decline in the proliferation

Table 3. Duncan's test of 6-BA and KT in callus induction of *Cyclocodon lancifolius*.

Factors	Level	Mean value	0.05 level	Factors	Level	Mean value	0.01 level
KT	0.05	85.56	a	6-BA	0.5	92.22	A
	0.1	78.89	ab		1.0	80.00	AB
	0.01	75.56	b		1.5	67.78	B

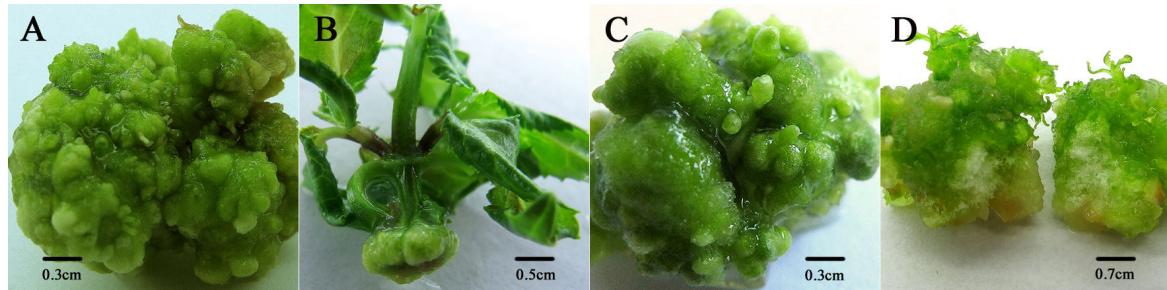


Figure 2. Results of callus induction of stem segments with nodes in *Cyclocodon lancifolius* by C04 test. A. The tight callus. B. The formed callus at the base. C. The expanded callus. D. The callus after subculture.

coefficient of adventitious shoot. After repeated experiments, the seeding from the MS medium with $0.2 \text{ mg}\cdot\text{L}^{-1}$ TDZ were transferred into blank MS medium, the proliferation coefficient of callus and clusters buds did not change and the growth of cluster bud was well-grown (Figure 4F). Thus, MS medium with $0.2 \text{ mg}\cdot\text{L}^{-1}$ TDZ and MS medium were used alternately as the culture method for the differentiation and proliferation of adventitious shoot in this study.

Rooting culture. Main seedlings (3-4 cm) from the MS medium with $0.2 \text{ mg}\cdot\text{L}^{-1}$ TDZ and blank MS medium were excised for complete combination experiment. As can be seen from Table 5, the rooting rate of adventitious shoots from different differentiation medium was significantly different. The differentiated adventitious shoots by blank MS medium had higher rooting rate. When $1.0 \text{ mg}\cdot\text{L}^{-1}$ NAA and $0.1 \text{ mg}\cdot\text{L}^{-1}$ 6-BA were added to the MS medium, the rooting rate reached 100 %. In these medium, adventitious roots were differentiated from the base of stem segment after 15 days (Figure 5A-B) and the seedlings grew healthily and had developed roots system after 30 days (Figure 5C-D). In contrast, differentiated adventitious seedlings directly from TDZ not only had a lower rooting rate, but also produced a small number of calluses, from which adventitious roots were formed.

The seedlings with 5-6 cm high and 3-4 cm root were transplanted into the outdoor with natural light for 7 days.

Then culture remnants on the root were carefully washed and transplant it into peat soil matrix, and the survival rate was 95 % after 30 days (Temperature: 20-25 °C, Humidity: 75 - 95 %) (Figure 5E-F).

Discussion

In general, perennial herbs with strong woody stems mostly achieve the purpose of proliferation through axillary bud germination. In these species, although the explants have strong dedifferentiation ability, their redifferentiation ability is very weak (Cao *et al.* 2017, Wang *et al.* 2018). Hence, the method of direct organogenesis was considered in this experiment. However, in the single factor experiment, axillary buds rarely germinated from stem segments with nodes and were vitrified (Figure 1C and G). As reported by Frank *et al.* (1995), the vitrified shoots have a high activity of superoxide dismutase which leads to hydrogen peroxide accumulation. Meanwhile, the occurrence rate of adventitious bud was still very low after many combination experiments. On the other hand, the somatic embryo is one of the most ideal and commonly used receptors in genetic engineering, because of the strong regeneration ability. The somatic embryo is also a good way of plant regeneration. At present, many plants have been reported to induce somatic embryos *in vitro* with different explants (Choffe *et al.* 2000, Tanaka *et al.* 2000, Sagare *et al.* 2000). Therefore, the method of inducing embryonic

Table 4. Effect of TDZ on adventitious shoot differentiation of *Cyclocodon lancifolius*.

