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INDUCTION OF ADVENTITIOUS BUDS AND ESTABLISHMENT OF AN EFFICIENT REGENERATION SYSTEM IN *Polygonatum cyrtonema* HUA

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ABSTRACT

Polygonatum cyrtonema Hua is not only a widely used traditional Chinese medicine, but also processed into food and cosmetics. However, its large-scale cultivation and application are limited by the low reproduction rate for traditional artificial propagation technology. Therefore, the aim of this study was to establish an efficient and stable adventitious buds induction system for *P. cyrtonema* by using one bud rhizome segment as initial explants. The results showed that the optimal medium for the rhizome growth, adventitious buds occurrence and proliferation was MS medium supplemented with 3.0 mg·L^{-1} BA, 1.0 mg·L^{-1} NAA and 1.0 mg·L^{-1} KT, with a proliferation coefficient of 6.5. The best rooting medium was found to be half strength of MS medium with 0.5–1.0 mg·L^{-1} NAA. The survival rate was higher than 95%. Finally, an asexual rapid propagation system of *P. cyrtonema* was established, which can provide the technical basis for the development of nursery culture.

Key words: adventitious buds, orthogonal experiment, *Polygonatum cyrtonema*, proliferation coefficient, rhizome

INTRODUCTION

Polygonatum cyrtonema Hua is a perennial herb in the genus *Polygonatum* of the Liliaceae. In Flora of China, this herb is also referred as *P. henrys* Diels, *P. martini* Levy, *P. brachynema* Handel-Mazzetti, *P. brachynema* Hand. and *P. multiflorum* Allioni var. longi-folium Merrill. The species is a typical representative of monocotyledons of the Liliaceae, which mainly distributes in southern China and usually grows under forests, bushes or shady parts of slopes at an altitude of 500–2100 m [Chinese Academy of Sciences China flora editorial board 2000]. The rhizome is used as a medicine, and it is recognized as the original plant of commodity medicinal materials of sealwort in China together with *P. kingianum* Coll. et Hemsl and *P. sibiricum* Red. [Chinese Pharmacopoeia Commission 2020].

The main chemical components of *P. cyrtonema* include steroidal saponins, poly-saccharides, flavonoids, alkaloids with the efficacy of anti-inflammatory, anti-aging, anti-tumor, immune adjustment, anti-bacterial, anti-viral, hypoglycemic and lipid [Tang et al. 2019, Zhang et al. 2019, Zhao et al. 2019]. This

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plant has not only high medicinal value, but also edible, ornamental, beauty and other functions. In addition, some researchers had studied the food made with *P. cyrtonema*, and the results showed that its main ingredient was polygona-polysaccharose, and they believed that this composition could also be added into some functional food as an additive [Feng et al. 2019, Yelithao et al. 2019].

In nature, *P. cyrtonema* mostly carries on sexual reproduction (seed reproduction) with occasional asexual reproduction (rhizome reproduction). Nursery propagation is still carried out in the above two methods, but seed propagation is more common. In spite of the large number of available seeds and the ease of nursery sowing, seeds frequently suffer from physiological after-ripening dormancy due to structural defects of seeds and endogenous inhibitors, resulting in a very low natural germination rate [Xie and Fan 1996]. Therefore, a large number of seedlings can only be obtained if there is sufficient supply of seeds. Usually, the rhizome which grows from seed to a commercial size needs to four to five years, so seeds reproduction is not economically efficient. When conducting vegetative propagation, rhizome growth is quick but each rhizome produces only one bud per year. So, large-scale production and application for pharmaceutical purposes are limited by this low reproduction rate. In this situation, plant tissue culture technology offers many advantages such as short growth cycle, high proliferations coefficient, maximum retention of the desirable characteristics of the mother plant, and the lack of restrictions due to room availability and seasonal growing. In this study, our main target is to develop a rapid propagation system *in vitro* of *P. cyrtonema* through direct organogenesis method with one bud rhizome segment as explants, and we will screen an efficient regeneration system suitable for large-scale industrial production.

MATERIALS AND METHODS

Plant material and establishment of aseptic system. Twenty potted plants of *Polygonatum cyrtonema* Hua were provided by Professor Chang-Chun Ding (Yunnan Wenshan University, Kunming, China), and transplanted into the experimental greenhouse of Yunnan University of Chinese Medicine in March 2018.

