

Basal stem cluster bud induction and efficient regeneration for the Tibetan endemic medicinal plant *Swertia conaensis*

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Abstract

The artificial rapid propagation system for *Swertia conaensis* T. N. Ho et S. W. Liu was explored to screen the appropriate plant regeneration method and to provide an efficient propagation mode, useful for artificial breeding technology or for further research and development of the Tibetan endemic medicinal plant. In this study, the most suitable explant and hormone were chosen according to single factor test. Next, the effects of different hormone combinations on basal stem cluster bud induction, callus induction, adventitious bud occurrence and plant regeneration were investigated by using complete combination and orthogonal experiment. The obtained results showed that the explants suitable for *in vitro* of *S. conaensis* were stem tips with leaves, which were regenerated through the method of basal stem cluster bud occurrence in the MS medium with 2.0 mg·L⁻¹ 6-BA, 0.5 mg·L⁻¹ NAA, but the proliferation coefficient was low, only 3.16 after 40 days of culture. Subsequently, the proliferation coefficient failed to improve, irrespective of change of the concentration ratio of 6-BA and NAA. Therefore, in the orthogonal experiment of adding ZT, the MS medium with 1.0 mg·L⁻¹ ZT, 0.5 mg·L⁻¹ NAA and 2.5 mg·L⁻¹ 6-BA induced a large number of callus green and compact, with 86.30% callus occurrence rate. After 40 days of culture, the rate of adventitious bud occurrence was 96.55% and the proliferation coefficient was high (10.37). The rooting rate was 100% in the 1/2MS medium with 0.5 mg·L⁻¹ NAA. The survival rate of regenerated plants was more than 95%. Indirect organogenesis was more efficient than direct organogenesis in *in vitro* culture of *S. conaensis*. In this study, the efficient and stable regeneration system of *S. conaensis* was achieved through the method of explant to callus to adventitious buds, which provided an effective way to an endangered species.

Keywords: adventitious buds; basal stem cluster buds; callus; proliferation coefficient; *Swertia conaensis*

Introduction

Swertia is a genus in Gentianinae family, with a total of more than 150 species, distributed in Europe, Asia, North America and Africa belonging to the type of disjunct distribution of the north temperate and south temperate zones. Most species of this genus are found in Asia, a few in North America and Africa, and only one

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species has spread to Europe (He *et al.*, 1994). In China, 79 species have been recorded and mainly distributed in southwest provinces, and the plant of this genus is a famous folk herbal medicine with high economic value (Cao *et al.*, 2015; Khanal *et al.*, 2015; Kshirsagar *et al.*, 2015; Ahluwalia *et al.*, 2018).

Swertia conaensis T. N. Ho et S. W. Liu is a perennial herb, mainly distributed in Xizang region of China. It grows among rocks on hilltops over an altitude of 4,000 m, main root is developed; stem is extremely shortened; leaves are clustered in the shape of an open lotus, base gradually narrow, margin with short ciliate, apex obtuse to rounded; petiole flat, as long as leaf; calyx green, nearly as long as corolla; corolla dark blue, densely pubescent (Chinese Academy of Sciences China flora editorial board, 1988). The research group found that the whole plants of *S. conaensis* are used as medicine for the treatment of jaundice hepatitis, dysentery, pneumonia, tonsillitis, gynaecological inflammation and other diseases in Tibetan areas when we collected materials. However, this plant is easy to be identified in the flowering stage. During this stage, this species was picked in large number by the herdsman, which leads to its failure to complete the life cycle for low sexual reproduction efficiency and a sharp decline in the wild population and individual number, and it was now difficult to find the species in the origin of type specimens.

Currently, there is a lack in relevant studies on *S. conaensis*, with only partial reports on classification (Chen *et al.*, 2014). The biology of reproduction, particularly in biological engineering, is completely unreported. Therefore, for *S. conaensis* which has limited studies, it is necessary to use plant culture technology with the reproducible and replaceable characteristics to establish the sustainable utilization and mass reproduction system. However, in plant tissue culture, the type of explant is one of the main factors for the effect of *in vitro* culture. Different explants and even different organs of the same plant have different responses for *in vitro* induction and their own redifferentiation ability, so different explants have obvious differences in proliferation method and effect (Liu *et al.*, 2010; Song *et al.*, 2011). Meanwhile, propagation methods play an important role in *in vitro* regeneration of plants. Generally, the plants obtained by direct organogenesis are stronger and the transplanting survival rate is higher, but the proliferation coefficient usually can not meet the requirements of rapid propagation. Indirect organogenesis has a higher proliferation coefficient and can obtain a large number of plants in a short time. In *Swertia* plants, the leaves, stem segments and stem tips were reported as explants. Among them, the leaves regeneration was mainly through indirect organogenesis (Huang, 2005; Mahendran and Bai, 2017); the stem tips were mostly regenerated by direct organogenesis (Kshirsagar *et al.*, 2015b); while the above two methods in stem segments with buds culture regeneration have been reported (Huang *et al.*, 2016).

In this study, with different organs of *S. conaensis* as materials, the suitable explants were firstly screened out, and then the most effective methods were selected from a variety of propagation methods. Finally, the efficient rapid propagation system of *S. conaensis* was achieved, and its proliferation coefficient was higher than previous reports in the same genus. The results can provide a theoretical basis for the protection of natural resources of *S. conaensis*. Meanwhile, it can also provide experimental basis for artificial mass planting, germplasm conservation and genetic transformation research.