Hormone	Concentration ($\text{mg}\cdot\text{L}^{-1}$)	Occurrence rate of adventitious shoot (%)
TDZ	0.05	86.67 ± 2.0 b
	0.1	90.0 ± 0.9 ab
	0.2	100 ± 0.0 a
	0.3	80.00 ± 1.5 b

Note: Data are mean \pm SE; different lowercase letters in the same column mean significant difference ($P < 0.05$)

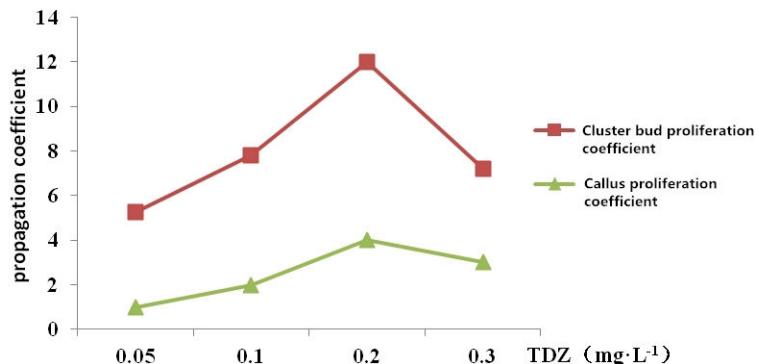


Figure 3. Effects of TDZ on callus and adventitious shoots proliferation in *Cyclocodon lancifolius*.

callus was considered in this study to establish a plant regeneration system of *C. lancifolius*. Interestingly, when the callus was inoculated on MS medium with TDZ, no embryonic callus was observed, only a large number of adventitious buds were differentiated. This suggested that TDZ played a decisive role in the redifferentiation of herbs with woody stems in addition to its obvious effect in inducing embryonic callus, which has also been reported in *Vaccinium vitis-idaea* L. (Debnath 2005).

The dedifferentiation ability of different tissues and organs of the Campanulaceae plants is different, so the choice of explant is also different. Similarly, there were differences in the calluses between leaves and stem

segments with nodes. According to Peng et al. (2010) and Tang et al. (2010), the induced callus from stem segments of *Codonopsis lanceolata* (Sieb. et Zucc.) Trautv. did not grow as well as that from leaves. On the contrary, as reported by Liu (2004), stem segments of *Platycodon grandiflorus* (Jacq.) A. DC. were more beneficial to callus induction, and its texture was more compact than induced callus from the leaves, which was like the results of our study. At the same culture conditions, the induced calluses from various explant not only have differences in morphology (callus from stem segments with nodes had a compact texture and bright green color, while callus from leaf was loose and yellow-green) but also have significant