Well-grown plants with buds and rhizomes were selected, and the soil of the rhizome surface was washed out. After that, rhizomes were soaked for 10 min with 10% washing powder solution (*w/v*), and rinsed for 30 min with running water. Afterwards, the washed rhizomes were treated with 75% ethanol solution (*v/v*) for 5 s, followed by 0.1% $HgCl_2$ (*w/v*) in the aseptic bench. Five-time treatments (5, 6, 8, 10, and 12 min) of $HgCl_2$ were assayed. After disinfection, the rhizomes were rinsed with sterile water for unless 3 min three times. Finally, they were placed on a sterile absorbent paper, to dry off the water excess, and then one bud rhizoma segment was cut into 0.5 × 0.5 cm^2 with a sterile scalpel and excess tissue was removed. The rhizoma segment was placed on MS medium added with 1.5 $mg \cdot L^{-1}$ Zeatin (ZT) for initiate culture. After 60 days, the contamination and survival rates were registered. Several subcultures on this medium were needed until enough material was available to initiate the proliferation experiments.

Culture conditions. Except for rooting medium, MS was used as the basal medium for all stages. Different plant growth regulators (PGRs) at several concentrations were added according to the particular experiment. In addition, all media were supplemented with 30 $g \cdot L^{-1}$ sucrose, 4.8 $g \cdot L^{-1}$ agar, and the pH value was adjusted to 5.8–6.0 before autoclaving. The reagents and PGRs were purchased from Beijing Dingguo Biotechnology Co. Ltd (Beijing, China). The media were autoclaved at 121°C for 22 min for later use. The growth room temperature was controlled at (23 ±2)°C, with 10 h d^{-1} photoperiod and 1500~2000 lx light intensity.

For the establishment of the culture, 10 vials (50 mL conical vials) filled with 18 mL of medium and with one explant per vial were used for each disinfection treatment. For proliferation culture and rooting stages, 500 mL culture vessels were used with 10 explants per vessel. 10 bottles per treatment were used in the single factor experiment and 20 bottles in the orthogonal and rooting experiments. Each bottle was filled with 50 mL of medium. Each treatment in every experiment was repeated 3 times.

Single factor expertiment. To screen for the suitable PGRs and concentration range, one bud rhizomes segments (0.5 × 0.5 cm^2 in size) were cultured in Murashige and Skoog (MS) medium supplemented with

Benzylaminopurine (BA: 2.0, 2.5, 3.0, 3.5, 4.0, and 5.0 mg·L^{-1}), Kinetin (KT: 1.0, 2.0, 2.5, and 3.0 mg·L^{-1}), Indole-3-butyric acid (IBA: 0.5, 1.0, 1.5, and 2.0 mg·L^{-1}), α-naphthaleneacetic acid (NAA: 0.5, 1.0, 1.5, and 2.0 mg·L^{-1}) and 2,4-dichlorophenoxyacetic acid (2,4-D: 0.01, 0.05, 0.1, 0.5 mg·L^{-1}), respectively. After 45 days of culture, the growth of each treatment was estimated.

The occurrence and proliferation culture for adventitious bud. According to the results of the single factor preliminary experiment, BA (2.0, 2.5, and 3.0 mg·L^{-1}), NAA (0.5, 1.0, and 1.5 mg·L^{-1}) were selected to be used in a combination experiment. Then, KT (1.0, 2.0, and 2.5 mg·L^{-1}) and 2,4-D (0.01, 0.05, and 0.1 mg·L^{-1}) were tested in two L_9 (3^4) orthogonal experiment along with BA (2.0, 2.5, and 3.0 mg·L^{-1}) and NAA (0.5, 1.0, and 1.5 mg·L^{-1}), respectively. The material was the rhizomes with buds (0.5 × 0.5 cm^2 in size) from a single factor experiment. After 50 days of culture, proliferation coefficient was evaluated.

Rooting and acclimatization of the plantlets. Different concentrations of NAA (0, 0.5, 1.0, 1.5, and 2.0 mg·L^{-1}) were supplemented either to MS medium or half strength of MS medium for rooting culture and the material was individualized shoot with 2–3 leaves (2–3 cm in length) coming from proliferation cultures. After 40 days of culture, the rooting rate was measured.