Materials and Methods

Plant materials

Swertia conaensis were sampled from Quzhuomu village, Cona county, Tibet autonomous region, China (long. 91°45' E, lat. 28°15' N; Alt: 4,200 m) in June 2017. The plants with soil were transplanted in an experimental greenhouse of Qiucheng breeding company limited, Lijiang city, Yunnan province, China (long. 100°17' E, lat. 26°83' N; Alt: 3,150 m). After that, the leaf, petiole and stem tip with the leaves (1.5-2.0 cm) were obtained from 25 *S. conaensis* samples, washed for 30 min with tap water after soaked 5 min by 10% detergent solution (w/v). Next, the samples were placed on a sterile operating platform, stirred for 10 s in 75% ethanol solution (v/v), and then surface-sterilized by 0.1% HgCl₂ (w/v), in which the leaf and petiole for 6 min,

stem tip with the leaves for 8 min. After removing the $HgCl_2$, the materials were rinsed for 6 times with sterile water and each time not less 3 min. Finally, they were placed in sterile filter paper to eliminate moisture (Xi *et al.*, 2020).

Basal medium

The basal medium for all stages was MS (Murashige and Skoog) medium (rooting culture was 1/2MS medium), and different concentrations of 6-BA (6-benzylaminopurine), ZT (zeatin), KT (kinetin), NAA (α -naphthaleneacetic acid), 2, 4-D (2, 4-dichlorophenoxyacetic acid), and IBA (indole-3-butyric acid) were added as required. In the study, the concentrations of all hormones were mass concentrations. The amount of sucrose added in basic medium was 3%, and agar was 0.55%. The medium pH was adjusted with 1 N HCl to 5.6-5.8 before the autoclaving at 122 °C for 22 min.

Initial culture medium

After surface disinfection, petiole and stem tip in length were cut into 1.5 cm and 1.0 cm respectively, and the leaves in size were cut into about 0.5×0.5 cm. These materials were inoculated on basal medium using 6-BA, ZT, KT, NAA, 2, 4-D or IBA, respectively. Herein, the concentrations of 6-BA, ZT, KT were set at 0.1, 0.5, 1.0, 2.0, 3.0 $mg \cdot L^{-1}$ and the concentrations of NAA, 2, 4-D, IBA were set at 0.05, 0.1, 0.5, 1.0, 1.5 $mg \cdot L^{-1}$ for conducting single-factor pre-experiment. Thus, the hormone types and concentration range suitable for growth of *S. conaensis* were screened.

Basal stem cluster bud occurrence and proliferation culture medium

According to the results of initial culture, the stem tips with leaves were used as materials, 6-BA (1.5, 2.0 and 2.5 $mg \cdot L^{-1}$) and NAA (0.1, 0.5 and 1.0 $mg \cdot L^{-1}$) were used as factors for complete combination experiment. The bud formation at the base of the material was used as the standard for statistics, after 40 d of culture, the occurrence rate and proliferation coefficient of basal stem cluster buds were counted.

Synchronous culture medium for callus induction, adventitious buds' differentiation and proliferation

On the basis of initial culture, the stem tip with leaves was used as the experimental material for L_9 (3^4) (a matrix with 9 rows and 4 columns; L: Latin; 9: nine combinations of levels; 3: three levels; 4: four factors) orthogonal test. The factors of orthogonal selection were ZT (0.5, 1.0 and 1.5 $mg \cdot L^{-1}$), 6-BA (1.0, 2.0 and 2.5 $mg \cdot L^{-1}$), NAA (0.1, 0.5 and 1.0 $mg \cdot L^{-1}$). The callus induction rate and cluster bud's occurrence rate were counted after 40 d of culture.

Rooting culture medium

Based on the results of single factor pre-experiment, the main buds with length of 3-4 cm were cut and then cultured in 1/2 MS or MS medium supplemented with NAA (0.1, 0.5 and 1.0 $mg \cdot L^{-1}$). The rooting rate and root growth condition were observed and recorded regularly for 45 d.

Culture conditions and inoculation methods

The temperature of the culture room was controlled at 20 ± 2 °C, the illumination was 10 $h \cdot d^{-1}$ with 1500-2000 lx the light intensity. For initial culture, each treatment consisted of 5 bottles with 2 materials in each bottle. For complete combination, orthogonal experiment and rooting culture, each treatment consisted of 10 bottles with 2 materials in each bottle. The above experiments were repeated 3 times with the exception of initial culture, and if there was pollution, materials were taken again to supplement the number of inoculated in each treatment.

Rooting and the plant transplantation

After 40 d of rooting culture, the bottle plants with caps were exposed under natural light for 7 d, and then the bottle caps were opened to adapt to environment for 3 d. Subsequently, the test tube plant was carefully removed from the bottle and the residual medium on the root was washed out. The root was soak with carbendazim solution (w/v) for 5 min and then the rooting plant were transplanted into a soil mixture disinfected with 0.1% formaldehyde (ratio: humus: fine river sand = 3: 2). After transplantation, the temperature of growth chamber was controlled at 18-22 °C and the humidity was 70%. The survival rate of the plants was counted after 30 d of culture.

Statistical analysis

The obtained data were processed and analysed by Excel and SPSS (19.0) software. The data was calculated as follows:

Survival rate of explants = (the number of explants with pollution-free and viable / the total number of explants inoculated) ×100% (1)

The occurrence rate of basal stem cluster bud = (the number of materials with buds / the number of initial inoculation) ×100% (2)

Callus induction rate = (the number of materials with callus / the number of initial inoculation) ×100% (3)

Cluster bud occurrence rate = (the number of callus with buds / the number of initial inoculation) ×100% (4)

Proliferation coefficient = the number of inoculation in subculture / the total number of initial inoculation (5)

Rooting rate = (the number of single buds with adventitious roots / the total number of single buds inoculation) ×100% (6)

The survival rate of plants = (the number of survival plants / the total number of transplantations) ×100% (7)

