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Callus induction and *in vitro* plant regeneration of *Primula denticulata* subsp. *sinodenticulata*

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ABSTRACT: *Primula denticulata* subsp. *sinodenticulata* is an endemic medicinal plant from China; however, its resources are extremely scarce due to a lot of obstacles such as scattered distribution, long natural renewal cycle, and overexploitation for medicinal value. In this study, a rapid propagation protocol *in vitro* of *P. denticulata* subsp. *sinodenticulata* was established, hoping to provide a possible way for resource protection. The leaves and petioles of different ages of the plant were selected as explants, and Murashige and Skoog (MS) medium were used as the basic medium. The appropriate types and concentrations of hormones for growth were selected via a single factor, and then the effects of types and concentrations of different hormones on callus induction and cluster bud occurrence were investigated by a complete combination experiment. The study showed that the callus with a strong redifferentiation capacity was obtained from 8- to 10-day old leaves and 10- to 20-day old petioles in MS medium with 0.5 mg/l 6-benzylaminopurine (BA) and 1.0 mg/l α-naphthaleneacetic acid (NAA) after 30 to 40 days of culture. Subsequently, green cluster bud started to appear with 100% occurrence rate. The obtained cluster bud was subcultured in the aforementioned medium with 12.67 proliferation coefficient. The optimal rooting medium was the hormone-free 1/2 MS medium; the rooting rate was 100%, and the survival rate was over 95%. In this study, micropropagation of *P. denticulata* subsp. *sinodenticulata* was established, which can provide an optional method for the protection of wild resources.

KEYWORDS: cluster bud, NAA, bud organogenesis, petiole, leaf

INTRODUCTION

Primula denticulata subsp. *sinodenticulata*, a perennial herb in *Primula* genus of Primulaceae, is a subspecies that stretches into Yunnan, Sichuan, and Guizhou Province of China. This plant is not markedly different in character from its original species. However, compared with the original species, flowering scape is more robust, usually 3–6 times as long as the leaf rosette, and the corolla is slightly larger. Furthermore, the distribution area is not connected with that of the original species. Florescence is in March to April, and fruit period is in April. It is an endemic medicinal plant of China, mainly produced in Cangshan of Dali city Yunnan Province, Luding of Sichuan Province, and Leishan of Guizhou Province, in which it usually grows in hillsides, meadows, or bushes at an altitude of 1500–3000 m [1].

P. denticulata subsp. *sinodenticulata* is commonly known as Mi San Hua or Bao Chun Hua in China. Its root is used as a traditional medicine with the efficacy of restoratives, curing malnutrition, and lactagogue. It is usually used to treat a cough caused by physical weakness, physical weakness after illness, emaciation, and abdominal swelling for infant, and little or no milk for postpartum mothers [2]. In addition, there is also literature pointing out that it has the function for promoting blood circulation, eliminating skin discomfort, and relieving pain. Local inhabitants generally use the root of this plant as medicine for treating stomachache, abdominal pain, gout, fractures, bruises, and skin diseases [3].

In the original habitat, *P. denticulata* subsp. *sinodenticulata* is easy to be recognized during flowering because of beautiful attractive flowers. As a result, the flower of the species is illegally picked by tourists, resulting in incomplete the life cycle and low reproduction rate. Furthermore, due to its remarkable medicinal value, a large number of the wild plants were excavated, thereby resulting in destruction of its habitat, low wild population, and the number of individuals. At present, some habitats are hard to find, which seriously limits its research and development work. In a word, the direct harvesting of natural resources cannot fundamentally solve the problem of resource shortage due to the factors of scattered distribution, long natural renewal cycle, and excessive mining in *P. denticulata* subsp. *sinodenticulata*. Thus, the rapid artificial propagation and cultivation with scientific and rational sustainable use of resources is a better choice.

According to related reports, there are two main methods used on plant regeneration of *Primula*. One is indirect callus or embryonic callus-mediated bud

organogenesis from leaf, petiole, flower, or stem tip explants [4, 5], and other is direct bud organogenesis from seed, stem segment, or shoot tip explants [6, 7]. For *P. denticulata* subsp. *sinodenticulata*, due to the plant morphology, dense leaves grow in basal rosette; *in vitro* culture via shoot tip as explants is difficult to achieve. Moreover, the number of shoot tips produced by each plant is limited, and the use of shoot tips will seriously damage the female plant. Using inflorescences as explants is seasonal. Petioles and leaves are abundant and readily available without destroying the parent plant. Therefore, in this study, the leaves and petioles of different leaf ages were used as explants to establish an efficient system of callus induction and plant regeneration, and the propagation coefficient fully achieved the purpose of large-scale rapid propagation. This technique can provide an optional method for protecting the wild resources and developing artificial cultivation of *P. denticulata* subsp. *sinodenticulata*.