After 40 days from the beginning of rooting phase, culture vessels with rooted plantlets were placed under natural light for 5 days, and then after 2 more days, the sealing film closure of the culture flask was removed. After that, the rooting plantlets removed the residual medium washed off the root and the root soaked 0.1% carbendazim (*w/v*) for 5 min. These rooted plantlets were transplanted to high-temperature sterilized humus soil for 60 days under a controlled environment (temperature: 20–25°C, humidity: 70%) for acclimatization, and then planted in the field were transferred.

Statistical analysis. The obtained data were processed and analyzed by SPSS software (19.0). The calculation formula was as follows:

Contamination rate = (total number of contaminated explants / total number of cultured explants) × 100%,

Survival rate of explants = (total number of explants with growth potential / total number of cultured explants) × 100%,

Proliferation coefficient = the number of valid culture in subculture / the number of initial cultures,

The rooting rate (%) = (the number of rooted plants / the number of plants cultured) × 100%,

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

RESULTS

The establishment of aseptic system and the initial culture. Disinfection time influenced the contamination and survival rate of explants (Tab. 1). Among the treatments, disinfection time of 5 to 6 min was not adequate. Although the buds grew with only 5 to 6 min of sterilization, the contamination rate was relatively high (54.2% and 45.6%, respectively). When the disinfection time was extended to 8–10 min, the contamination rate decreased significantly, and the survival rate of explants increased with it, reaching the highest 67.64%. Sterilization for more than 10 min resulted in the lowest contamination rate, but the survival rate of explants also decreased significantly. In all treatment,

Table 1. Effects of different sterilization times on percentage of contamination and survival of explants

Sterilization time / min	Number of explants	Contamination rate / %	Survival rate / %
5	30	53.16 ±2.12 a	34.25 ±1.09 a
6	30	45.60 ±1.98 b	42.15 ±2.54 b
8	30	21.25 ±3.72 c	67.64 ±1.35 c
10	30	18.30 ±3.14 c	60.28 ±1.58 c
12	30	10.02 ±2.49 d	39.75 ±3.48 d

Except for dead explants. Data are mean ± SE; different lower case letters in the same column mean significant difference ($P < 0.05$)

there was a certain number of death explant due to necrosis of the tissues related to the tenderness of initial explants.

After 50 days from the establishment of the culture, swollen rhizomes and bud cluster were obtained (Figs. 1A–C). These bud clusters were subcultured several times (each subculture of 60 days length) until enough material was available for conducting single factor experiment. The subculturing was performed on MS medium supplemented with 1.5 mg·L^{-1} KT, with a mean proliferation rate of 2.5.

Single factor experiment. Growth of *P. cyrtonema* rhizome was observed in all treatment (BA, KT, 2,4-D, NAA or IBA). Among them, basal rhizome enlargement was observed in all BA treatments, especially in the 2.0 and 3.0 mg·L^{-1} treatments (Fig. 2A). For KT treatments, rhizome enlargement was not that obvious, but more foliar buds appeared (Fig. 2B). When KT concentration was higher than 3.0 mg·L^{-1}, abnormal leaf thickening occurred. For NAA treatments, the formation of new adventitious bud and root induction were significantly affected within the range of 0.5–1.5 mg·L^{-1}, with a rooting rate of 41.52%, and strong and well-developed rooting system (Fig. 2C). The effect of IBA was similar to that of NAA, so, as it has an unstable activity and a high price its use was abandoned. 2,4-D significantly promoted the growth of the buds on the rhizome, no callus or swelling of rhizomes were observed (Fig. 2D). When the concentration of 2,4-D was higher than 0.1 mg·L^{-1}, the leaves of the shoots were irregularly curled and not fully expanded.