Results

Explants screening and single factor experiment results

The obtained results showed that all treatment could promote the growth of three explants. Calluses were produced from incision of the leaf and petioles in 6-BA, ZT, KT or 2, 4-D treatment, especially in 6-BA or ZT. However, no adventitious bud differentiation, only callus proliferation and aging were observed (Figures 1A, B). For stem tip with leaves as materials, in 6-BA (1.0-2.0 mg·L⁻¹) and ZT (0.5-1.0 mg·L⁻¹) treatment, with the elongation of basal leaves, the base had obviously cluster bud occurrence (Figure 1C), while the treatment of KT has no similar phenomenon. In NAA, 2, 4-D or IBA treatment, the difference in growth states was not significant. Although stem tip growth was observed, no significant elongation of leaves was found, only the expansion of the part in contact with the medium was observed and then callus appeared, especially in the NAA (0.1-0.5 mg·L⁻¹) treatment growth states was best (Figure 1D). Therefore, stem tips with leaves were used as explant, and 6-BA / NAA were selected for the next complete combination experiment.

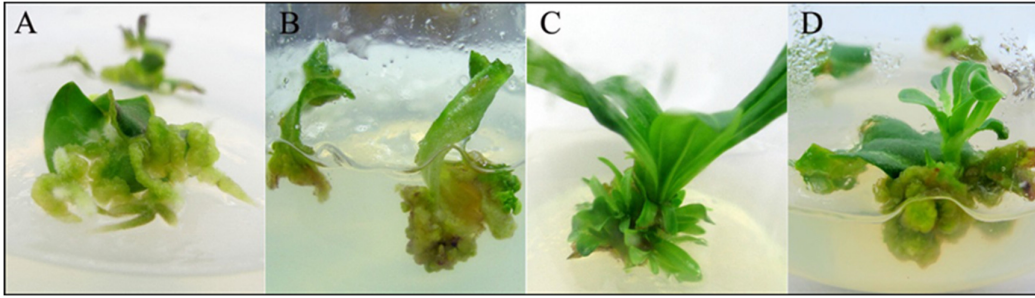


Figure 1. Result of single factors test of *Swertia conaensis* (A) Growth condition of leaves in the MS medium with 2.0 mg·L⁻¹ 6-BA; (B) Growth condition of petiole in the MS medium with 1.5 mg·L⁻¹ 6-BA; (C) Growth condition of stem tip with leaves in the MS medium with 1.0 mg·L⁻¹ 6-BA; (D) Growth condition of stem tip with leaves in the MS medium with 0.5 mg·L⁻¹ NAA

Basal stem cluster buds occurrence and proliferation

There was a great difference between the hormone treatment and the blank control (CK, Table 1). The plants in CK culture medium did not stop growing completely, presumably because the stem tips at this stage were mostly from single factor experiments, which had accumulated some hormones. The obtained results showed that the occurrence rate of basal stem cluster bud was 100% in all treatment, but the proliferation coefficient had a significant difference. When NAA concentration remained unchanged, 6-BA was in the range of 1.5 to 2.0 mg·L⁻¹, which was a positively correlated with the proliferation coefficient. When 6-BA concentration was 2.0 mg·L⁻¹, the proliferation coefficient was the highest, which was significantly different from other treatments ($P < 0.05$). When 6-BA concentration exceeded 2.0 mg·L⁻¹, the proliferation coefficient began to decrease. When the concentration of 6-BA was kept unchanged, the comparison between the 04, 05 and 06 treatments that the proliferation coefficient was related to the concentration of NAA, and there were significant differences between treatments. In the 05 treatment, the occurrence of basal stem cluster bud had a great response and the whole process recorded no obvious callus. Stem tip with one leaf was cultured in above treatment (Figure 2A). After 15 d of culture, new leaves began to appear, and its basal nodes had new buds' occurrence (Figure 2B). After 40 d of culture, with elongation of basal leaves, new leaves at the top continued to occur, while the basal nodes were significantly enlarged, and cluster buds on the basal node further grew, but the proliferation coefficient was only about 3.2 (Figure 2C).

Table 1. Effects of 6-BA / NAA combination on culture of stem tip with leaves of *Swertia conaensis*

Medium	Hormone (mg·L ⁻¹)		Proliferation coefficient
	6-BA	NAA	
CK	0	0	0.37 ± 0.05 h
01	1.5	0.1	2.59 ± 0.19 g
02	1.5	0.5	2.62 ± 0.90 ef
03	1.5	1.0	2.61 ± 0.10 fg
04	2.0	0.1	2.8 ± 0.10 c
05	2.0	0.5	3.16 ± 0.09 a
06	2.0	1.0	3.00 ± 0.15 b
07	2.5	0.1	2.63 ± 0.20 e
08	2.5	0.5	2.77 ± 0.28 d
09	2.5	1.0	2.76 ± 0.21 d

Note: CK: blank control; 6-BA: 6-benzylaminopurine; NAA: α -naphthaleneacetic acid; Data are mean ± SE; different lowercase letters in the same column mean significant difference ($P < 0.05$).

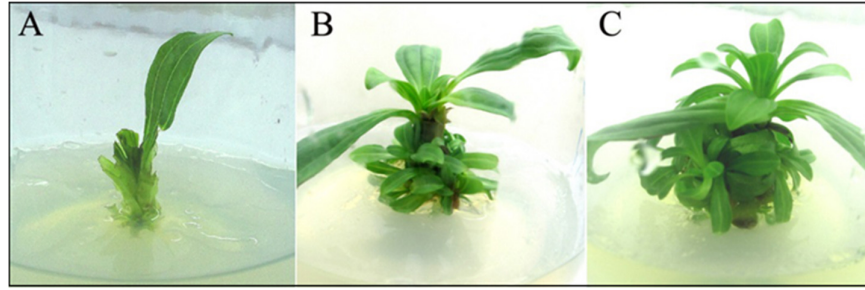


Figure 2. The basal stem cluster bud occurrence and proliferation of stem tip with leaves of *Swertia conaensis* (A) Initial state; (B) Growth condition after 15 d of transfer; (C) Growth condition after 40 d of transfer

Synchronous culture for callus induction, adventitious buds' differentiation and proliferation

In order to further explore the proliferation method of *S. conaensis* and improve the proliferation coefficient, ZT was introduced to conduct orthogonal experiment according to the single factor experiment results (Table 2).