MATERIALS AND METHODS

Plant materials

Thirty mother plants (*Primula denticulata* subsp. *sinodenticulata*) were collected from Cangshan valley, Dali city, Yunnan Province, China (25°36′ N; 100°10′ E; Alt: 2515 m) in March 2018. Twenty-five plants with soil were transplanted in Yunnan Breeding and Cultivation Research and Development Center of Endangered and Daodi Chinese Medicinal Materials, Yunnan University of Chinese Medicine. Leaves and petioles were collected from these plants for establishing a sterile system.

Establishment of aseptic system

Firstly, these materials were soaked with 10% detergent solution (w/v) for 5 min and then were rinsed thoroughly with tap water for 30 min. Subsequently, on a sterile operating platform, they were surface sterilized in 75% ethanol solution (v/v) for 10 s, next in 0.1% $HgCl_2$ (w/v) for 8 min, and rinsed with sterile water for 6 times, each time for no less than 3 min. Ultimately, sterile filter paper was used to absorb the surface moisture of these materials for further initial culture.

Basal medium

In addition to rooting culture, the basic medium at all stages was the MS (Murashige and Skoog) medium with 3% sucrose (w/v) and 0.55% agar (w/v), and different concentrations of 6-BA (6-benzylaminopurine), KT (6-furfurylaminopurine), NAA (α-naphthaleneacetic acid), 2,4-D(2,4-dichlorophenoxyacetic acid), and IBA (indole-3-butyric acid) were added according to the actual requirements. The reagents and hormones used in the experiment were purchased from Dingguo Biotechnology (Beijing, China). The pH of the medium was adjusted to 5.6–5.8 with 1 N of HCl before autoclaving (121 °C, 22 min).

Initial culture medium

Basal medium supplemented with different types and concentrations of plant hormones was used for single factor experiment. Among them, the concentrations of 2,4-D and KT were 0.01, 0.05, 0.1, and 0.5 mg/l. The concentrations of NAA, IBA, and 6-BA were 0.1, 0.5, 1.0, and 1.5 mg/l. Thus, the types and concentrations of the hormones suitable for the growth of the leaf and petiole were obtained for further experiment.

Selection of the suitable age of explants and its culture medium

The leaves at the age of 1 to 7, 8 to 10, 11 to 20, and more than 20 days old and petioles at the age of 1 to 9, 10 to 20, 21 to 30, and more than 30 days old were selected for the experiments. These materials were cultured in MS medium supplemented with 0.1 and 0.5 mg/l of NAA or 0.1 and 0.5 mg/l of 6-BA. After 40 days of culture, the callus induction rate was counted.

Synchronous medium for callus induction, cluster bud occurrence, and proliferation

According to results of above experiments, 8- to-10-day old leaves and 10- to 20-day old petioles were used as explants, and 6-BA and NAA were used as factors (0.1, 0.5, and 1.0 mg/l, respectively) for the complete combination experiments (B01–B10). After 40 days of culture, the callus induction rate and cluster bud occurrence rate were calculated. The materials used in the proliferation phase were defined as the callus with 3 to 4 bud points, whose size was about 1.0 cm × 1.0 cm. Meanwhile, the leaves and petioles were excised from strong young buds and subcultured into the optimal medium for callus induction and cluster bud occurrence. After 40 days of culture, the proliferation coefficient was counted.

Rooting culture medium

The main buds growing to 3 to 5 cm were excised from cluster bud for rooting culture. Rooting medium was the 1/2 MS medium with 3% sucrose (w/v) and 0.55% agar (w/v), which included NAA at different concentrations. The concentration of NAA was set to 0.0, 0.1, 0.5, 1.0, and 1.5 mg/l for 5 treatments (R01–R05). After 40 days of culture, the rooting rate and root growth states were counted.

Culture conditions and inoculation methods

The temperature of the culture chamber was controlled at 22 ± 1 °C. The illumination time was 10 h/day with 1500–2000 lx illumination intensity. For initial culture and selection of the suitable age of explants, every treatment consisted of 5 bottles with 3 materials (leaf

or petiole) per bottle. For complete combination and rooting culture, each treatment was inoculated with 20 bottles of 3 materials in each bottle (culture of callus induction and cluster bud occurrence: 8- to 10-day old leaf or 10- to 20-day old petiole; proliferation culture: callus with 3 to 4 bud points; rooting culture: the main buds with 3 to 5 cm height). In addition to the initial culture, the above experiments were repeated for 3 times, and the number of materials in each treatment was replenished if contamination occurred.