The occurrence and proliferation culture for adventitious bud. According to the single factor experiment results, the best growth was obtained with BA and NAA as cytokinin and auxin, respectively. In a preliminary multifactor experiment, a combina-

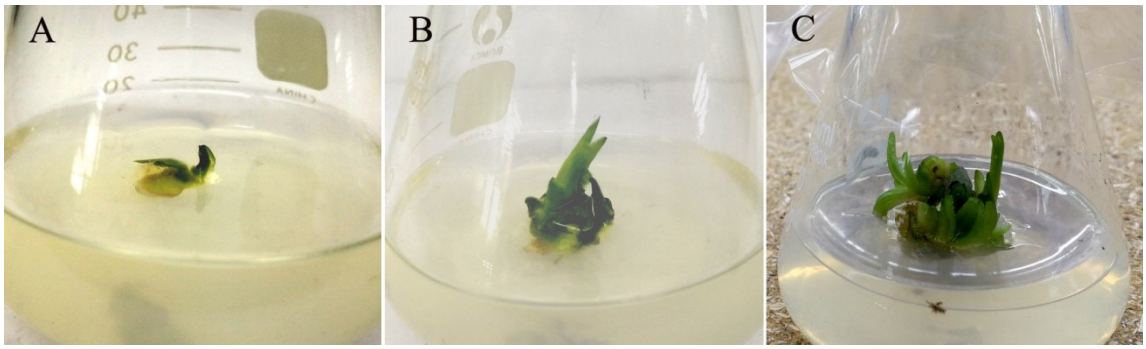

Fig. 1. Establishment of aseptic culture. (A) 15 days after the establishment: the bud of rhizome was beginning to grow; (B) 30 days after the establishment: the base of the rhizome expanded, new buds points appeared in the enlarged rhizome; (C) 50 days after the establishment: the rhizome continued to grow and the new buds began to grow

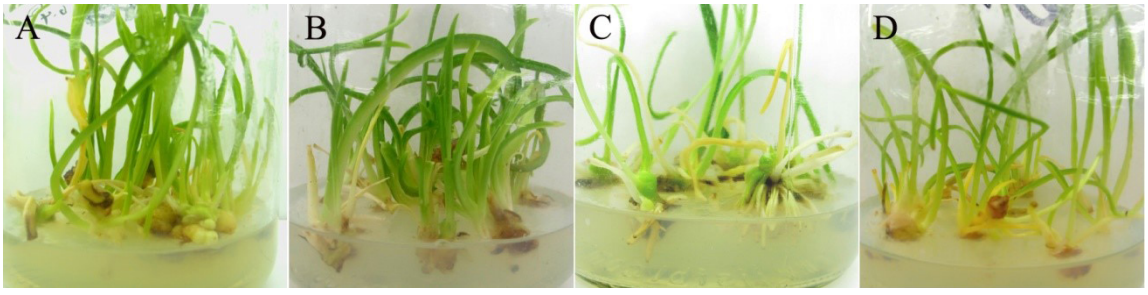

Fig. 2. Single factor experiment. The growth condition in (A) 3.0 mg·L^{-1} BA treatment; (B) 1.0 mg·L^{-1} KT treatment; (C) 1.0 mg·L^{-1} NAA treatment; and (D) 0.01 mg·L^{-1} 2,4-D treatment

tion of BA and NAA alone produced a proliferation coefficient (3.31) under the expected, therefore KT and 2,4-D were introduced in the assay and two L_9 (3^4) orthogonal experiments were conducted.

Table 2 shows the results of the orthogonal experiment. Rang (*R*) analysis with $R_{BA} > R_{KT} > R_{NAA} > R_{Error}$, indicates that BA was the main factor influencing the formation of new buds and therefore proliferation coefficient, followed by KT and NAA. The *R* values of all three PGRs were greater than that of the error (0.33), indicating that these three factors were all effective for the proliferation of *P. cyrtonema*. According to the analysis of variance (Tab. 3), BA had a highly significant effects on the proliferation of *P. cyrtonema* ($P < 0.01$), while KT and NAA had no significant effects ($P > 0.05$). The optimal combination of PGRs was $A_3B_2C_1$ (3.0 mg·L^{-1} BA + 1.0 mg·L^{-1} NAA + 1.0 mg·L^{-1}1 KT) by means of average value analysis (Tab. 2).