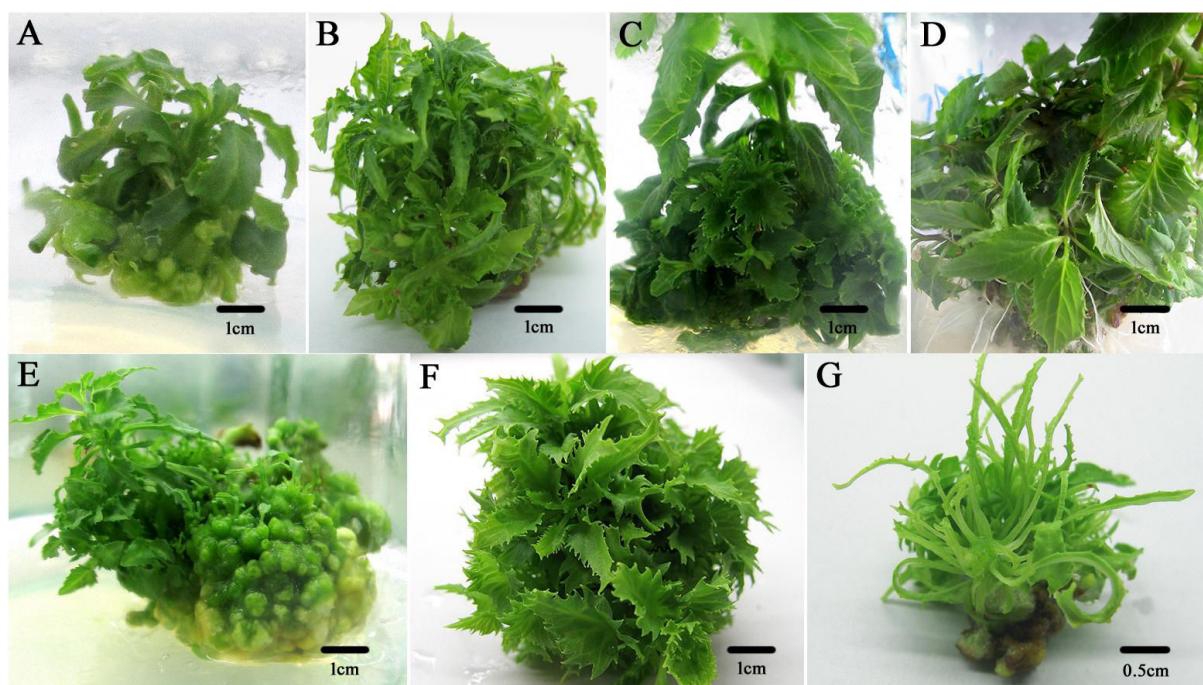


Figure 4. Callus proliferation and adventitious buds differentiation in *Cyclocodon lancifolius*. A. The conical bud clusters in the MS medium with $0.2 \text{ mg}\cdot\text{L}^{-1}$ TDZ. B. Adventitious buds cluster. C. The main buds and cluster buds of the base. D. Cluster buds after 30 days. E. Callus began to turn yellow in the subculture. F. The well-grown cluster bud in the blank MS medium. G. The malformed adventitious bud.

Table 5. Effect of different combination between 6-BA and NAA in root differentiation of *Cyclocodon lancifolius*.

NO.	Hormone combination		Rooting rate of the differentiated seedlings from blank MS (%)	Rooting rate of the differentiated seedlings from TDZ (%)
	NAA (mg·L ⁻¹)	6-BA (mg·L ⁻¹)		
R01	0.0	0.00	0	0
R02	0.5	0.05	76.67 ± 4.0 c	51.11 ± 7.2 de
R03	0.5	0.1	83.33 ± 2.9 b	60.00 ± 7.2 ab
R04	0.5	0.5	80.00 ± 3.3 bc	53.33 ± 7.6 bd
R05	1.0	0.05	93.33 ± 1.7 a	66.67 ± 5.8 a
R06	1.0	0.1	100 ± 0.0 a	67.78 ± 5.8 a
R07	1.0	0.5	86.67 ± 1.7 b	63.34 ± 6.0 ab
R08	1.5	0.05	70.00 ± 4.4 cd	44.42 ± 7.8 e
R09	1.5	0.1	66.67 ± 4.7 d	38.96 ± 9.3 f
R10	1.5	0.5	70.00 ± 3.8 cd	47.78 ± 8.8 de

Note: Data are mean ± SE; different lowercase letters in the same column mean significant difference ($P < 0.05$).

differences in redifferentiation ability. In this study, the incidence of adventitious buds from stem segments with nodes was higher than that from the leaves. Whereas, the induced callus from leaves produced adventitious buds belong to the accidental phenomenon. It was speculated that the morphology and texture of callus had some effect on the differentiation of adventitious buds. The indirect effect of the selection of explants on the differentiation efficiency of adventitious buds was further explained (Dayal *et al.* 2003), so stem segments with nodes were the optimal explant for rapid propagation of *C. lancifolius* *in vitro*.

For studies on indirect organogenesis using leaves and veins as materials, some researchers believe that induced callus from leaves originated from two types of cells, mesophyll cells, phloem parenchyma in small vascular bundles and bundle sheath cell, whereas induced callus from veins are derived from vascular bundles, sclerenchyma and parenchyma cells (Peng *et al.* 2007). In our study, different from *Amorphophallus dunnii* Tucher (Luo *et al.* 2006) and *Nicotiana tabacum* L. (Peng *et al.* 2004), which adopted leaf veins to induce callus and then differentiate and form regenerated plants, the occurrence of adventitious buds from the leaf veins of *C. lancifolius* was more similar to the direct organogenesis. This phenomenon has not been reported in other species and deserved further study.

PGRs types and concentrations are two kinds of external factors in tissue culture. TDZ is active substituted urea cytokinins, which is beneficial for the proliferation of adventitious buds and cluster buds in a few woody plants (Ledbetter & Preece 2004, Radhika *et al.* 2006), its most important physiological function is to induce somatic embryogenesis (Gallo-Meagher *et al.* 2000). In this study,

