Eur. J. Hortic. Sci. | ISSN 1611-4426 print, 1611-4434 online | https://doi.org/10.17660/eJHS.2022/029 | © ISHS 2022

Original article

In vitro organogenesis of basal stem cluster buds and callus culture of *Passiflora edulis* (*P. edulis* f. *edulis* Sims × *P. edulis* f. *flavicarpa* Degener)

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Summary

***Passiflora edulis* is a species among the third generation of emerging fruit lianas, and it has huge economic value and broad market prospects for China. However, owing to low awareness of the breeding of favorable varieties, coupled with its late domestication and subsequence artificial selection, there is a lack of favorable cultivars grown in China compared with other countries. The aim of this study was to establish an efficient regeneration method for maintaining the excellent characteristics of the F_1 hybrid between *P. edulis* f. *edulis* Sims and *P. edulis* f. *flavicarpa* Degener. In this study, the influence of explant type, medium composition, and plant growth regulators for *in vitro* propagation of *P. edulis* were studied with single factorial, complete combination, and orthogonal experimental designs. The results revealed an efficient regeneration protocol for *P. edulis* adventitious buds via two propagation methods, namely indirect callus-mediated bud organogenesis from leaf explants and direct basal stem cluster bud organogenesis from nodal segment explants. Although Olive medium (OM) supplemented with 1.5 mg L^{-1} 6-BA, 0.3 mg L^{-1} IBA, and 1.5 mg L^{-1} KT, easily generated adventitious buds from leaf explants through dedifferentiation and redifferentiation, this propagation method had some problems such as the length of the procedure, the poor quality of adventitious buds obtained and its low efficiency of bud formation. However, on OM supplemented with 1.0 mg L^{-1} 6-BA and 0.1 mg L^{-1} IBA, direct bud organogenesis from nodal explants overcame these problems, with a regeneration coefficient of 7.0. In addition, etiolation of leaves occurred after three generations in proliferation culture. To solve this problem, the OM was modified by increasing ferric salt content by 0.5 times. Adventitious root induction was accomplished on OM by adding 0.3 mg L^{-1} IBA, yielding a 98% rooting rate. The survival rate was more than 95%. An effective and stable regeneration system was determined in the present study, providing a technical foundation for *P. edulis* propagation and maintaining crosses with favorable characteristics.**

Keywords
adventitious bud, basal medium, leaves, nodal segment, proliferation coefficient, regeneration method

Significance of this study

What is already known on this subject?

- Tissue culture of *P. edulis* has already been achieved. However, owing to the different materials and methods used by the researchers, the results across studies are quite different.

What are the new findings?

- In this study, the most suitable basal medium was OM, while nodal explants were the most suitable explants. A cluster system was formed with both basal adventitious cluster buds and upper axillary buds growing, with a higher effective proliferation coefficient achieved via a method using basal stem cluster buds. To date, a regeneration system utilizing basal stem cluster buds has not been reported in *P. edulis*.

What is the expected impact on horticulture?

- These results provide a technical foundation for *P. edulis* propagation and maintaining crosses with excellent characteristics.

Introduction

Passiflora edulis is a species of fruit liana with extremely high economic value among the third generation of emerging fruit lianas (Dornelas et al., 2006). The fruits of *P. edulis* are not only eaten fresh but can also be processed into juice, fruit wine, fruit tea, and fruit vinegar, among other products, earning its reputation as the "king of juice" (Talcott et al., 2003). In addition to its consumption as a food, the fruit is also a traditional Chinese medicine with diverse medical and health-promoting properties (Dhawan et al., 2004; Boeira et al., 2010; Patel et al., 2011; Singh et al., 2012). However, there are still many obstacles in the actual agricultural production of *P. edulis*, including the cultivation of a single variety with low yield and poor resistance, among other issues. However, seed breeding also has disadvantages, such as a low seed germination rate, long recovering stage, low survival rate, and relatively low vigor, which ultimately lead to low yields. In addition, *P. edulis* field crops need to be rotated every three years because they are prone to several bacterial and viral diseases (Lombardi et al., 2007; Fischer and Rezende, 2008). Cultivation of cuttings from seedlings still has some prob-

lems, including long growth cycle, low survival rate, and the difficulty of long-term asexual reproduction. This method also enables the accumulation of viruses, leading to the gradual disappearance of excellent characters, declines in quality, and yield decreases from year to year (Zhang et al., 2000b). These unfavorable factors have restricted the development of industrial-scale planting and processing of *P. edulis*, which has limited the competitiveness of Chinese *P. edulis* in the global market.

Studies on *P. edulis* in markets outside of China are also relatively early, though its successful tissue culture was reported in the 1980s (Winks et al., 1988; Faria and Segura, 1997). The artificial propagation of *P. edulis* in China began in the early 21st century, and so far, there have been many related reports on this development (Zhang et al., 2000a, b; Feng et al., 2006; Sun et al., 2018). However, owing to differences in the materials and methods used by researchers, the results reported across various studies have differed considerably (Hall et al., 2000; Lombardi et al., 2007; Pinto et al., 2010). In particular, characterization of the most effective propagation methods, propagation coefficients, and growth cycle are still the key factors restricting cultivation of *P. edulis* at an industrial scale.

So far, Yunnan, China has been unable to not attain industrial scale *in vitro* propagation of *P. edulis* plants to meet the needs of developing this industry. The Xishuangbanna area in the south of Yunnan province has unique natural conditions for *P. edulis* cultivation. Accordingly, the cultivation of *P. edulis* is an emerging agricultural industry in Yunnan, and this species is one of the major bioengineering projects in this province. The hybrid of *P. edulis* f. *edulis* Sims and *P. edulis* f. *flavicarpa* Degener shows obvious heterosis, strong disease resistance, a more than 70% fertile fruit rate, and high sweetness (no application for new varieties). However, this hybrid was unstable and could not be maintained for its aforementioned excellent traits by means of sexual reproduction. Therefore, various organs of the hybrid F_1 generation were selected as materials to conduct systematic research for callus induction, occurrence of adventitious buds, axillary bud formation, rooting, and transplanting. This systematic methodology enabled the identification of efficient proliferation methods and the establishment of an efficient and stable regeneration system for *P. edulis*.