Acclimatization and transplant

The bottles with thick roots and rooting plant 8 to 10 cm height (30-day old plant) were exposed to natural light. After 3 days, the sealing film was removed for adapting to the environment for 2 days. Then, the plants were taken out, and the adhered agar at the root was washed carefully. After that, the root system of those plants was soaked with 0.1% carbendazim (w/v) for 5 min and transplanted into plastic containers with sandy soil. The transplanted plantlets were covered by a clear plastic bag for maintaining a 20 to 25 °C temperature and 70% relative humidity. The plastic bag was opened for the 1 to 2 openings (2.0 cm^2) with a frequency of once every 2 days and then removed after 10 days. The survival rate of plantlet was computed after 30 days of transplantation.

Statistical analysis

The obtained data were processed and analyzed by SPSS software (IBMCorp, Armonk, USA). The calculation methods of the data were as follows: Callus induction rate (%) = (the number of explants with callus)/(the number of explants) × 100%; Cluster bud occurrence rate (%) = (the number of callus with adventitious buds)/(the number of callus inoculations) × 100%; Proliferation coefficient = (the number of cluster bud in subculture)/(the number of initial inoculations); The rooting rate (%) = (the number of rooted plantlets)/(the number of plantlet inoculations) × 100%; and The survival rate (%) = (the number of survival plantlets)/(the number of transplantations) × 100%.

RESULTS

Single factor experiment

The obtained results showed the responses of two explants were similar in all treatments. In the 2,4-D treatment, only partial expansion of the material was observed with a small amount of callus at the incision edge, but no adventitious bud differentiation was observed (Fig. 1a). In the KT treatment, almost whole material was chlorosis, but occasionally, green bud spots were observed at the incision without obvious callus (Fig. 1b). An almost similar response occurred in both NAA and IBA treatments, in which the explants grew well and induced small green conical bud points with tight texture, especially at the concentration of 0.5 to 1.0 mg/l (Fig. 1c and 1d). In the 6-BA treatment, both materials could induce green and compact callus with adventitious cluster bud formation, and the appropriate concentration was 0.1 to 0.5 mg/l (Fig. 1e and 1f). To sum up, 6-BA and NAA were suitable hormones for *in vitro* culture of *P. denticulata* subsp. *sinodenticulata*.

Selection of different explant ages

In single factor experiment, it was found that the same explant type at different ages showed unresponsiveness and low response, which proved that the selection of the time of collecting the explants of *P. denticulata* subsp. *sinodenticulata* was an important factor. Therefore, the response ability of explants of different ages was researched. As seen in Fig. 2, the callus induction rate of 8- to 10-day old new leaves was up to 25.5%. Eleven- to twenty-day old leaves were put into the same medium; most of the leaves turned yellow after 7 days, and callus induction was only 14.5%. More than 20-day old leaves could hardly induce callus and gradually withered. Using petioles as explants, 10 to 20 days old had the highest callus induction rate (30.33%). However, petioles above 30 days old could hardly induce callus, and all of them withered and died with the extension of culture time. Thus, the suitable explants for rapid propagation *in vitro* of *P. denticulata* subsp. *sinodenticulata* were 8- to 10-day old leaves and 10- to 20-day old petioles.

Callus induction and cluster bud occurrence

In the complete combination experiments, except CK treatment, both 8- to 10-day old leaves and 10- to 20-day old petioles could induce callus with adventitious bud differentiation, but occurrence frequency was significantly different between treatments (Table 1). As can be seen from Table 2, when NAA concentration was constant, the callus induction rate and cluster bud occurrence rate within the range of 0.1 to 0.5 mg/l of 6-BA increased with the increase the concentration of 6-BA, reacing the maximum value at 0.5 mg/l. At more BA concentration than 0.5 mg/l, the callus induction rate and cluster bud occurrence rate were falling fast. In addition, it was observed that the response ability of petiole explants was slightly higher than that of leaves in callus induction, but there was no significant difference in cluster bud occurrence.

