Plant Regeneration through Somatic Embryo in *Herpetospermum pedunculosum*, an Endangered Tibetan Medicinal Herb

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Abstract: **Objective** An effective reproducible protocol for complete plant regeneration *via* somatic embryogenesis has been developed for *Herpetospermum pedunculosum*, an endangered Tibetan medicinal herb. **Methods** The cotyledon explants used in this study were excised from seedlings germinated *in vitro*. Callus was induced from cotyledon explants on Murashige and Skoog's medium, supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D, 0.1–1.0 mg/L) alone or in combination with 6-benzylaminopurine (BA, 0.5, 1.0, and 2.0 mg/L). **Results** The calli showed differentiation of globular embryos after three weeks of incubation on MS medium supplemented with various combinations of BA and NAA. Sixty-two percent of the embryogenic calli produced somatic embryos in MS basal medium supplemented with BA (1.0 mg/L) + NAA (2.0 mg/L). The addition of KN (0.5 mg/L) to MS medium containing both BA and NAA (2.0 mg/L each) significantly increased the frequency of somatic embryogenesis. The maximum percentage of embryogenic calli formation was 83%, and globular embryos formed and germinated successfully in this medium. Then, transferring the regenerated plants from this medium to hormone-free MS medium will further enhanced the development of the plants, and the healthy plantlets are formed successfully within four weeks. The plantlets were transferred to soil to acclimatize under greenhouse conditions and 75% survived. **Conclusion** Somatic embryogenesis protocol as reported here can play a key role in the propagation and conservation of this endangered species.

Key words: callus induction; cotyledon; *Herpetospermum pedunculosum*; plant regeneration; somatic embryogensis
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Introduction

With its varied climates, the Qinghai-Tibet Plateau has a rich diversity of medicinal herbs, which have long been used in traditional Tibetan medical formulations. The forest contains a wealth of plant species, but large scale exploitation has been responsible for the rapid loss of medicinal plants. Now many valuable medicinal plants are in danger of extinction. Pharmaceutical companies depend largely upon materials procured from naturally occurring plants, which are being rapidly depleted. Therefore, special attention needs to be given to propagate and conserve exploited species. Plant tissue culture has been proven to be an alternate and effective means in the propagation of endangered species (Wochok, 1981), because it offers the possibility of propagating plants at higher rates than those obtained through traditional procedures. Micropropagation of several medicinal plants from the Qinghai-Tibet Plateau, including *Podophyllum hexandrum* Royle (Nadeem *et al*, 2000), *Saussurea involucrata* Kar. et Kir. ex Maxim. (Jia *et al*, 2005), *Swertia mussotii* Franch. (Xiang *et al*, 1996), *Przewalskia tangutica* (Xu *et al*, 2009), and *Cuminum cyminum* L. (Tawfik and Noga, 2002) have been reported. It must be emphasized that plants raised *in vitro* need to be carefully hardened and acclimatized. The overall success depends upon efficient field transfer,

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continued monitoring of growth, regeneration ability of plantlets, and periodic estimation of active ingredients. These considerations are necessary to further validate the usefulness of *in vitro* techniques. Currently efforts have been initiated in this direction.

Herpetospermum pedunculosum (Ser.) Baill. is an important medicinal plant belonging to the cucurbitaceae family. It is popularly known as *Bolenggua* and grows in the highlands at altitudes ranging from 2300 to 3500 m in the Southwest China, Nepal, and the Northeast of India (Li *et al*, 2005). Seeds have been widely used by herbalists and traditional doctors in Tibet for the preparation of formulations used in the treatment of liver and gall diseases, fever, indigestion, jaundice, and cholecystitis (Li *et al*, 2005). In the search for herbal medicine to treat hepatitis B virus (HBV) infections, experimental results showed that the ethanol extract of the seeds was efficacious against the virus, tumor, and experimental hepatic injury (Tan and Shi, 2005). Previous chemical studies have revealed that this genus accumulates a large amount of lignans (Kaouadji, Favre, and Mariotte, 1978, 1979; Kaouadji and Favre, 1983, 1984a, 1984b; Kaouadji and Pieraccini, 1984; Zhang *et al*, 2006). The active constituents, especially herpetin, have been found to exhibit effective inhibitory effects on HBV-DNA and the replication and expression of hepatitis B surface antigens (HBsAg and HBeAg), offering wide research and development prospects (Yuan *et al*, 2005).

The natural resources of *H. pedunculosum* have been declining in recent years as large quantities of its fruit have been harvested to meet the ever increasing demand for the crude drug, without leaving sufficient seeds to maintain its populations. This species has been listed as endangered by local governments and further harvesting has been prohibited in some regions (Li *et al*, 2002). In order to protect the species, organized cultivation in agricultural areas at low altitudes has been carried out in Tibet since 2000. The mode of regeneration of *H. pedunculosum* in nature is by seed. However, this method of propagation is very inefficient since the seed set is poor and dormancy is not easily broken. In addition, a major factor hampering the mass propagation of this species is the lack of knowledge of its taxonomy, reproductive biology, genetics, agronomic, and quality traits. To date, almost no significant research has been undertaken on the breeding of *H. pedunculosum.* Still, domestication and the establishment of commercial plantations are the best alternatives to conserve the species and offset the pressure on the natural populations. This, however, requires (1) adequate propagation systems, (2) more homogeneous, high yielding materials, and (3) appropriate agricultural practices for the management of plantations (Arce-Montoya1 *et al*, 2006).