Materials and methods

Plant materials

The plantlet chosen for this study results from a cross between *P. edulis* f. *edulis* Sims (a native purple species from Longfei farm, Xishuangbanna, Yunnan) and *P. edulis* f. *flavicarpa* Degener (a yellow species from Taiwan). The experimental plant materials were collected from an annual F_1 hybrid with excellent characteristics at Longfei farm in Xishuangbanbana, Yunnan, China. The stem tip, nodal segments, and leaves of vigorously growing F_1 hybrids were used as explants for rapid *in vitro* propagation. These materials were soaked in 10% (*v/v*) mild detergent for 10 min and washed for 30 min under running tap water. Then, the washed explants were immersed into 75% (*v/v*) ethyl alcohol for 10 s and surface sterilized using 0.1% $HgCl_2$ (*w/v*), with the stem tips and nodal segments surface sterilized for 10 min and the leaves for 5 min. Finally, these materials were washed six times with sterile water and then inserted into the medium.

Single factor experiment

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), Olive medium (OM) (Wang et al., 2019), White medium and Woody Plant medium (WPM) (Lloyd and McCown, 1981) were used as basal media to investigate the suitable factors for the growth of different *P. edulis* explants (leaves, stem tips, and nodal segments) by adding different kinds of plant growth regulators (PGRs). Basal medium was supplemented with 6-benzylaminopurine (6-BA: 0.1, 0.5, 1.0, 1.5, and 2.0 mg L^{-1}), kinetin (KT: 0.05, 0.1, 1.0, 1.5, and 2.0 mg L^{-1}), α-naphthaleneacetic acid (NAA: 0.1, 0.5, 1.0, 2.0, and 3.0 mg L^{-1}), 3-indolebutyric acid (IBA: 0.05, 0.1, 0.3, 0.5, and 1.0 mg L^{-1}) or 2,4-dichlorophenoxyacetic acid (2,4-D: 0.01, 0.03, 0.05, 0.1, and 0.5 mg L^{-1}). Explant growth for each of the treatments was recorded after 45 d.

Basal culture medium and culture condition

The basal media were MS medium, OM, White medium, and WPM with 20 g L^{-1} sucrose (sucrose content in MS medium was 30 g L^{-1}) and 4.6 g L^{-1} agar (Ali et al., 2009). The pH value of all media was 5.4–5.6.

The growth chamber temperature was maintained at 21–23°C with a 12 h d^{-1} photoperiod at 1,500–2,000 lx irradiance provided by cool-white fluorescent tubes. Five explants (stem tips or nodal segments) in each bottle and three replicates were prepared for each treatment. For the leaf culture and single factor experiments, 10 culture bottles of eight materials per bottle were used, with three replicates per condition.

Callus induction, adventitious bud differentiation, and proliferation culture from leaf explants

According to a single factor experimental design, leaves were used as explants and OM as the basal medium to test callus culture induction, adventitious bud induction and the effect of several concentrations of three distinct PGRs, 6-BA (0.5, 1.0, and 1.5 mg L^{-1}), IBA (0.1, 0.3, and 0.5 mg L^{-1}), and KT (1.0, 1.5, and 2.0 mg L^{-1}). A three-level, four-factor factorial experiment, i.e., with $3^4 = 81$ treatments, with three replicates (243 experimental units), is difficult to conduct. Therefore, an L_9 (3^4) orthogonal experiment was developed to reduce the number of combinations, while still achieving the objective of conducting a comprehensive experiment (Wang et al., 2019). The callus induction rate and differentiation rate of adventitious buds were calculated after 35 d of *in vitro* culture.

Occurrence and proliferation culture of axillary buds and basal stem cluster buds

Stem tips and nodal segments were used as explant materials to study the effects of different combinations of PGRs on axillary bud formation, as well as on proliferation of basal stem clusters of buds. Basal OM medium supplemented with combinations of 6-BA (0.5, 1.0, and 1.5 mg L^{-1}) and IBA (0.1, 0.3, and 0.5 mg L^{-1}) was tested. The regeneration coefficient was calculated after 45 d.

Optimization of culture medium to mitigate leaf etiolation

To prevent the etiolation of leaves of cultured materials after three generations in proliferation culture, the optimized OM was modified further by increasing the amount of ferric salt by 0.5, 1.0, and 1.5 times. The regeneration coefficient was calculated, and the etiolation of the leaves was observed after 45 d.

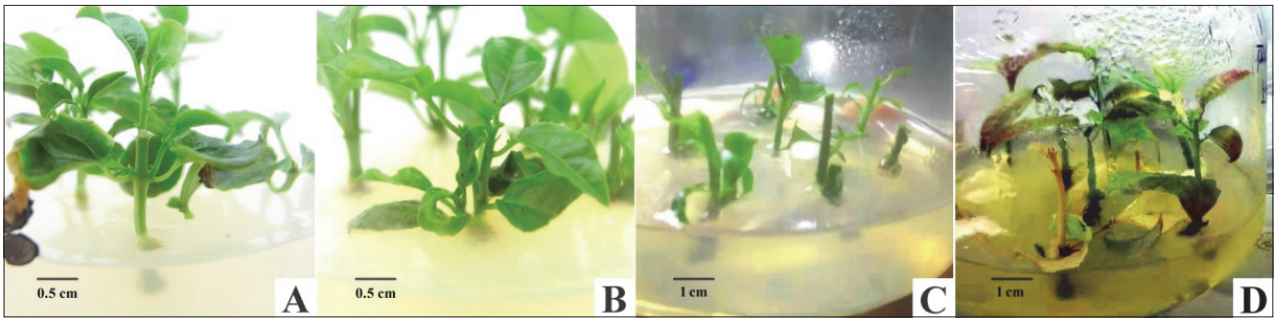


Figure 1. The results of the basal media. The growth situation: (A) Stem tip in OM medium after 20 d of culture. (B) Nodal segments in MS medium after 20 d of culture. (C) Nodal segments in White medium after 20 d of culture. (D) Nodal segments in WPM after 30 d of culture.

Rooting and transplantation

Based on the single factor experimental results, stem tips and nodal segments about 3 cm in height and with 2–3 leaves were selected as materials and inserted into OM medium with 0.0, 0.1, 0.3, or 0.5 mg L^{-1} IBA for rooting. The rooting rate was calculated after 45 d.