After 10 days of 8- to 10-day old leaf culture in B07 medium (MS + 6-BA 0.5 mg/l + NAA 1.0 mg/l), the edges began to curl, and green callus appeared from the incisions (Fig. 3a). After 20 days, green callus appeared at the whole leaf edge, and the callus from an incision began to differentiate into bud points (Fig. 3b). After 30 days, the callus at the leaf edge began to differentiate into bud points, and the bud points at the incision site formed green conical buds

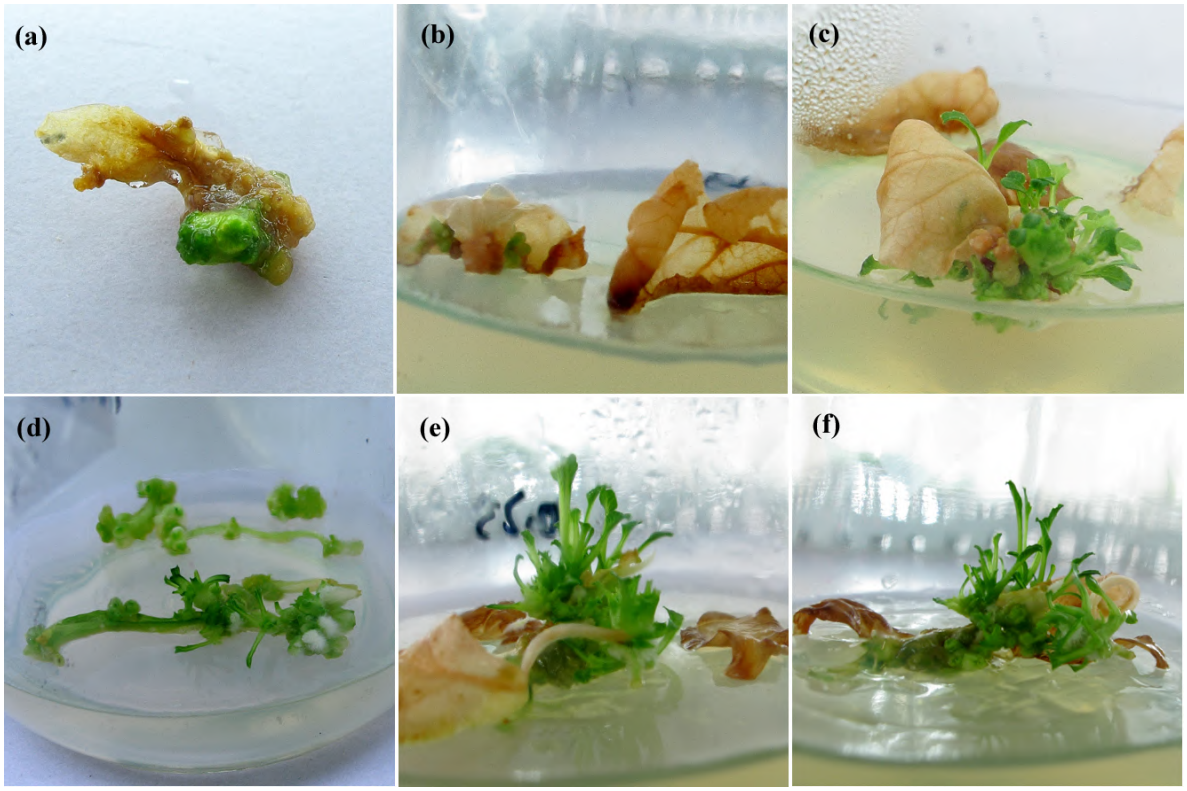

Fig. 1 Single factor experiments of *P. denticulata* subsp. *sinodenticulata*. (a) The growth condition of the leaf in the 2,4-D treatment. (b) The growth condition of the leaf in the KT treatment. (c) The growth condition of the leaf in 1.0 mg/l NAA. (d) The growth condition of the petiole in 1.0 mg/l NAA. (e) The growth condition of the leaf in 0.5 mg/l 6-BA. (f) The growth condition of the petiole in 0.5 mg/l 6-BA.

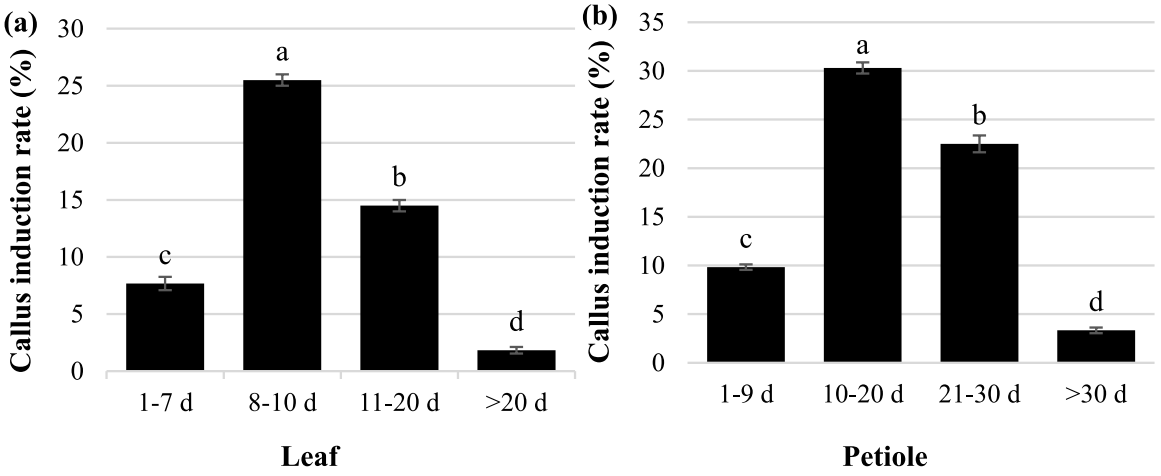

Fig. 2 Screening of different explant ages of *P. denticulata* subsp. *sinodenticulata*.