The hormone in descending order was $R_{ZT} > R_{6-BA} > R_{NAA} > R_{blank}$, and the range of three hormones was all higher than that of the blank column (Table 2). This indicated that three hormones for callus and cluster bud differentiation were effective, among which ZT for callus induction and cluster buds occurrence had the best response, followed by 6-BA and NAA. According to the results of variance analysis (Table 3), for callus induction and cluster bud differentiation, ZT had a statistical significance, while 6-BA and NAA had no statistical significance ($P > 0.05$). Duncan test in 3 levels of ZT for callus induction (Table 3) showed that level 2 ($1.0 \text{ mg}\cdot\text{L}^{-1}$) had the best response, which was significantly different from level 1 ($0.5 \text{ mg}\cdot\text{L}^{-1}$) and level 3 ($1.5 \text{ mg}\cdot\text{L}^{-1}$). In terms of cluster bud occurrence, there were significant differences between level 2 and 3 ($1.0 \text{ mg}\cdot\text{L}^{-1}$ and $1.5 \text{ mg}\cdot\text{L}^{-1}$) and level 1 ($0.5 \text{ mg}\cdot\text{L}^{-1}$). The optimal hormone combination of *S. conaensis* for callus induction and cluster bud differentiation was $A_2B_2C_3$, namely in the MS medium with $1.0 \text{ mg}\cdot\text{L}^{-1}$ ZT, $0.5 \text{ mg}\cdot\text{L}^{-1}$ NAA and $2.5 \text{ mg}\cdot\text{L}^{-1}$ 6-BA. In this medium, after 15 d of culture, compact and white callus were produced at the contact site of stem tip and medium (Figure 3A). After 25 d of culture, with the proliferation of callus, obvious signs of bud growth were observed (Figure 3B). After 30 d of culture, green bud points appeared continuously on the surface of callus (Figure 3C). After 40 d of culture, cluster buds were formed and conical buds were found everywhere on the surface of callus. The callus induction rate was 86.30%, the cluster bud occurrence rate was 96.55%, and the proliferation coefficient was over 10.0 (Figure 3D).

The above medium could also be used as cluster buds proliferation medium. Callus with green buds and compact texture induced by stem tip were cut into $1.5 \times 1.5 \text{ cm}$ in size and inoculated into this medium (Figure 3E). After 20 d of culture, cluster buds gradually differentiated and grew (Figure 3F). After 25 d of culture, cluster buds began to proliferate in large number, and callus were covered by cluster buds (Figure 3G). After 30 d of culture, a cluster bud system was formed (Figure 3H).

Table 2. Result of callus induction and adventitious bud occurrence of *Swertia conaensis* by L₉ (3⁴) orthogonal test

Medium	Hormone / (mg·L ⁻¹)				Callus induction rate (%)	Cluster bud occurrence rate (%)	
	ZT	NAA	6-BA	blank			
C1	0.50	0.10	1.00	(1)	26.07	50.67	
C2	0.50	0.50	2.00	(2)	40.67	60.27	
C3	0.50	1.00	2.50	(3)	48.29	64.80	
C4	1.00	0.10	2.00	(3)	56.30	86.13	
C5	1.00	0.50	2.50	(1)	86.30	96.55	
C6	1.00	1.00	1.00	(2)	84.80	90.20	
C7	1.50	0.10	2.50	(2)	59.50	85.10	
C8	1.50	0.50	1.00	(3)	57.88	83.79	
C9	1.50	1.00	2.00	(1)	50.99	70.60	
Callus induction rate	K1	38.343	47.290	56.250	54.453	-	-
	K2	75.800	61.617	49.320	61.657	-	-
	K3	56.123	61.360	64.697	54.157	-	-
	R	37.457	14.327	15.377	7.500	-	-
Cluster bud occurrence rate	K1	58.580	73.967	74.887	72.607	-	-
	K2	90.960	80.203	72.333	78.523	-	-
	K3	79.830	75.200	82.150	78.240	-	-
	R	32.380	6.236	9.817	5.916	-	-

Note: L: Latin; 9: nine combinations of levels; 3: three levels; 4: four factors; K: the mean value; R: range; C: treatment group for callus induction and adventitious bud occurrence; ZT: zeatin; NAA: α -naphthaleneacetic acid; 6-BA: 6-benzylaminopurine.

Table 3. The variance analysis of callus induction and cluster bud occurrence in *Swertia conaensis*

Factors	Source	Type III sum of squares	DF	Mean square	F value	Significance
Callus induction	ZT	2106.301	2	1053.151	19.462	$P < 0.05$
	NAA	403.284	2	201.642	3.726	$P > 0.05$
	6-BA	355.813	2	177.906	3.288	$P > 0.05$
	Error	108.226	2	54.113	-	-
Cluster bud occurrence	ZT	1623.904	2	811.952	16.921	$P < 0.05$
	NAA	65.450	2	32.725	0.106	$P > 0.05$
	6-BA	155.642	2	77.821	0.266	$P > 0.05$
	Error	66.822	2	33.411	-	-

Note: DF: degree of freedom; ZT: zeatin; NAA: α -naphthaleneacetic acid; 6-BA: 6-benzylaminopurine.