When the adventitious shoots grew to a height of about 6–8 cm, the rooting bottle was placed at room temperature (to avoid direct sunlight) for 3 d in order to plantlets adapt to the environment. The plants were removed from the culture medium, and the residual culture medium was removed, and plantlets transplanted into sterilized humus for thermal preservation and to moisturize the culture (temperature: 20–25°C; relative humidity: 75–95%). The survival rate and growth status of plants were calculated after 45 d.

Statistical analysis

Data were submitted to one-way analysis of variance using SPSS software (IBM Corp., Armonk, U.S.A.). The significance of the parameters tested was analysed, mean values were compared and declared significant at a probability level of $P \leq 0.05$ to determine the minor differences among various treatment methods. Similarly, the level of significance of mean values were subjected to Duncan's test using SPSS software. All measured data were calculated according to the following formulas.

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

Differentiation rate of adventitious buds = (the number of callus with buds / the total number of leaves with callus) × 100%;

Regeneration coefficient = the number of inoculation in subculture / the total number of initial inoculations;

Rooting rate = (the number of plants with adventitious roots / the total number of plants inoculation) × 100%;

Survival rate = (the number of surviving plants / the total number of transplantations) × 100%.

Results

Basal media culture experiment

The growth of *P. edulis* differed among basal media. The leaves of stem tips and nodal explants were green and grew normally in both OM and MS medium, while the growth rate in OM was significantly better than that in MS medium (Figure 1A and 1B). The plant material cultured in White medium and WPM turned red and then partly brown without obvious growth (Figure 1C and 1D). Thus, OM and MS medium were determined to be suitable media for rapid *in vitro* propagation of *P. edulis*.

Effects of concentration of individual PGR on adventitious bud formation from distinct explants

Callus tissue was induced from leaves under all PGRs treatments, but adventitious buds were only occasionally observed under the 1.0 mg L^{-1} 6-BA or 0.3 mg L^{-1} IBA treatments. A few axillary buds formed from nodal segments under the 6-BA, IBA, NAA, or KT treatment, with the base swelling under the 6-BA or KT treatments noticeably. The stem tip grew vigorously with adventitious roots occurring under IBA or NAA treatments, and the number and thickness of adventitious roots under the IBA treatment were particularly substantial. Thus, 6-BA, IBA, and KT were used as exogenous PGRs for rapid *in vitro* propagation of *P. edulis* in this study.

Callus induction, adventitious bud differentiation, and proliferation culture from leaf explants

Callus tissue was successfully induced from leaves in all experimental treatments, indicating that the leaves of *P. edulis* were easily dedifferentiated. However, the quality and redifferentiation ability of the calli were greatly affected by exogenous PGRs. Range (*R*) analysis of the occurrence rate of adventitious buds showed that 6-BA had the best response followed by those of KT and IBA, whereas the *R* value of the error had the lowest response, as shown in Table 1. This means three PGRs were found to be effective in inducing adventitious bud differentiation. Analysis of variance (Table 2) showed that 6-BA had a significant impact on adventitious bud differentiation ($P < 0.05$), which was positively correlated with the differentiation rate at concentrations of 0.5 to 1.5 mg L^{-1}. IBA and KT had no significant effect on adventitious bud differentiation ($P > 0.05$). As shown in Table 3, Duncan's test among calli treated with 6-BA showed that 1.5 mg L^{-1} 6-BA had a strong influence on the occurrence rate of adventitious buds, which was significantly different from the rate under 1.0 mg L^{-1} and 0.5 mg L^{-1} 6-BA treatments. The optimal PGRs combination for adventitious bud induction from the calli of leaf explants of *P. edulis* was 1.5 mg L^{-1} 6-BA, 0.3 mg L^{-1} IBA, and 1.5 mg L^{-1} KT according to mean value analysis.

Table 1 also shows that *R* analysis ranked the factors, in descending order, as follows: 6-BA, KT, Error, and IBA. This indicated that 6-BA was also the main factor that influenced the differentiation coefficient of adventitious buds. The concentration of 6-BA within the range of 0.5 to 1.5 mg L^{-1} was positively correlated with the differentiation coefficient of adventitious buds. *R* of 6-BA (1.660) and KT (1.000) exceeded the error term (0.577), suggesting that the effect of these two factors on the differentiation coefficient of adventitious buds was reliable. However, *R* value of IBA (0.284) was lower than that of the error term (0.577), suggesting that its effect

Table 1. The results of L_9 (3^4) mean number of callus with buds occurrence and differentiation from leaf explants in *P. edulis*.

No.		PGRs (mg L^{-1}) A 6-BA	B IBA	C KT	D Error	Occurrence rate of adventitious buds (%)	Differentiation coefficient of adventitious buds
L01		0.5	0.1	1.0	(1)	35.31 ± 0.11	1.57 ± 0.05
L02		0.5	0.3	1.5	(2)	56.85 ± 0.09	2.04 ± 0.07
L03		0.5	0.5	2.0	(3)	41.23 ± 0.18	1.36 ± 0.03
L04		1.0	0.1	1.5	(3)	72.81 ± 0.14	2.12 ± 0.05
L05		1.0	0.3	2.0	(1)	70.45 ± 0.08	1.66 ± 0.07
L06		1.0	0.5	1.0	(2)	66.43 ± 0.15	1.32 ± 0.04
L07		1.5	0.1	2.0	(2)	81.00 ± 0.17	2.47 ± 0.08
L08		1.5	0.3	1.0	(3)	87.24 ± 0.16	3.15 ± 0.06
L09		1.5	0.5	1.5	(1)	90.67 ± 0.14	4.33 ± 0.05
Occurrence rate of adventitious buds	K_1	44.463	63.040	62.993	65.447		
	K_2	69.897	71.513	73.443	68.093		
	K_3	86.303	66.110	64.227	67.093		
	R_1	41.840	8.473	10.450	2.646		
Optimal combination		$A_3B_2C_2$ (1.5 mg L^{-1} 6-BA + 0.3 mg L^{-1} IBA + 1.5 mg L^{-1} KT)					
Differentiation coefficient of adventitious buds	K_1	1.657	2.053	2.013	2.520		
	K_2	1.700	2.283	2.830	1.943		
	K_3	3.317	2.337	1.830	2.210		
	R_2	1.660	0.284	1.000	0.577		
Optimal combination		A_3C_2 (1.5 mg L^{-1} 6-BA + 1.5 mg L^{-1} KT)					

No.: The group number of the experiment (L01–L09); $K_{1\text{-}3}$: the mean value of different concentration of different PGRs; *R*: range; Error: a blank column, reflects the error caused by random factors.