(Fig. 3c). After 40 days, with the increase of bud points, the former buds rapidly grew into dense green cluster bud (Fig. 3d). In B07 medium, the callus induction rate from 8- to 10-day old leaf explants was 90%. Surprisingly, as long as callus was present, the occurrence rate of the cluster bud would be 100%.

Like bud formation mediated by callus on leaf explants, 10- to 20-day old petiole was cultured in B07 medium for 10 days, and the edges began to wrinkle and bulge with green callus occurrence (Fig. 3e). After 20 days, callus appeared in the entire edge of the material with the appearance of obvious bud points

Table 1 Effects of different hormone combinations on callus induction rate and cluster bud occurrence rate of two explants of *P. denticulata* subsp. *sinodenticulata*.

Medium	Hormone (mg/l)		Leaf		Petiole	
	6-BA	NAA	Callus induction rate (%)	Cluster bud occurrence rate (%)	Callus induction rate (%)	Cluster bud occurrence rate (%)
B01	0	0	0.00	0.00	0.00	0.00
B02	0.10	0.10	46.67 ± 1.22^{h}	71.43 ± 0.12^{d}	50.00 ± 1.36^{e}	73.33 ± 0.05^{e}
B03	0.10	0.50	50.00 ± 1.18^{gh}	80.00 ± 0.09^{c}	56.67 ± 1.28^{de}	77.78 ± 0.11^{de}
B04	0.10	1.00	53.33 ± 1.22^{fg}	81.25 ± 0.13^{c}	60.00 ± 1.14^{cd}	82.35 ± 0.14^{cd}
B05	0.50	0.10	66.67 ± 1.51^{c}	90.00 ± 0.05^{ab}	80.00 ± 1.21^{b}	91.67 ± 0.14^{b}
B06	0.50	0.50	76.67 ± 1.36^{b}	91.30 ± 0.12^{ab}	83.33 ± 1.46^{b}	90.12 ± 0.07^{b}
B07	0.50	1.00	90.00 ± 1.47^{a}	100.00 ± 0.00^{a}	97.33 ± 1.17^{a}	100.00 ± 0.00^{a}
B08	1.00	0.10	56.67 ± 1.25^{ef}	88.24 ± 0.07^{b}	78.94 ± 1.05^{b}	87.50 ± 0.07^{b}
B09	1.00	0.50	60.00 ± 1.05^{cde}	88.89 ± 0.07^{b}	66.67 ± 1.35^{c}	88.00 ± 0.10^{bc}
B10	1.00	1.00	58.33 ± 1.33^{cde}	86.47 ± 0.05^{b}	63.33 ± 1.10^{cd}	85.00 ± 0.16^{c}

B01–B10: culture medium number; 6-BA: 6-benzylaminopurine; NAA: α-naphthaleneacetic acid; Data are mean ± SE; different lowercase letters in the same column mean significant difference ($P < 0.05$).

Table 2 Effects of different concentrations of NAA on adventitious root induction of *P. denticulata* subsp. *sinodenticulata*.

Medium	NAA(mg/l)	The number of root	The rooting rate (%)	The root system status
R1	0.0	33.50 ± 1.95^{a}	100.00 ± 0.00^{a}	The root system was robust and developed; the base had no callus.
R2	0.1	26.33 ± 1.25^{b}	76.67 ± 1.95^{b}	The root system was well developed; the base had occasional callus and cluster bud.
R3	0.5	23.23 ± 1.44^{bc}	66.65 ± 1.81^{c}	The roots were long and robust with a few calluses and cluster bud at the base.
R4	1.0	18.16 ± 1.87^{c}	60.00 ± 1.36^{cd}	The root system was slender with many shorter branches, and the base had the callus and cluster bud.
R5	1.5	17.62 ± 0.97^{c}	56.35 ± 1.50^{d}	The roots were few and thin with numerous callus and cluster bud at the base.

R1–R5: culture medium number; NAA: α-naphthaleneacetic acid; Data are mean ± SE; different lowercase letters in the same column mean significant difference ($P < 0.05$).