Table 4. Duncan's test of three levels of ZT

Factors	Mean	0.05 level	Factors	Mean	0.05 level
Callus induction	38.343	c	Cluster bud occurrence	58.580	b
	75.800	a		90.960	a
	56.123	b		79.830	a

Note: ZT: zeatin.

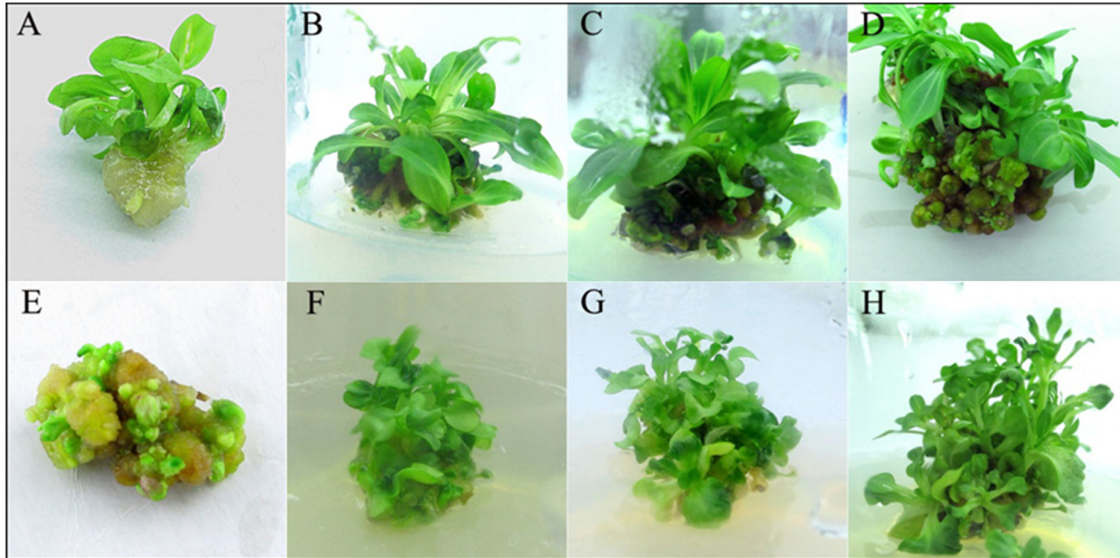


Figure 3. Synchronous culture for callus induction, cluster bud differentiation and proliferation of stem tip with leaves of *Swertia conaensis* (A) Callus of the base of stem tip with leaves after 15 d of culture; (B) Callus proliferation with a larger number of adventitious buds; (C) Green buds points appeared on the surface of callus after 30 d of culture; (D) Cluster buds formation after 40 d of culture; (E) Callus with green buds points; (F) Callus covered by a larger number of buds; (G) Growth condition of callus after 30 d of culture; (H) Growth condition of callus after 40 d culture

Rooting culture and the plant transplantation

The rooting of *S. conaensis in vitro* was relatively easy. Rooting rate of 67.50% and 42.16% were found in CK treatment (1/2MS medium and MS medium), but the roots of the plant were small and the plants were thin (Table 5). It can be noted that the 1/2 MS medium was superior to the MS medium for adventitious root induction and growth. The rooting rate in NAA treatment was significantly higher than that of the blank control treatment, indicating that NAA had a significant effect on root induction. In the range of the concentration of NAA between 0.1 and 0.5 mg·L⁻¹, the rooting rate increased in proportion with the increase of NAA concentration. However, when the NAA concentration exceeded 1.0 mg·L⁻¹, the rooting rate began to decrease; in other words, high auxin had an inhibitory effect on the occurrence of adventitious roots. The optimal medium for adventitious roots induction was the 1/2MS medium containing 0.5 mg·L⁻¹ NAA, with 100% rooting rate. In this medium, white root tips began to appear after 15 d of culture (Figure 4A). After 25 d of culture, significant root elongation and increased diameter were seen (Figure 4B). After 35 d of culture, the old leaves elongated, new leaves appeared continuously and adventitious roots elongated further (Figure 4C). After 45 d of culture, the root system was developed and sturdy for domestication and transplantation (Figure 4D). After 40 d of transplantation, the survival rate reached over 95% (Figures 4E-H).

Table 5. Effects of different medium and different concentration NAA on adventitious roots

Medium	NAA (mg.L ⁻¹)	Rooting rate (%)	Growth condition
1/2MS (CK)	0.0	67.5 ± 0.13 d	Pale green leaves; root with small amount and thin; plants with weak and slow growth; no callus
1/2MS	0.1	86.3 ± 0.15 b	Green leaves; root with stout and long; plants with well-grown; no callus
1/2MS	0.5	100.0 ± 0.10 a	Lots of dark green leaves; root with dense and stout; no callus
1/2MS	1.0	79.4 ± 0.12 c	Bright green leaves; root with stout and well-grown; a small number of callus
MS (CK)	0.0	42.1 ± 0.07 h	A few green leaves; root with small amount and thin; plants with weak and poor; no callus
MS	0.1	56.2 ± 0.14 f	Fewer new green leaves; a large number of root with thin; plants with weak; a small number of callus
MS	0.5	58.8 ± 0.12 e	Green leaves with slow grown; a large number of root with thin; the base with obvious callus
MS	1.0	54.6 ± 0.09 g	A few leaves with slow grown; root with less developed; a large number of callus

Note: MS: Murashige and Skoog; CK: blank control; NAA: α -naphthaleneacetic acid.