Table 2. Analysis of variance for occurrence rate and of mean number of callus with buds in *P. edulis*.

Source	Sum of square	DOF	Mean square	*F* value	Significance
6-BA	2,666.619	2	1,333.309	25.272	$P<0.05$
IBA	110.418	2	55.209	0.115	$P>0.05$
KT	195.671	2	97.835	0.211	$P>0.05$
Error	10.461	2	5.230	0.011	$P>0.05$

DOF: degree of freedom.

Table 3. Duncan's test in three concentrations of 6-BA for occurrence rate of mean number of callus with buds in *P. edulis*.

PGRs concentration	Mean value	0.05 level
3 (1.5 mg L^{-1})	86.303	a
2 (1.0 mg L^{-1})	69.897	b
1 (0.5 mg L^{-1})	44.463	c

Different lowercase letters in the same column indicated the significant difference at $P<0.05$.

Table 5. Duncan's test in three concentrations of 6-BA for differentiation coefficient of mean number of callus with buds in *P. edulis*.

PGRs concentration	Mean	0.05 level
3 (1.5 mg L^{-1})	3.317	a
2 (1.0 mg L^{-1})	1.700	b
1 (0.5 mg L^{-1})	1.657	b

Different lowercase letters in the same column indicated the significant difference at $P<0.05$.

Table 4. Analysis of variance for differentiation coefficient of mean number of callus with buds in *P. edulis*.

Source	Sum of square	DOF	Mean square	*F* value	Significance
6-BA	5.371	2	2.686	6.897	$P<0.05$
IBA	0.136	2	0.068	0.054	$P>0.05$
KT	1.701	2	0.850	0.849	$P>0.05$
Error	0.500	2	0.250	0.208	$P>0.05$

DOF: degree of freedom.

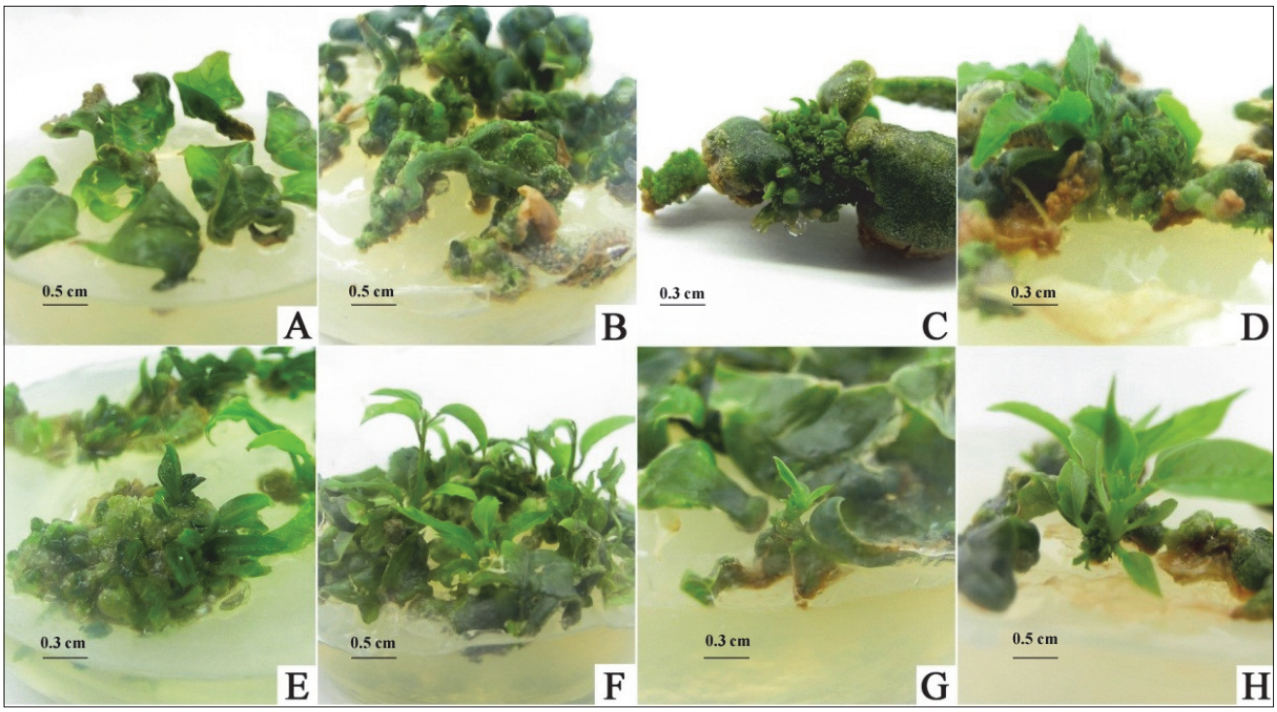


Figure 2. Indirect callus-mediated buds organogenesis from leaf explants. (A) The edge of leaves began to form callus after 7 d of culture. (B) Much callus after 14 d of culture. (C) Many adventitious bud points from callus after 21 d of culture. (D) and (E) More adventitious bud points occurrence with callus proliferation after 28 d of culture. (F) Adventitious bud elongation after 45 d of culture. (G) Individual adventitious bud after 3–5 d of callus formation. (H) Cluster buds at the base of the stem appeared with the growth of individual adventitious bud.

on differentiation coefficient of adventitious bud was not reliable. Analysis of variance (Table 4) showed that 6-BA had a significant effect on differentiation coefficient of adventitious bud ($P < 0.05$), while IBA and KT had no significant effect ($P > 0.05$). Duncan's test across three concentrations of 6-BA showed that 1.5 mg L^{-1} 6-BA had an enormous influence on the differentiation coefficient of adventitious buds, significantly differing from the coefficients under 1.0 mg L^{-1} 6-BA and 0.5 mg L^{-1} 6-BA (Table 5), while its mean value was also significantly higher than that of the other two concentrations. Based on mean value analysis, the optimal PGRs combination based on the differentiation coefficient of adventitious buds was 1.5 mg L^{-1} 6-BA and 1.5 mg L^{-1} KT.