(Fig. 3f). After 30 days, the callus proliferated rapidly, and bud point grew into cluster bud (Fig. 3g). After 40 days, the entire petiole was covered by a mixture of callus and adventitious cluster bud (Fig. 3h). In this medium, the callus induction rate from 10- to 20-day old petiole explants and the occurrence rate of adventitious cluster bud was 97.33% and 100%, respectively.

Proliferation culture

Callus with 3 to 4 bud points was transferred into newly configured B07 medium. After 10 days, the callus increased significantly, and a large number of bud points appeared on it (Fig. 4a and 4b). After 20 days, with the growth of early buds, cluster bud occurred in large numbers, and the accumulation and superposition of callus showed stratification phenomenon (Fig. 4c and 4d). After 30 days, the buds grew rapidly into clusters (Fig. 4e and 4f). After 40 days, while the cluster bud grew further, new callus and buds appeared continuously in the base (Fig. 4g and 4h). At this time, using callus with 3 to 4 bud points for proliferation culture, the proliferation coefficient reached 12.67; using the obtained leaf and petiole from strong buds for proliferation culture, the proliferation coefficient was over 20.0.

Adventitious root induction and domestication transplanting

The occurrence rate of adventitious roots was calculated after 40 days of rooting culture (Table 2). Rooting response of *P. denticulata* subsp. *sinodenticulata* was the best in the hormone-free 1/2 MS medium, and NAA significantly inhibited the occurrence of adventitious roots. After the addition of NAA, adventitious roots became thinner, two ends of the leaf curled upward, and there was a certain amount of callus and bud proliferation, which was not conducive to the later acclimation and transplanting. Therefore, the optimal medium for rooting was the 1/2 MS medium. After 10 days of culture, white adventitious roots began to appear at the base of petiole, namely the basal stem (Fig. 5a). After 20 days, new leaves began to appear as the number of adventitious roots increased (Fig. 5b). After 30 days, adventitious root growth was obvious, and the whole root system was robust and developed

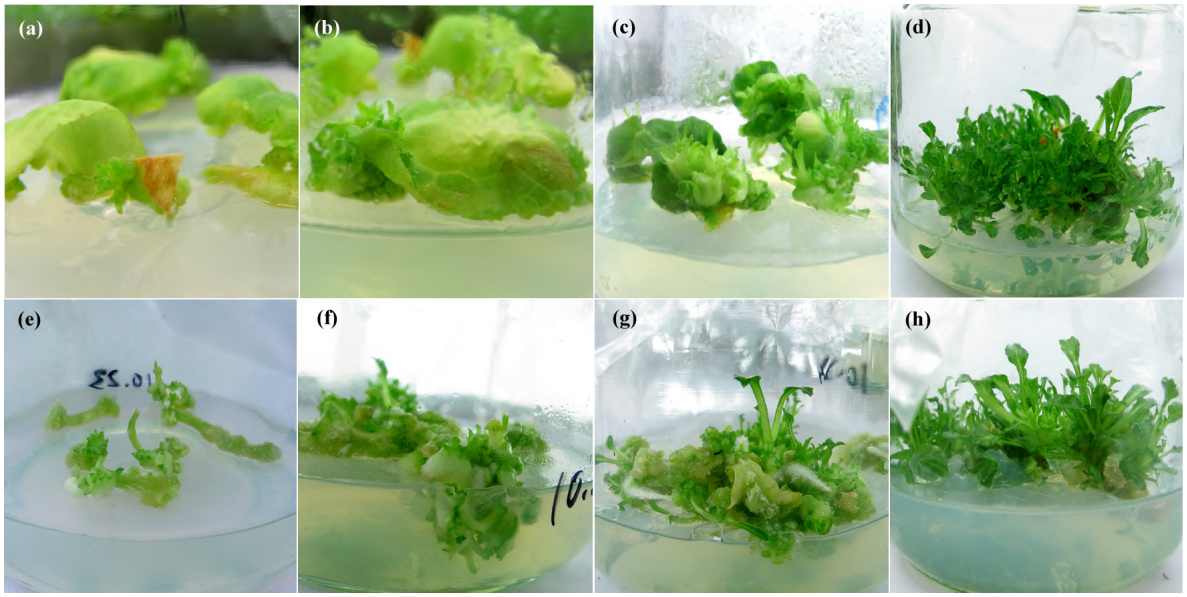

Fig. 3 Callus induction and adventitious cluster bud occurrence in B07 medium of two explants of *P. denticulata* subsp. *sinodenticulata*. The culture of 8- to 10-day old leaf explants: (a) The leaf edges began to curl, and green callus appeared from the incisions after 15 days. (b) The callus from an incision began to differentiate into bud points after 20 days. (c)The bud points at the incision site formed green conical buds after 30 days. (d) Cluster bud formed after 40 days. The culture of 10- to 20-day old petiole explants: (e) The petiole edges began to wrinkle and bulge to form green callus after 10 days. (f) The obvious bud points appeared after 20 days. (g) Cluster bud formed after 30 days. (h) A mixture of callus and cluster bud formed after 40 days.