Comparing the results of the two L_9 (3^4) orthogonal experiments and the variance analysis of their data, BA/NAA/KT combination produced better than BA/NAA/2,4-D combination in proliferation as it produced more adventitious buds. Therefore, MS medium supplemented with 3.0 mg·L^{-1} BA, 1.0 mg·L^{-1} NAA and 1.0 mg·L^{-1} KT was selected as the medium for the proliferation of *P. cyrtonema* rhizome cultures. In this medium, 10 days after transplant, buds began to grow (Fig. 3A). After 20 days, the number of adventitious buds increased and they grew rapidly with the enlargement of rhizomes (Fig. 3B). After 30 days, adventitious buds grew even faster, and the rhizomes expanded further and became lumped (Fig. 3C). After 40 days, more bud spots appeared on the rhizome (Fig. 3D). After 50 days of culture, a 6.50 proliferation coefficient was registered (Fig. 3E). Longer culture led to a slow down in growth due to the limitation of growth space. At this time, subculture of cultures could be performed (Fig. 3F).

Table 2. Number of proliferated adventitious buds of *P. cyrtonema* with different combinations of BA, NAA and KT or 2,4-D: two L_9 (3^4) orthogonal experiment

Medium		Factor (mg·L^{-1})					Proliferation coefficient	
		A (BA)	B (NAA)	C		D (error)	KT	2,4-D
				KT	2,4-D			
1		2.0	0.5	1.0	0.01	(1)	3.58 ±0.72	4.63 ±0.25
2		2.0	1.0	2.0	0.05	(2)	3.05 ±0.83	3.30 ±0.18
3		2.0	1.5	2.5	0.10	(3)	3.24 ±0.57	3.02 ±0.43
4		2.5	0.5	2.0	0.05	(3)	3.08 ±0.62	3.42 ±0.17
5		2.5	1.0	2.5	0.10	(2)	3.92 ±0.85	3.01 ±0.23
6		2.5	1.5	1.0	0.01	(1)	4.32 ±0.12	3.98 ±0.37
7		3.0	0.5	2.5	0.10	(2)	4.87 ±0.4 6	4.77 ±0.45
8		3.0	1.0	1.0	0.01	(3)	6.50 ±0.33	5.14 ±0.41
9		3.0	1.5	2.0	0.05	(2)	5.35 ±0.50	4.36 ±0.28
KT	K_1	3.29	3.84	4.80		4.42		
	K_2	3.77	4.49	3.83		3.95		
	K_3	5.57	4.30	4.01		4.27		
	R	2.28	0.65	0.97		0.33		
2,4-D	K_1	3.65	4.27	4.58		4.32		
	K_2	3.47	3.82	3.69		3.69		
	K_3	4.76	3.79	3.60		3.86		
	R	1.29	0.49	0.89		0.63		

BA – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; KT – kinetin; 2,4-D – 2,4-dichlorophenoxyacetic acid; *Kn* – the mean value for n level of the factor; *R* – range

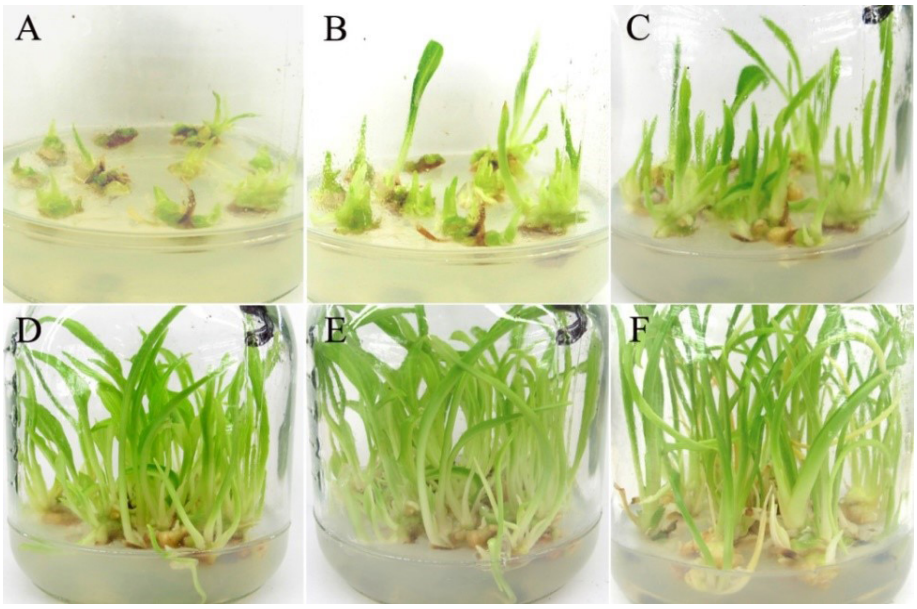

Fig. 3. Adventitious buds development on proliferation stage. (A) 10 days of culture; (B) 20 days of culture; (C) 30 days of culture; (D) 40 days of culture; (E) 50 days of culture; (F) 60 days of culture