This study determined that the induction rate of adventitious buds was more important than the differentiation coefficient via the L_9 (3^4) orthogonal test. Herein, OM with 1.5 mg L^{-1} 6-BA (A_3), 0.3 mg L^{-1} IBA (B_2), and 1.5 mg L^{-1} KT (C_2) was used as the synchronous medium for induction and differentiation of adventitious buds of *P. edulis.* The edges of leaves produced callus tissue after 7 d in this medium (Figure 2A), and calli increased significantly after 14 d with a 100% rate of callus induction (Figure 2B). Many adventitious buds appeared on calli after 21 d (Figure 2C). Then, the number of adventitious buds increased significantly, with a proliferation of calli occurring by 28 d (Figure 2D and 2E). After 35 d, adventitious buds began to grow into plantlets successively with a 91.33% occurrence rate of adventitious buds and a 4.30 differentiation coefficient of adventitious buds (Figure 2F; the result of optimal combination), which was not significantly different from the results achieved under the best combination of treatments L09 ($A_3B_3C_2$: OM with 1.5 mg L^{-1} 6-BA, 0.5 mg L^{-1} IBA and 1.5 mg L^{-1} KT). In addition, 9.57% of experimental materials presented individual adventitious bud at the vein incision shortly after the formation of calli (Figure 2G). Cluster buds at the bases of stems appeared with the growth of individual adventitious bud (Figure 2H).

It was noteworthy that indirect callus-mediated bud organogenesis from leaf explants did not form a whole proliferation cycle at 35–45 d. During this time, only 2–3 adventitious shoots with a small amount of callus tissue were used for subculture owing to the adventitious shoots being extremely thin. In the next subculture, although the calli continued to proliferate and a large number of adventitious bud points appeared, their growth was slowly accompanied by vitrification. Even when the culture period was extended to 60 d, only a few adventitious buds developed into plantlets, with a 3.36 effective proliferation coefficient.

Occurrence and proliferation culture of axillary buds and basal stem cluster buds

After callus-mediated bud organogenesis from leaf explants was established, the same orthogonal experiment was conducted for nodal explants, but the culture effect was not optimal. Axillary buds began to form from the top to the bottom in OM with 1.5 mg L^{-1} 6-BA, 0.3 mg L^{-1} IBA, and 1.5 mg L^{-1} KT at about 10 d (Figure 3A). Then, secondary lateral buds also appeared with the growth of axillary buds after 20 d (Figure 3B). A system with many branches and axillary buds was formed with a 4.07 regeneration coefficient after 45 d (Figure 3C). There was no swollen base and no callus in all experimental treatments for the culture of nodal segments during the whole culture cycle.

In Table 6 combined experiment, the bases of all the materials were swollen to produce calli with the occurrence of basal stem cluster buds occurring under all treatments other than the blank treatment. When IBA concentration remained unchanged, the regeneration coefficient increased at first and then decreased as 6-BA concentration increased (Table 6). The regeneration coefficient was the highest when 6-BA concentration was 1.0 mg L^{-1}, and significantly differed

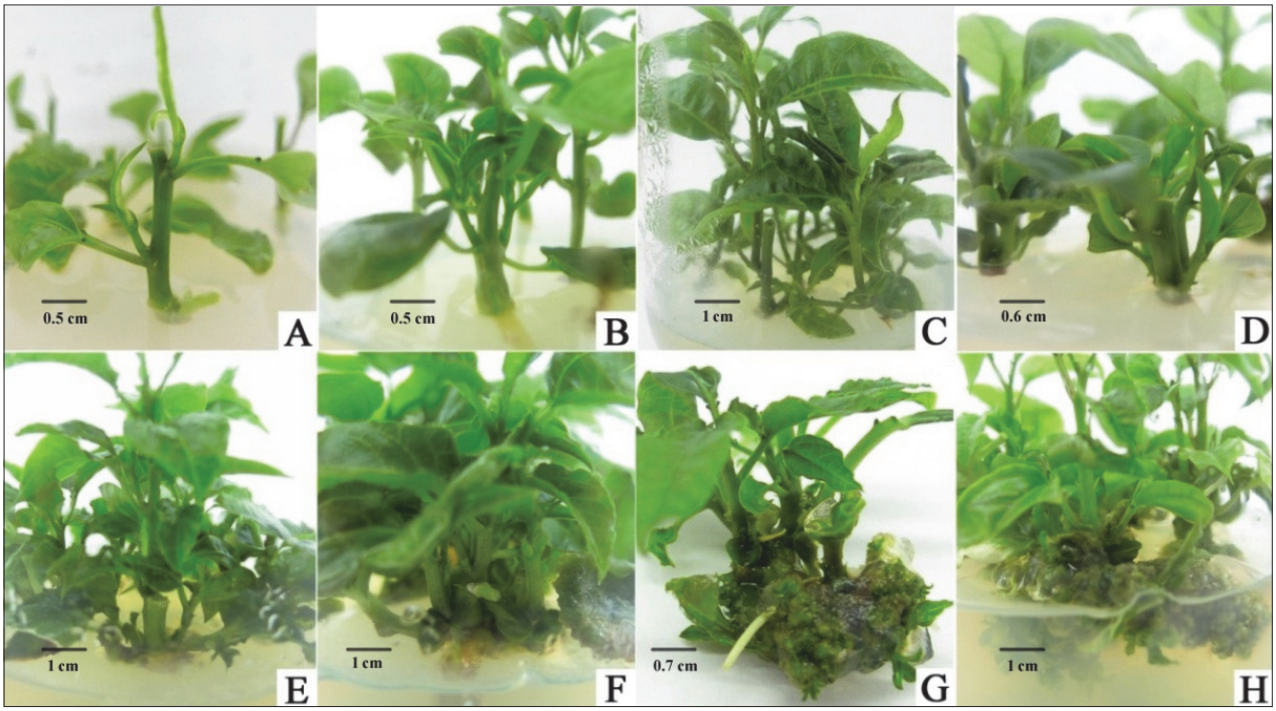


Figure 3. Direct basal stem cluster buds organogenesis from nodal explants. (A) Axillary buds started to form in the culture medium for callus induction from leaves after 10 d. (B) The secondary lateral bud appeared after 20 d of culture. (C) Growth condition after 30 d. (D) After 10 d of culture in S05 medium, axillary buds formed and the base swelled. (E) Sturdy basal stem cluster buds of stem with tight callus after 20 d of in S05 medium. (F) Basal stem cluster buds and more calli after 25 d of in S05 medium. (G) and (H) Calli began to differentiate into adventitious bud with the growth of basal stem cluster buds after 35 d of culture.