the maximum proliferation coefficient of the cluster buds was 12.0 with the help of TDZ after 30 days. Next, the main bud was used as the rooting material and the rest callus with buds cultured in the MS medium with TDZ 0.2 mg·L⁻¹ after 30 days. The proliferation coefficient of the callus with buds was higher than the single callus, which was estimated that synthesized auxin from the bud tip during the growth process was transported to the callus at the base due to the downward transport, and then combined with exogenous cytokinins (TDZ) to promote its proliferation. This phenomenon has been reported in *Gentiana rigescens* Franch. ex Hemsl. (Xi *et al.* 2018). While stem segments with nodes were cultured on this medium individually (MS + 0.2 mg·L⁻¹ TDZ), the rate of callus induction was lower with malformed adventitious buds (Figure 4G). Therefore, in the medium with TDZ, the callus with buds was the optimum explants for bud differentiation culture. This may be related to the fact that a low concentration of TDZ can induce the biosynthesis of cytokinin and inhibit the degradation of endogenous auxin to promote the proliferation and redifferentiation of callus (Chen *et al.* 2006). In the subsequent subculture, the callus with buds decayed and the proliferation coefficient of adventitious bud gradually decreased. However, when the callus with buds was transferred into blank MS medium, the proliferation coefficient began to upturn, which indicated that the accumulation of endogenous hormone to a certain extent will inhibit the occurrence of buds (Bairu *et al.* 2011). It was guessed that the residual TDZ activity in the regenerated plants was reduced to the critical value of suitable cluster bud differentiation after the MS medium culture, and then the cluster bud differentiation was

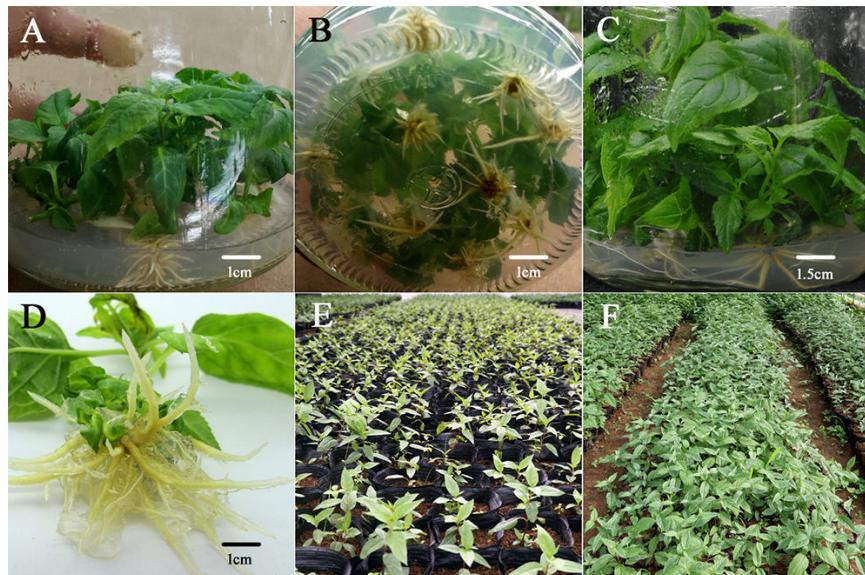


Figure 5. The plantlets from blank MS medium maintained on R06 medium to rooting (A-D) and transplanting of rooting plants (E-F). A-B. Adventitious roots after 15 days. C-D. Well-rooted plantlets after 30 days. E-F. The transplanted plants.

resumed. The proliferation coefficient of the adventitious bud was more than 12.0 in the alternate culture of MS medium with $0.2 \text{ mg} \cdot \text{L}^{-1}$ TDZ and the blank MS medium. Although it is unclear that the mechanism of TDZ on the endogenous hormone of *C. lancifolius*, the efficient shoot formation system of *C. lancifolius* can be established by selecting the appropriate TDZ concentration, which provides a reference for species that are difficult to redifferentiate cluster bud after the formation of callus.

In rooting culture of *C. lancifolius*, the differentiated seedlings from TDZ had few callus and part of the root system were differentiated from callus. Such adventitious roots were not attached to the stem by vascular bundles, and the root system was easily separated from the plant during the transplanting process. This may be due to the synergistic effect between the activity of TDZ hormone left in adventitious buds cultured by TDZ and the addition of other exogenous hormones, which promoted callus induction, making its rooting rate was not as good as differentiated seedlings from blank MS medium. Therefore, the differentiated adventitious shoots by blank MS medium were more suitable as rooting materials. Furthermore, the formed root-adventitious by the synergism of the hormones $1.0 \text{ mg} \cdot \text{L}^{-1}$ NAA and $0.1 \text{ mg} \cdot \text{L}^{-1}$ 6-BA was better than that by NAA alone (Fulzele et al. 2002, Yu et al. 2006). It indicated that the low concentration of cytokinin could promote the formation of the root-adventitious of *C. lancifolius*.

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