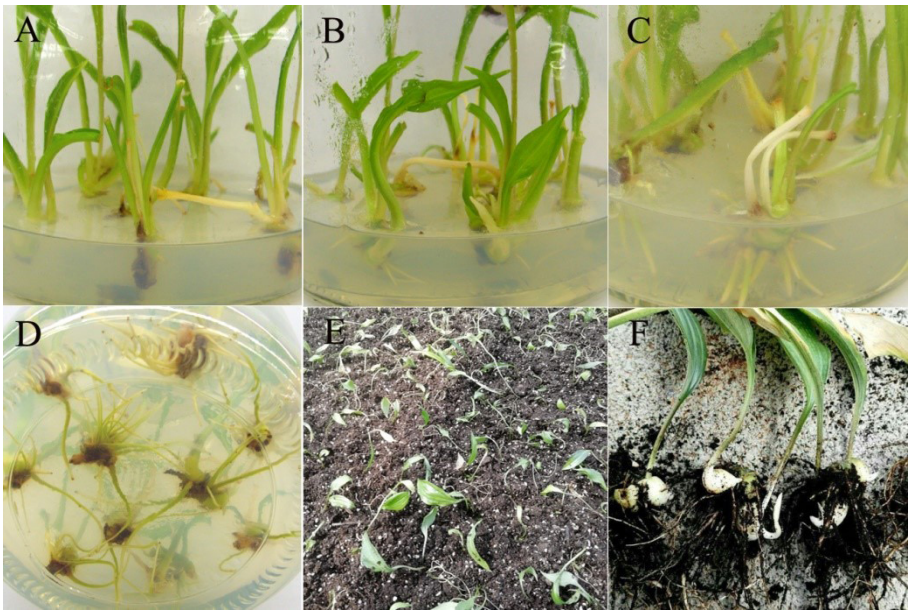

Fig. 4. Adventitious roots induction and acclimatization. (A) 20 days of culture on rooting medium: adventitious roots began to appear with the growth of new leaves; (B) 40 days of culture on rooting medium: the base of *in vitro* plants was enlarged, and adventitious roots grew rapidly; (C) 60 days of culture on rooting medium: plantlets had strong and thick roots; (D) Detail of the roots after 60 days of culture; (E) newly transplanted *in vitro* plants; (F) acclimated *in vitro* plants 60 days after transplanting

Adventitious roots induction and domestication acclimatization. Rooting experimental results are shown in Table 4. Using half of salts strength of MS medium produced significantly better rooting rate than full strength of MS medium for the same NAA concentration. Rooting rate was almost 100% in the range of 0.5–1.0 mg·L^{-1} NAA, while it decreased rapidly with higher concentration of NAA. Therefore, the best medium for induction of adventitious roots was half strength of MS medium supplemented with (0.5–1.0) mg·L^{-1} NAA, achieving the highest rate (100%) (Figs. 4A–D).

Rooted plantlets were transplanted into humus soil (Fig. 4E), and swollen rhizomes were observed 60 days later, with an acclimatization survival rate higher than 95% (Fig. 4F).

DISCUSSION

In vitro propagation and plant regeneration using tissue culture technology is of great significance for the protection and utilization of medicinal plants with long growth cycles and low reproductive capacity

Table 3. Variance analysis of proliferation coefficient produced by the combination of BA and NAA with KT or 2,4-D in the orthogonal experiment

PGR	Source	Type III sum of square	DF	Mean square	*F*	Significance
KT	A(BA)	8.687	2	4.344	11.097	$P < 0.01$
	B(NAA)	0.718	2	0.359	0.192	$P > 0.05$
	C(KT)	0.713	2	0.357	0.511	$P > 0.05$
	D(Error)	0.079	2	0.039		
2,4-D	A(BA)	2.913	2	1.456	3.869	$P < 0.05$
	B(NAA)	0.445	2	0.222	0.283	$P > 0.05$
	C(2,4-D)	0.627	2	0.314	1.558	$P > 0.05$
	D(Error)	0.044	2	0.022		