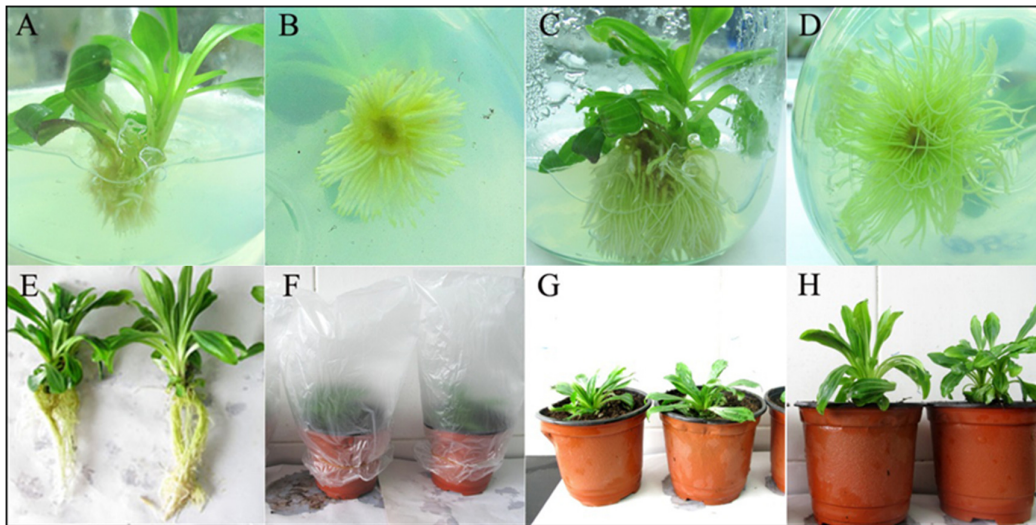


Figure 4. Adventitious roots induction and transplantation of *Swertia conaensis* (A) Adventitious roots began to appear in large number after 15 d of culture; (B) Growth condition of adventitious roots after 25 d of culture; (C) Growth condition of adventitious roots after 35 d of culture; (D)-(E) Rooting plants after 45 d of culture; (F) The tube plants covered by plastic bags after 15 d of transplanting; (G) The tube plants after 30 d of transplanting; (H) The tube plants after 40 d of transplanting

Discussion

Effects of explants on the rapid propagation of S. conaensis in vitro

In this study, the stem tip with leaves, leaf and petiole were used as explants and all could induce callus, but the ability of dedifferentiation had significant differences. The calluses induced from leaf were loose in texture and proliferated very fast. However, after the combination of various hormones, no adventitious buds were produced. The calluses differentiated from petiole were compact in texture, and even with adventitious buds, the growth was malformed and vitrified seriously, thereby lost next culture value. However, the calluses

induced from stem tip with leaves, with strong ability of redifferentiation, were easy to induce adventitious buds. These obtained results showed that the ability of dedifferentiation and redifferentiation of different organs in the same plant was significantly different (Matt and Jehle, 2005; Nagalakshmi *et al.*, 2014; Lin *et al.*, 2016). It was speculated that endogenous hormones and organization in different organs of the same plant were different, leading to different dedifferentiation abilities, similar to the study of *Wrightia arborea* (Dennst.) Mabb. and *Vernicia fordii* (Hemsl.) Airy Shaw (Nagalakshmi *et al.*, 2014; Lin *et al.*, 2016).

Different proliferation methods of S. conaensis

It has been reported that the tissue culture methods of *Swertia* genus plants are divided into direct and indirect organ types. For *S. conaensis*, in 6-BA/NAA combination, the base of stem tip with leaves could induce adventitious cluster buds, namely the basal stem cluster buds. Different researchers have different views for the basal stem cluster buds. Wang *et al.* (2018) considered that the basal stem cluster buds of *Codonopsis bulleyana* Forrest ex Diels were adventitious buds occurring on the callus, that was, indirect organogenesis. In another study, Li *et al.* (2020) observed that the essence of basal stem cluster buds of *Lycium ruthenicum* Murr was axillary bud germination, belong to direct organogenesis. The occurrence of basal stem cluster buds in this study was similar to that of above two species. The morphology of the basal stem cluster bud occurrence of *S. conaensis* was similar to that of the adventitious buds from the calluses, but it was found to be completely different after anatomical comparison. In generally, buds that grow in a certain position on a branch are called normal bud, while buds grow from the internodes of the stem, root or leaf, without a certain position, are called adventitious buds (Li *et al.*, 2012). The stem of *S. conaensis* is extremely short, there may be many nodes at the base of contact with the medium, and then germinated at the specific location, which are easily regarded as adventitious buds due to their large number. On the other hand, in the process of cultivation, callus was not observed except basal node expansion, and there was obvious vascular bundle connection between cluster buds and the main buds during the transfer, so it could be judged as the normal bud. Therefore, in this study, it was considered that the essence of the basal stem cluster buds proliferation methods of *S. conaensis* was axillary bud germination, that was, the direct organogenesis, but the proliferation coefficient of this method was low (3.16), which could not reach the base requirement of rapid propagation.

After the introduction of ZT on the basis of 6-BA/NAA combination, the base of stem tip with leaves could induce organogenic callus, and then differentiated a large number of adventitious buds in a short time. Furthermore, in the process of adventitious buds' differentiation, the callus continuously proliferated and differentiated, and a method of callus to cluster buds to callus to cluster buds was repeated. Using this propagation mode, the proliferation coefficient of one culture cycle could more than 10.0, achieving the purpose of rapid reproduction. At present, most reports suggested, with the stem tip as initial explant, the plant regeneration via direct and indirect method were very hard acted on simultaneously. Because the formation of callus at the base of stem tip is mainly attributed to the accumulation of auxin at the base, which initiates cell proliferation in the presence of exogenous cytokinins to form callus. Meanwhile, the basal transport of auxin also inhibits the formation of lateral buds (Preece *et al.*, 1991; Debnath, 2005). Noticeably, in this study, stem tip with leaves could be directly proliferated and indirectly regenerated. It was speculated that the addition of different exogenous hormones may trigger different regeneration methods of stem tip with leaves, which also indicated that different explants of the same plant, or even the same explants of the same plant, can be regenerated in different methods due to different exogenous hormones. However, such reports are rare.