Table 6. Effects of a combination of 6-BA and IBA on nodal segment culture in *P. edulis*.

No.	PGRs (mg L^{-1})		Regeneration coefficient
	6-BA	IBA	
S01	0	0	1.09 ± 0.05 g
S02	0.5	0.1	3.55 ± 0.19 d
S03	0.5	0.3	3.25 ± 0.90 d
S04	0.5	0.5	2.21 ± 0.10 f
S05	1.0	0.1	7.53 ± 0.20 a
S06	1.0	0.3	6.13 ± 0.28 b
S07	1.0	0.5	5.45 ± 0.21 c
S08	2.0	0.1	4.21 ± 0.10 c
S09	2.0	0.3	3.09 ± 0.09 de
S10	2.0	0.5	2.67 ± 0.15 ef

No.: The group number of the experiment (S01–S10). Different lowercase letters in the same column indicated the significant difference at $P<0.05$.

from coefficients under other experimental treatments ($P<0.05$). The comparison of treatments S05 (OM with 1.0 mg L^{-1} 6-BA and 0.1 mg L^{-1} IBA), S06 (OM with 1.0 mg L^{-1} 6-BA and 0.3 mg L^{-1} IBA) and S07 (OM with 1.0 mg L^{-1} 6-BA and 0.5 mg L^{-1} IBA) indicated, the regeneration coefficient decreased from 7.53 to 5.45 as IBA concentration increased while 6-BA concentration was constant. When IBA concentration was 0.1 mg L^{-1}, the differences among the three treatments were significant, yielding the highest regeneration coefficient. Axillary buds formed and the base began to swell after 10 d in S05 medium (Figure 3D). Next, axillary buds at the tops of nodal segments grew rapidly, and the robust basal stem cluster buds appeared with tight calli after 20 d (Figure 3E). A large number of basal stem cluster buds were observed, and the calli increased after 25 d (Figure 3F). After 35 d, many adventitious buds began to differentiate from calli. The culture cycle lasted approximately 45 d, and the regeneration coefficient exceeded 7.0 (Figure 3G and 3H).

Optimization of culture conditions

The quality of *in vitro* propagated plants of *P. edulis* was inversely related to the number of proliferation subcultures. The leaves showed different degrees of etiolation beyond the

Table 7. Results of proliferation and growth in the different improved OM in *P. edulis*.

Media*	Regeneration coefficient	Growth condition
0.5 times	7.05	Etiolation of the leaves was significantly improved, with the leaves hypertrophy; plant well-grown and the growth cycle shorten.
1.0 times	6.42	Etiolation of the leaves was also improved, but the basal stem cluster buds were decreased.
1.5 times	5.11	Etiolation of the leaves was significantly improved, but the basal stem cluster buds were significantly reduced with long growth cycle.

* Increased volume of ferric salt on OM.

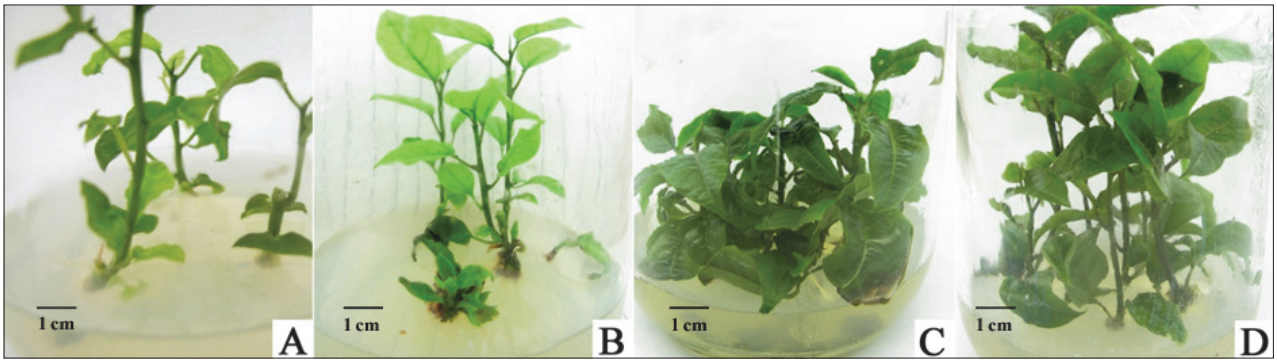


Figure 4. Etiolation of leaves and its reduction in *P. edulis*. (A) Etiolation in OM medium. (B) Etiolation in MS medium. (C) Growth condition in OM increasing the ferric salt content by 0.5 times. (D) Growth condition in OM increasing the ferric salt content by 1.5 times.

third generation. Meanwhile, these plants grew slowly, and their leaves gradually fell off as the number of generations increased, which seriously affected the regeneration coefficient and the quality of plantlets. This phenomenon was not effectively alleviated even when the basal medium type was changed (Figure 4A and 4B). In this study, OM was improved by comparing the basal medium components for the same PGRs. The culture results are summarized in Table 7.

Table 7 shows that the etiolation of the leaves was significantly reduced by using OM with additional ferric salt as the basal medium. Among the treatments tested, plants grew quickly with large fleshy leaves, with a growth cycle shortened to about 35–40 d and a regeneration coefficient that was not significantly decreased by increasing ferric salt by 0.5 times (Figure 4C). Increasing ferric salt by 1.5 times also effectively improved etiolation symptom of the leaves. However, the growth was slow, with a growth cycle prolonged to approximately 60 d, and the basal stem cluster buds were significantly reduced (Figure 4D).