KT – kinetin; 2,4-D – 2,4-dichlorophenoxyacetic acid; DF – degree of freedom

Table 4. Effects of MS media and concentrations of NAA on rooting

Number	Basic medium	NAA (mg·L^{-1})	Inoculation bottles	Rooting rate (%)
CK-1	1/2MS	0	60	40.58 ±1.25 de
1		0.5	60	100.00 ±0.00 a
2		1.0	60	97.52 ±0.57 a
3		1.5	60	78.34 ±1.93 b
4		2.0	60	52.18 ±0.88 c
CK-2	MS	0	60	31.25 ±1.06 e
5		0.5	60	43.70 ±0.67 cd
6		1.0	60	39.10 ±0.80 d
7		1.5	60	38.65 ±0.96 de
8		2.0	60	35.46 ±1.17 e

NAA – α-naphthaleneacetic acid; CK – blank control; MS – Murashige and Skoog. Data are mean ± SE; different lower case letters in the same column mean significant difference ($P < 0.05$)

[Beena et al. 2003, Gonçalves et al. 2008]. Previous works on culture micropropagation of *Polygonatum* use rhizomes (with or without buds), seeds, tender stems, terminal buds, leaves, and anthers as initial explants, being rhizomes with buds and seeds the most frequently reported [Zhao et al. 2003, Bisht et al. 2012, Tiwari and Chaturvedi 2018, Zhao et al. 2019]. Some researchers pointed out that only adventitious bud could regenerate the plants of *Polygonatum*, as the high degree of differentiation of rhizomes, stems and leaves, makes it difficult to dedifferentiate and finally produce *in vitro* regeneration [Zhao et al. 2003]. In this study, adventitious bud proliferation on the nodes of the rhizome was inducted in the absence of callus, resulting in the establishment of an *in vitro* propagation system and plant regeneration of *P. cyrtonema*. This method of adventitious bud propagation was similar to the induction of basal stem bud cluster of *Lycium ruthenicum* Murr [Li et al. 2020] and *Halocnemum strobilaceum* (Pall.) Bieb. [Ti et al. 2016]. These adventitious buds formed on extremely shortened rhizome nodes. The larger of the rhizome and the more nodes it had, the more adventitious buds it would produce. Superficially, such adventitious buds were similar to adventitious buds produced by callus, but the nature of it was adventitious buds developed directly from on nodes rather than indirect organogenesis. In preliminary study, sterile leaves, petioles, and rhizomes were used as initial explants to try to induce callus formation through multiple combinations of exogenous PGRs, with no success. Therefore, the results of this study supported the views of Zhao et al. [2003] and Bisht et al. [2012] stating that only adventitious buds explants could regenerate plants of *Polygonatum*. Moreover, differences in *in vitro* regeneration methods (direct or indirect organogenesis) were reported for *Polygonatum* at present [Zhao et al. 2009, Tiwari et al. 2018, He et al. 2019], which might be explained by the view of regional differences. In the same way, differences on axillary shoot production were also reported for *Agave* plants from different geographical regions when the same parts of the plants were used as explants for *in vitro* propagation, showing obvious regional differences [Ramírez-Malagón et al. 2008]. Something similar happened when culturing 7 species of *Daphne* L. [Noshad et al. 2009]. Significant differences between rhizomes shape of *Polygonatum* plants from northern China and from south of the Yangtze River have also been reported [Ding and Zhao 1991]. Therefore, it could be inferred that the difference in existing reported methods of *in vitro* regeneration might be caused by the different sources of experimental materials.