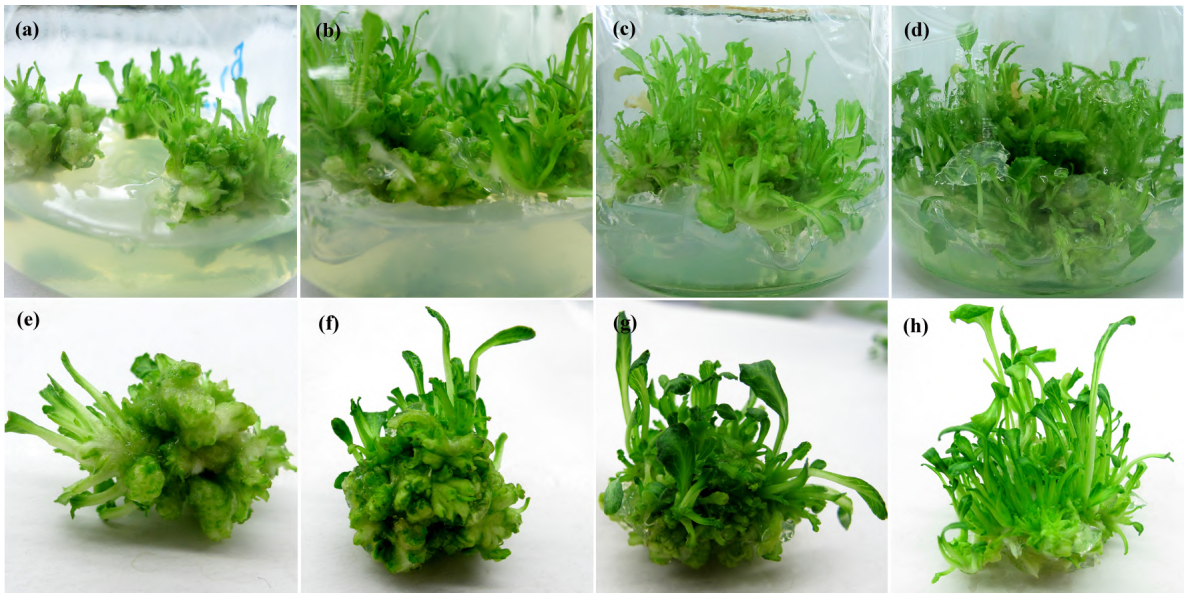

Fig. 4 Proliferation culture of *P. denticulata* subsp. *sinodenticulata*. (a) and (b) After 10 days of culture, the callus increased significantly, and a large number of bud points appeared on it. (c) and (d) After 20 days of culture, cluster bud occurred in a large number, and stratification phenomenon occurred. (e) and (f) The buds grew rapidly into clusters after 30 days. (g) and (h) New callus and buds appeared continuously in the base after 40 days of culture.

Fig. 5 Adventitious root induction and domestication transplanting of *P. denticulata* subsp. *sinodenticulata*. (a), (b), (c), and (d) The process of adventitious root induction. (e), (f), and (g) The process of domestication transplanting.

(Fig. 5c). After 40 days, new leaves grew rapidly, the root system was further robust and accompanied by white root hairs (Fig. 5d), and the rooting rate reached 100%. After transplanting for 30 days, the survival rate was over 95% (Fig. 5e-g).