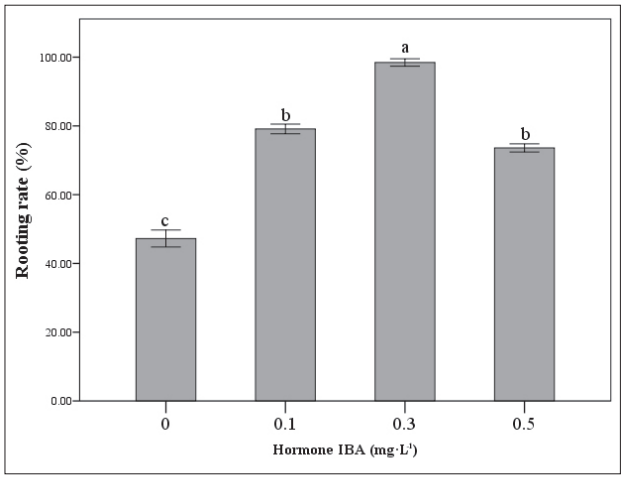


Figure 5. Effects of different IBA concentrations on rooting in *P. edulis*.

Rooting and acclimatization

The *in vitro* rooting results showed that adventitious roots were more easily produced from *in vitro* propagated *P. edulis* plants, and the rooting rate in the blank medium was 47% (Figure 5). The addition of IBA was more conducive to the growth of rooting plantlets, but the concentration should not be higher than 0.3 mg L^{-1}. The thickness of roots and plantlets increased in direct proportion to the increase in IBA concentration within the range of 0.1 to 0.3 mg L^{-1} IBA (Figure 6A). When the IBA concentration reached 0.5 mg L^{-1}, the rooting rate began to decline, and the roots became thinner and less numerous. Thus, the most suitable culture combination tested was OM with 0.3 mg L^{-1} IBA, in which rooting vigorously growing plantlets with thick roots were obtained within 45 d (Figure 6B). After acclimatization, plantlets were transplanted into humus soil, and new leaves grew after 25 d (Figure 6C). The survival rate at 45 d exceeded 95% (Figure 6D).

Discussion

Effect of basal medium types on rapid *in vitro* propagation of *P. edulis*

In previous studies on tissue culture of *P. edulis*, the basal media were mostly MS and 1/2 strength MS media (Faria and Segura, 1997; Zhang et al., 2000a; Feng et al., 2006; Sun et al., 2018), while Miller and White media (Li, 2018) were also occasionally used. In this study, OM and WPM, which have

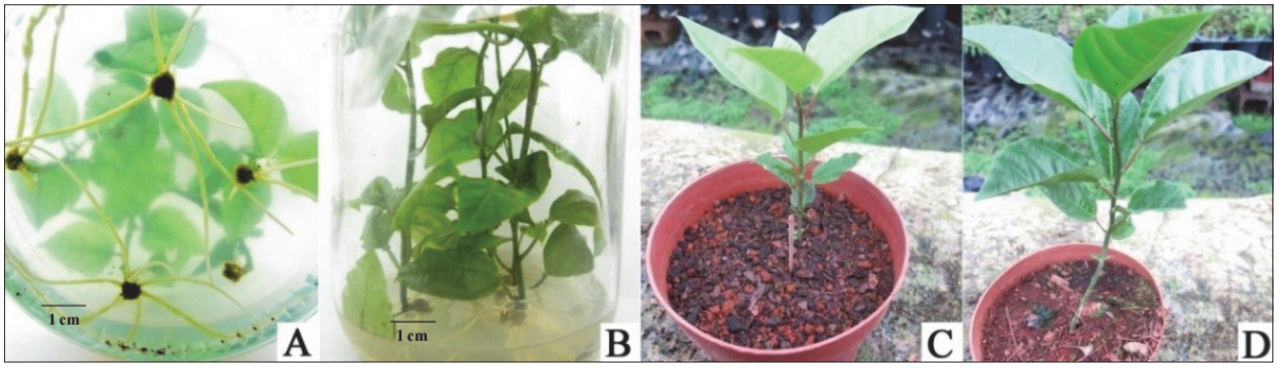


Figure 6. Rooting and transplanting in *P. edulis*. (A) Rooting plants after 25 d. (B) Rooting plants after 45 d. (C) Growth condition 25 d after transplanting to soil. (D) Growth condition after 45 d.

not been used for tissue culture of *P. edulis*, were utilized as well. Thus, OM was determined to be the most suitable basic medium for culture of *P. edulis*. Accordingly, the plant leaves yielded with OM were larger than those with other media, and the growth rate of axillary buds and basal stem cluster buds were also significantly higher than those achieved with other media (Figure 1). Likewise, MS medium was also suitable for the cultivation of *P. edulis*, which was consistent with most reports (Faria and Segura, 1997; Zhang et al., 2000a; Feng et al., 2006; Sun et al., 2018). However, the growth of plants in WPM and White medium was slow, with red leaves, leaf abscission at the base, and withering buds at the tops of plants, indicating they were not suitable for culture of *P. edulis*. MS medium has been widely used to culture a large number of plants, including some woody plants, because of its high concentration of inorganic salts and ions, especially nitrate, compared with other media. OM has more abundant magnesium, phosphorus, glutamine, and trace elements, thus obtaining better culture results for some woody plants (Ali et al., 2009). Contrasting with the results of other researchers, this study confirmed that OM, with its higher inorganic elements content, was more conducive to *in vitro* culture of *P. edulis*. OM significantly reduced the reddening of branches and leaves caused by insufficient N, P, K, Zn, S, and other elements. In addition, OM includes compounds not found in other media, such as biotin, folic acid, and especially glutamine. The main function of glutamine is to transport and store ammonia nitrogen, which indicated that the absorption and utilization of ammonia nitrogen in *P. edulis* may be important for *in vitro* culture of this *P. edulis*.

The results of this study showed that the basic culture medium had a great influence on the rapid *in vitro* propagation of *P. edulis*. If a particular basal medium is not suitable for plant growth, irrespective of the types or concentrations of PGRs used, the growth of *in vitro* propagated plants would not be ideal. Thus, the effects of the types and concentrations of PGRs on plant growth can be shown only when the medium is suitable.