Types and concentration of exogenous PGRs were important factors affecting adventitious bud formation, proliferation and regeneration of *P. cyrtonema*. This species showed growth in all tested PGRs, but the growth differed and no single treatment induced satisfactory proliferation. Cytokinin was superior to auxin in terms of proliferation potential. Both BA and KT alone showed signs of the rhizomes enlargement, accompanied by the emergence of new buds. NAA was beneficial to adventitious root induction. In the 1970s and 1980s, when tissue culture technology was developing rapidly, many researchers pointed out that in plants, cytokinins existed in a free form, and their main synthesis site was root tips with the effect of stimulating RNA synthesis and regulating cell cycle [Torrey 1972, Van-Staden and Davey 1979, Chen et al. 1985, Sundberg et al. 1991]. Auxin can not only promote cell elongation, but also stimulate vascular bundle differentiation, and participate in the differentiation of buds and roots. For example, plants grow fastest in spring, mainly because the buds produce auxin that stimulates the division of cells in the cambium [Aloni 2010, Xi et al. 2020]. Therefore, in plant tissue culture, cytokinin and auxin should be added at the same time in the general proliferative culture stage, and only appropriate auxin should be added in the rooting culture. The synergistic effects of PGRs combinations on the promotion of adventitious bud development and proliferation of this medicinal genus have been fully confirmed, such as the effect of the combination of BA and NAA for *P. verticillatum* (L.) All. [Bisht et al. 2012], BA and 2,4-D for *P. cyrtonema* Hua [Zhao et al. 2003], and other species, such as the combination of BA, KT and NAA for *Cyclocodon lancifolius* (Roxb.) Kurz [Xi et al. 2020], and ZT, NAA and KT for *Vaccinium corymbosum* L. [Wang et al. 2019]. Also, in this study, the synergistic effect of the combination of two cytokinins (BA and KT) and single auxin (NAA) on adventitious bud development and proliferation, was superior to that of the combination of a single cytokinin (BA) and two auxins (NAA and 2,4-D), indicating that cytokinins stimulated adventitious bud development, even

with the extremely short nodes of *P. cyrtonema*. The combination of cytokinins and auxin might stimulate the peak of RNA synthesis, resulting in the enlargement of the rhizome and the formation of adventitious bud cluster from the node. This study proved that the combination of BA, NAA and KT induced the enlargement of the rhizome and the occurrence of adventitious buds from node of *P. cyrtonema*. This method was not only effective in *in vitro* culture, but also had a high proliferation coefficient, thus achieving the goal of efficient and rapid propagation.

Adventitious roots induction in *P. cyrtonema*, was obtained either on 1/2MS or MS medium with different concentrations of NAA. However, 1/2MS medium added with 0.5–1.0 mg·L^{-1} NAA produced the best with 100% rooting rate. Numerous, non-callused adventitious roots formed at the base of the rhizome. After transplanting, the survival rate was high. Rooting rates of *P. cyrtonema* in blank control were also 40.58% on half strength of MS medium and 31.25% on MS medium. Rooting rate on MS medium with 0.5 mg·L^{-1} NAA ($P < 0.05$) was significantly higher than the rooting rate of the control. This phenomenon might be attributed to the accumulation and conversion of a certain proportion of exogenous PGRs into endogenous hormones during the proliferation and growth of adventitious buds. Therefore, adding a slightly high concentration of auxin in the rooting medium inhibited the formation of adventitious roots, which was consistent with the research of Wang et al. [2021].

In addition, shoots selection and preparation of materials were also very important to achieve excellent rooting and acclimatization rates. Strong main shoots should be selected from bud cluster, excess of rhizomes tissue and buds should be pruned away, and leaves should be cut from the lower 1/3 of the base. If rooting culture was carried out by cutting, it was easy to transfer oversized rhizome or bud cluster into the medium, which would cause small bud cluster to appear at the base of *in vitro* shoot, resulting in a decreased rooting rate. Even if adventitious roots grew well, survival rate after transplant was diminished.

CONCLUSIONS

For single factor experiments, the response of cytokinin was better than that of auxin, and single PGR was not conducive to the growth of the rhizome. The optimal PGR combination to achieve a good proliferation coefficient was neither found by complete combination experiments therefore conducting L_9 (3^4) orthogonal experiment was necessary to define the optimal culture media. The results showed that BA was the main factor affecting the formation and proliferation of adventitious buds. The combination of KT, NAA, and 2,4-D with BA had a strong synergistic effect. The optimal medium for the rhizome growth, adventitious buds occurrence and proliferation was MS medium added with 3.0 mg·L^{-1} BA, 1.0 mg·L^{-1} NAA and 1.0 mg·L^{-1} KT, with a proliferation coefficient of 6.5. The best rooting medium was found to be half strength of MS medium with 0.5–1.0 mg·L^{-1} NAA. The survival rate was higher than 95%.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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