Effects of exogenous hormones on the rapid propagation of S. conaensis in vitro

In *in vitro* rapid propagation of *S. conaensis*, single factor, complete combination and orthogonal results showed that the culture effect of hormone combination was significantly higher than that of single hormone, indicating that the synergistic effect of multiple hormones was far greater than that of single hormone (Kishore *et al.*, 2011; Afshan *et al.*, 2014). Single factor experiments showed that both 6-BA and ZT could promote the occurrence of the basal stem cluster buds on stem tip with leaves. Because of the high cost of ZT,

6-BA and NAA were selected for the complete combination experiment, but satisfactory proliferation coefficient and method were not obtained. ZT, a natural cytokinin isolated from higher plants, is generally believed to have good effects on bud initial, regeneration and proliferation (Debnath and Mcrae 2002; Debnath *et al.*, 2005). In the previous reports of *Swertia* genus, exogenous auxin is an important factor for callus induction and adventitious bud differentiation (Xiang *et al.*, 1999; Huang *et al.*, 2016). In this study, ZT was the key factor for callus induction. In addition to the medium with ZT, white compact callus appeared quick, then rapidly proliferated and accompanied by a large number of cluster bud differentiation and growth. The proliferation coefficient was much higher than that of the method of basal stem cluster buds. It indicated that ZT can promote cell division and non-differentiated tissue differentiation *in vitro*, and can also strongly induce adventitious bud differentiated and growth. This finding was also found at *Actinidia polygama* Miq. and *Vaccinium macrocarpon* Ait. (Takahashi *et al.*, 2004; Debnath, 2008). In addition, this study considered that *S. conaensis* has a harsh habitat, and only by increasing endogenous auxin levels and accelerating growth can complete its life cycle in a short time. In terms of morphology, the stem is extremely shortened, and fleshy roots and basal leaves are developed, so as to relieve the obstacle caused by insufficient cytokinin on plants that cannot complete normal physiological activities, and thus produce a strong response to exogenous cytokinin.

Conclusions

This is the first study reporting *in vitro* rapid propagation of *S. conaensis*. The stem tips with the leaf were optimal explant, the artificial rapid propagation system of *S. conaensis* was achieved successfully via direct and indirect organ methods. Although both propagation method was able to regenerate intact plant, the direct organ method had a low propagation coefficient (3.16), which could not reach the base requirement of rapid propagation. However, in the indirect organ method, the callus to cluster buds to callus to cluster buds was repeated, resulting in a huge propagation coefficient (10.37). In addition, in rooting culture, the 1/2 MS medium was superior to the MS medium, the rooting rate was 100% in the 1/2MS medium with 0.5 mg·L⁻¹ NAA. After transplanting, the survival rate was more than 95%.