Different types of *P. edulis* explants and proliferation methods

The type of explant used is one of the main factors affecting plant tissue culture. Different plant species and different organs from the same plant can have different responses to external induction, so the proliferation methods and effects of different explants differ (Li, 1996). In the published *P. edulis* tissue culture literature, most axillary buds or adventitious buds obtained were directly induced from nodal explants (Biasi et al., 2000; Pipino et al., 2008; Garcia et al., 2011; Sun et al., 2018). The results of this study showed that leaf explants of *P. edulis* were easy to dedifferentiate and re-differentiate into adventitious buds under appropriate PGRs types and concentrations (Table 1; Figure 2). However, this method had some problems, such as its long culture period, the poor quality of adventitious buds obtained, and the low efficiency of plantlet formation, making this method only suitable for the establishment of a primary aseptic system of excellent species. Additionally, a large number of adventitious buds can be obtained in a short time by inducing calli from leaves to differentiate adventitious buds, but this required 3–4 generations of rejuvenation culture to obtain thick and strong stems or stem tips for rooting culture of regenerated plants. This may be one of the reasons most researchers abandoned utilizing leaves as explants.

In the process of culturing nodal segments, it was found that the stem tissue contacting the medium or near the medium was swollen, and then thick adventitious cluster buds occurred, which was not reported by previous studies (Figure 3D–H). Different researchers have different views of the basal stem cluster buds. Wang et al. (2018) noted that the basal stem cluster buds of *Codonopsis bulleyana* were adventitious buds inducted from calli through an indirect organogenesis method. Ti et al. (2016) concluded that the basal stem cluster buds of *Halocnemum strobilaceum* were axillary buds that occurred in direct organogenesis. In contrast to *C. bulleyana*, the basal stem cluster buds of *P. edulis* were directly produced from nodal segments at the base, and they were not connected to calli. *P. edulis* has alternate single leaves unlike *Halocnemum strobilaceum*. In general, there is only one axillary bud on each node. However, the basal stem cluster buds of *P. edulis* produced from indeterminate positions of nodal segments were in contact with the medium, so they were considered to be adventitious buds. Therefore, this study speculated that the cell division of potential meristems of nodal segments of *P. edulis* was strengthened and that the number of cells increased to form dedifferentiated cell masses under the action of exogenous and endogenous PGRs. This caused the observed swelling and thickening of the bases of nodal segments, which then produced adventitious buds and broke through the epidermis of the nodal segments to form basal stem cluster buds. While calli and their adventitious bud points observed in the culture process resembled indirect callus-mediated bud organogenesis from leaf explants, their quality was not as good as that of basal stem cluster buds, which were discarded during the subsequent proliferation. In this culture method, with the occurrence and growth of basal stem cluster buds, axillary buds at all levels were constantly formed and grown. Finally, a cluster system was formed with both basal adventitious cluster buds and upper axillary buds growing, yielding a higher effective proliferation coefficient.

Effects of exogenous PGRs on rapid *in vitro* propagation of *P. edulis*

In the single factor experiment, leaves were able to produce calli under any single PGR, and the stem tips and nodal segments also had different degrees of growth, indicating that *P. edulis* was extremely sensitive to exogenous PGRs. This may have been owing to the rapid growth of *P. edulis* in nature, resulting in the rapid consumption of endogenous PGRs in culture. The combination of 6-BA, IBA, and KT showed better synergistic effects on callus-mediated bud organogenesis from leaf explants, exceeding the effects of either compound alone or both combinations of compounds (Figure 2). Notably, the regeneration coefficient of basal stem cluster buds under the combination of three PGRs (6-BA, IBA, and KT) was much lower than that under combined 6-BA and IBA treatment (Figure 3). These results indicate that KT can promote cell division and induce bud differentiation and development, but it significantly inhibited the occurrence of adventitious buds in *P. edulis* tissue culture. Whether this phenomenon indicated that KT only acts on calli that had been dedifferentiated into meristem tissue, but had an inhibitory effect on the proliferation and differentiation of potential or existing meristem tissue, remains to be further verified.

Etiolation of leaves and its reduction

Etiolation of the leaves of plants from *in vitro* culture has been reported in *P. edulis* and some species, and most of these studies reduced this effect by increasing magnesium salt and

ferric salt in a basal medium (Monteiro et al., 2000; Pestana et al., 2012). However, the concentration of magnesium salt (731 mg L^{-1}) in OM was already much higher than that in other media. Therefore, this study reduced etiolation of leaves by increasing the concentration of ferric salt in OM. The results showed that the etiolation symptom of *P. edulis* leaves was completely reduced by increasing ferric salt content by 0.5 times. Notably, higher ferric salt content prolonged the growth cycle, with a decrease in the regeneration coefficient and other adverse consequences (Table 7).

Conclusion

In this study, the most suitable explant for indirect organogenesis was leaves, and the most suitable explant for direct organogenesis was nodal segments. Calli were induced from leaf explants with a 100% induction rate on OM with 1.5 mg L^{-1} 6-BA, 0.3 mg L^{-1} IBA and 1.5 mg L^{-1} KT, yielding a 91.33% occurrence rate and 4.30 differentiation coefficient of adventitious buds. Cluster systems were formed with both basal adventitious cluster buds and upper axillary buds growing on OM with 1.0 mg L^{-1} 6-BA and 0.1 mg L^{-1} IBA, with a 7.0 regeneration coefficient. Additionally, etiolation of leaves occurred after three generations in proliferation culture, but was addressed by increasing the ferric salt content of OM by 0.5 times. Induction of adventitious roots was accomplished on OM by adding 0.3 mg L^{-1} IBA, yielding a 98% rooting rate. The survival rate of plants grown on this medium for 45 d exceeded 95%.

Acknowledgment

The research was supported by Yunnan Breeding and Cultivation Research and Development Center of Endangered and Daodi Chinese Medicinal Materials (No. 2016DH011).

Conflict of interest

The authors declare that they have no conflict of interest.

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Received: Dec. 8, 2020
Accepted: Oct. 27, 2021

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