DISCUSSION

The explant type has been identified as one of the main factors affecting *in vitro* plant tissue culture. Different species and even different organs of the same plant have different responses to external induction and their own redifferentiation ability. Therefore, the proliferation mode and effect of different explants are obviously different [8]. The plants of Primulaceae family often used the leaf and petiole as explants because their stems were extremely short [4, 9]. There were many reports on the tissue culture of leaves, most of which were herbaceous plants of Lamiaceae [10, 11], Caprifoliaceae [12, 13], and Rosaceae [14, 15]. *P. denticulata* subsp. *sinodenticulata* was mainly distributed in the Cangshan valley with humid, rainy, and foggy weather. On the one hand, such habitat condition resulted in leaf thickening, loose arrangement of palisade tissue and sponge tissue cells, and large tissue space. On the other hand, this environment led to the development of veins, so that its ability to channel water increased. Therefore, under the stimulation of exogenous hormones, the parenchyma around the vein at the incision was easy into the state of dedifferentiation to rapid proliferation. The obtained callus was compact in texture and had strong redifferentiation ability, and once this occurred, it quickly differentiated into adventitious cluster bud. Noticeably, in synchronous culture, the differentiated buds had a unipolar structure and were directly connected to the callus, so this regeneration pathway was considered the indirect callus-mediated bud organogenesis from leaf and petiole explants. Meanwhile, the development stage of explants was crucial for the callus formation, and the dedifferentiation ability of explants with different physiological ages was different. Dedifferentiation ability of 1- to 7-day old leaves and 1- to 9-day old petioles was weak. It is speculated that this is because the morphological structure of leaf and petiole in this period has not been completed, resulting in insensitive to exogenous hormones [16, 17]. In contrast, 8- to 10-day old leaves and 10- to 20-day old petioles had the strongest dedifferentiation ability. However, the response ability of over 10-day old leaf and over 20-day old petiole decreased with the increase of explant age. This phenomenon is mainly due to the fact that young explants have more metabolically active cells, nutrients, and various hormones than older explants, thus showing stronger dedifferentiation ability [18–21]. Similarly, the results were observed in zea and coffee [22, 23]. In addition, petioles were more likely to induce callus than leaves, and adventitious cluster bud differentiated faster. From the site of originated and redifferentiated callus (the base), we speculated that because the petiole was an extension of the xylem and phloem of the stem, there was one or more layers of parenchyma cells without specific

function under the epidermis. These parenchyma cells had a strong ability to divide and were sensitive to plant growth regulators and environmental conditions. Thus, it was easier to dedifferentiate into callus than leaves.

In the process of plant tissue culture and rapid reproduction, the type and mass concentration of plant hormones considered the main factors affecting the culture and determining the culture method and quality of the materials [24]. In the rapid propagation of *P. denticulata* subsp. *sinodenticulata*, there were significant differences between the hormone treatments and the blank control treatments ($p < 0.01$), indicating that the addition of hormone was very important to its culture effect. The results of single factor and complete combination experiments showed that the culture effect of combined hormones was significantly higher than that of single hormone, indicating the synergistic effect of multiple hormones. Using auxin alone, 2,4-D could induce callus rapidly, but the redifferentiation ability was weak, so it was not suitable for simultaneous induction of callus and adventitious bud. NAA or IBA could rarely induce callus, but they had strong ability of redifferentiation. Although the culture time was longer, adventitious bud differentiation eventually occurred. Cytokinin alone was superior to auxin in both callus and adventitious bud differentiation, and satisfactory rapid propagation effect was achieved when the two were used together. From this result, it was estimated that the endogenous hormone level of the leaves in *P. denticulata* subsp. *sinodenticulata* was extremely low because it was far away from the growing point of stem tip and root tip, which made it extremely sensitive to exogenous hormones, either auxin or cytokinin. Therefore, it was easier to establish its rapid propagation system.

In the proliferation stage, there was an obvious phenomenon of callus to cluster bud. Moreover, in the B07 medium, the explant induced callus quickly differentiated into adventitious buds and continued to grow into cluster bud. By taking advantage of this feature, adventitious buds induced by callus were segmented continuously in the actual process of proliferation, which not only saved the time of callus induction, but also shortened the culture cycle, and greatly improved the propagation rate. Meanwhile, this proliferation method also greatly reduced the possibility of genetic variation in the process of *in vitro* culture of materials. In addition, the rooting rate was 100% in the 1/2 MS medium, and no callus was produced at the base. Adventitious roots mainly originated from the junction of petiole and basal stem, and the root vascular bundle was connected with the stem vascular bundle. This characteristic greatly improved the survival rate of the transplanting of *P. denticulata* subsp. *sinodenticulata*. This was consistent with the rooting of the primroses studied by Mizuhiro et al [25].

CONCLUSION

An efficient regeneration system of *P. denticulata* subsp. *sinodenticulata* was established by indirect callus-mediated bud organogenesis from the leaf and petiole explants. In this study, the suitable explants were 8- to 10-day old leaves and 10- to 20-day old petioles. The synchronous medium for callus induction and cluster bud differentiation was MS medium with 0.5 mg/l BA and 1.0 mg/l NAA with 100% occurrence rate and 12.67 proliferation coefficient of cluster bud. The optimal rooting medium was the hormone-free 1/2 MS medium; the rooting rate was 100%, and the survival rate was over 95%. The results of this study can provide an optional method for protecting the wild resources and developing artificial cultivation of *P. denticulata* subsp. *sinodenticulata*.

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