Authors' Contributions

Both authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

- Afshan N, Anwar S, Mohammad A (2014). Effect of adenine sulphate interaction on growth and development of shoot regeneration and inhibition of shoot tip necrosis under *in vitro* condition in adult *Syzygium cumini* L. -a multipurpose tree. Applied Biochemistry and Biotechnology 173:90-102. <https://doi.org/10.1007/s12010-014-0797-2>
- Ahluwalia V, Elumalai S, Kumar V, Kumar S, Sangwan RS (2018). Nano silver particle synthesis using *Swertia paniculata* herbal extract and its antimicrobial activity. Microbial Pathogenesis 114:402-408. <https://doi.org/10.1016/j.micpath.2017.11.052>
- Cao TW, Geng CA, Ma YB, Zhang XM, Zhou J, Tao YD, Chen JJ (2015). Chemical constituents of *Swertia mussotii* and their anti-hepatitis B virus activity. Fitoterapia 102:15-22. <https://doi.org/10.1016/j.fitote.2015.01.020>
- Chen YS (2014). Six new species of *Saussurea* (Asteraceae) from eastern Himalaya. Phytotaxa 177(4):191-206. <https://doi.org/10.11646/phytotaxa.177.4.1>
- Chinese Academy of Sciences, China flora editorial board (1988). Flora of China (Volume 62). Science Press, Beijing, China.
- Debnath SC (2005). Micropropagation of lingonberry: influence of genotype, explant orientation, and overcoming TDZ-induced inhibition of shoot elongation using zeatin. HortScience 40(1):185-188. <https://doi.org/10.21273/HORTSCI.40.1.185>
- Debnath SC (2008). Zeatin-induced one-step *in vitro* cloning affects the vegetative growth of cranberry (*Vaccinium macrocarpon* Ait.) micropropagules over stem cuttings. Plant Cell, Tissue and Organ Culture 93(2):231-240. <https://doi.org/10.1007/s11240-008-9366-0>
- Debnath SC, Mcrae KB (2002). An efficient adventitious shoot regeneration system on excised leaves of micropropagated lingonberry (*Vaccinium vitis-idaea* L.). Journal of Horticultural Science & Biotechnology 77:744-752. <https://doi.org/10.1080/14620316.2002.11511567>
- He TN, Xue CY, Wang W (1994). The origin, dispersal and formation of the distribution pattern of *Swertia* L. (gentianaceae). Acta Phytotaxonomica Sinica 32(6): 525-537. (in Chinese with English abstract)
- Huang HY (2005). Tissue culture of medicinal plant *Swertia mileensis*. Chinese Traditional and Herbal Drugs 36(2):261-265. (in Chinese with English abstract)
- Huang HY, Huang J, Wang MR, Ma XN, Xi YK (2016). Comparison on progeny regeneration capacity among different mating patterns and optimization of cultivation condition in *Swertia mileensis*. Chinese Traditional and Herbal Drugs 47(3):480-487 (in Chinese with English abstract). <https://doi.org/10.7501/j.issn.0253-2670.2016.03.022>
- Khanal S, Shakya N, Thapa K, Pant DR (2015). Phytochemical investigation of crude methanol extracts of different species of *Swertia* from Nepal. BMC Research Notes 8(1):1-9. <https://doi.org/10.1186/s13104-015-1753-0>
- Kishore KC, Arifullah M, Gayathri D, Rama GG (2011). *In vitro* shoot regeneration and control of shoot tip necrosis in tissue cultures of *Soymida febrifuga* (Roxb.) Plant Tissue Culture and Biotechnology 21(1):11-25. <https://doi.org/10.3329/ptcb.v21i1.9559>
- Kshirsagar P, Chavan J, Nimbalkar M, Yadav S, Dixit G, Gaikwad N (2015a). Phytochemical composition, antioxidant activity and HPLC profiles of *Swertia* species from Western Ghats. Natural Product Research 29(8):780-784. <https://doi.org/10.1080/14786419.2014.986124>
- Kshirsagar PR, Chavan JJ, Umdale SD, Nimbalkar MS, Dixit GB, Gaikwad NB (2015b). Highly efficient *in vitro* regeneration, establishment of callus and cell suspension cultures and RAPD analysis of regenerants of *Swertia lawii* Burkill. Biotechnology Reports 6:79-84. <https://doi.org/10.1016/j.btre.2015.03.003>
- Li N, Huang HY, Zeng B (2020). Cluster bud induction of base stem and establishment of high efficiency regeneration system of *Lycium ruthenicum*. Chinese Traditional and Herbal Drugs 51(13):3545-3553 (in Chinese with English abstract). <https://doi.org/10.7501/j.issn.0253-2670.2020.13.02>
- Li ZJ, Jiao PP, Zhou ZL, Li Q, Li JQ (2012). Morphological and anatomical features of root sucker propagation of *Populus pruinosa*. Chinese Bulletin of Botany 47(2):133-140 (in Chinese with English abstract). <https://doi.org/10.3724/SP.J.1259.2012.0013>
- Lin Q, Li Z, Zhang L, Tan XF, Long HX, Wu LL (2016). High-efficiency regeneration of seedlings from hypocotyl explants of tung tree (*Vernicia fordii*). International Journal of Agriculture and Biology 18:370-376. <https://doi.org/10.17957/IJAB/15.0097>

- Liu C, Allow P, Rowland LJ, Hancock JF, Song GQ (2010). Adventitious shoot regeneration from leaf explants of southern highbush blueberry cultivars. *Plant Cell, Tissue and Organ Culture* 103:137-144. <https://doi.org/10.1007/s11240-010-9755-z>
- Mahendran G, Bai VN (2017). Plant regeneration through direct somatic embryogenesis, antioxidant properties and metabolite profiles of *Swertia corymbosa* (Griseb.) Wight ex C.B. Clarke. *Plant Biosystems* 151(1):39-49. <https://doi.org/10.1080/11263504.2015.1064043>
- Matt A, Jehle JA (2005). *In vitro* plant regeneration from leaves and internode sections of sweet cherry cultivars (*Prunus avium* L.). *Plant Cell Reports* 24: 468-476. <https://doi.org/10.1007/s00299-005-0964-6>
- Nagalakshmi M, Vishwanath S, Viswanath S (2014). Adventitious shoot regeneration from hypocotyls of *Wrightia arborea* (Dennst.) Mabb.: an endangered toy wood species. *Journal Cell and Tissue Research* 14:4339-4344.
- Preece JE, Huetteman CA, Ashby WC, Roth PL (1991). Micro and cutting propagation of silver maple. I. Results with adult and juvenile propagules. *Journal of the American Society for Horticultural Science* 116:142-148. <https://doi.org/10.21273/JASHS.116.1.142>
- Song JY, Mattson NS, Jeong BR (2011). Efficiency of shoot regeneration from leaf, stem, petiole and petal explants of six cultivars of *Chrysanthemum morifolium*. *Plant Cell, Tissue and Organ Culture* 107(2):295-304. <https://doi.org/10.1007/s11240-011-9980-0>
- Takahashi W, Sugawara F, Yamamoto N, Bando E, Matsushita J, Tanaka O (2004). Plant regeneration in *Actinidia polygama* Miq. by leaf, stem, and petiole culture with zeatin, and from stem-derived calli on low-sucrose medium. *Journal of Forest Research* 9(1):85-88. <https://doi.org/10.1007/s10310-003-0053-z>
- Wang QQ, Wang YZ, Huang HY, Guo FM (2018). Rapid propagation system for tissue culture of *Codonopsis bulleyana*. *Journal of Chinese Medicinal Materials* 41(6):1262-1266 (in Chinese with English abstract). <https://doi.org/10.13863/j.issn1001-4454.2018.06.00>
- Xi YK, Wang Y, Zeng B, Huang HY, Yang WD (2020). Callus induction and adventitious bud differentiation of *Cyclocodon lancifolius* (Roxb.) Kurz. *Botanical Sciences* 98(4):534-544. <https://doi.org/10.17129/botsci.2609>
- Xiang FN, Xing MQ, Xia GM, Hu FZ, Li Y (1999). Tissue culture and antihepatitis constituent in calli of *Swertia franchetiana* H. Smith. *Bulletin of Botanical Research* 19(2):172-178 (in Chinese with English abstract).



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