The use of tissue culture techniques to assist in the selection and propagation plant materials which have consistent content of active component or have stable safety is an obvious option. There have been a number of reports on tissue culture and plant regeneration of cucurbitaceous species, which include organogenesis in *Cucurbita maxima* Duch. (Lee, Chung, and Ezura, 2003) and *C. pepo* (Krishnan *et al*, 2006); embryogenesis in *Cucumis sativus* L. (Malepszy, Niemirowicz, and Wiszniewska, 1982), *C. melo* L. var. *cantalou pensis* (Guis *et al*, 1997), and *C. melo* (Nakagawa *et al*, 2001; Tabei *et al*, 1991; Gray *et al*, 1993); embryo-rescue culture of *C. melo* var. *reticulatus* Ser. (Nuñez-Palenius *et al*, 2006); and callus formation for *C. sativus* L. (Malepszy and Nadolska, 1983). However, to our knowledge, no *in vitro* studies (organogenesis and somatic embryogenesis) on *H. pedunculosum* have been conducted so far.

The purpose of this investigation was to develop an efficient *in vitro* protocol that could maintain and propagate this endangered species. In this paper, we describe an effective *in vitro* protocol for plant regeneration of *H. pedunculosum via* somatic embryogenesis using cotyledon callus cells.

Materials and methods

Plant material

Mature fruits of *H. pedunculosum* were collected during September, 2005 from plants growing in and around Shannan city (29º16′N and 92º01′E, 4000 m above mean sea level; Tibet, China). Seeds were separated from the husks, washed under running tap water for 30 min, dried in the shade, and kept in paper bags at 4 ℃ until use. The authenticity of these seeds was identified by Professor WANG You-wei, a member of the pharmacy faculty of Wuhan University (Hubei Province, China). A voucher specimen (NO.96074) was

deposited at the same institute.

Seed germination and explant preparation

The shape of *H. pedunculosum* seed is flat rectangle, length 1–1.5 cm, width 4–7 mm, thickness 2–3 mm. About 60 seeds were used to initiate this study. We have developed a two-step sterilization procedure to avoid contamination of seed explants. Step 1: Intact seeds were sterilized with 70% ethanol for 1 min, rinsed with sterile distilled water three times, and then soaked in 0.1% $HgCl_2$ containing Tween-20 (one-two drops per 100 mL) for 10 min with gentle shaking. Seeds were rinsed thoroughly four times with sterile water and then steeped in 45 ℃ water for 12 h. Step 2: The seed coats were removed using forceps and a needle to dissect the embryos. The embryos were washed with sterile distilled water and were further treated with 0.1% $HgCl_2$ for 5 min, followed by five rinses in sterile distilled water. The embryos were then planted on the germination medium. Ten-day-old, aseptically grown embryos were used as the source of explants. Small segments (5 mm × 5 mm) of cotyledons were separated from the embryo axes and cultured on the callus induction medium with the adaxial side in contact with the medium.

Culture media and incubation conditions

Murashige and Skoog's medium (MS, 1962), supplemented with 3% sucrose and solidified with 0.5% agar (Sanland International Inc, Tokyo, Japan), was used as the basal medium throughout the study. The embryo germination medium used to obtain explants consisted of half-strength MS salts and MS vitamins (1/2 MS basal medium). Small segments of cotyledons (2–3 explants/vessel) were transferred to 250 mL screw-capped glass jars containing 50 mL MS medium. The medium was supplemented with different concentrations (0.1–4.0 mg/L) of 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4-D in combination with α-naphthalene acetic acid (NAA), 6-benzylaminopurine (BA), and kinetin (KT) to stimulate callus initiation, somatic embryogenesis and complete plant regeneration with shoot and roots.

The pH value of the media was adjusted to 5.80 before addition of the agar and was sterilized by autoclaving for 20 min under 1.1 kg/cm pressure at 121 ℃. All the cultures were incubated at (25 ± 2) ℃ under 16 h photoperiod with a light intensity of (2000–3000) lx, provided by cool white fluorescent bulbs.

Tissue culture procedures

Seed germination of *H. pedunculosum* on 1/2 MS basal medium was followed by callus formation. To induce callus formation, explants were incubated in the dark during the first week and thereafter in light under the same conditions. After initial callus formation, three replicates of twenty calli each were subcultured on the MS media formulations (Table 1), in order to determine the optimum medium for embryogenic callus induction. After three weeks, the best embryogenic callus, which was globular and compact in appearance, was selected and subcultured three more times at 3-week intervals on the same type of medium.

Table 1 Effect of various concentrations and combinations of BA, NAA, and KN on somatic embryo induction and mean number of somatic embryos per callus clamps ($\overline{X} \pm s$)

Growth regulators / ($mg \cdot L^{-1}$)	Response for somatic embryos induction / %	Number of embryos per callus clump
BA + NAA		
1.0 + 0.5	45 ± 5.0 f	9.9 ± 2.17 e
1.0 + 1.0	53 ± 6.3 ef	12.0 ± 2.33 cde
1.0 + 2.0	62 ± 6.3 de	13.5 ± 3.02 bcde
BA + NAA + KT		
1.0 + 2.0 + 0.2	63 ± 2.5 cd	11.0 ± 3.66 de
1.0 + 2.0 + 0.5	68 ± 3.8 bcd	12.9 ± 3.39 bcde
1.0 + 2.0 + 1.0	69 ± 7.6 bcd	9.3 ± 2.95 e
2.0 + 2.0 + 0.2	72 ± 8.8 bcd	17.0 ± 2.05 ab
2.0 + 2.0 + 0.5	83 ± 3.8 a	19.3 ± 2.60 a
2.0 + 2.0 + 1.0	77 ± 7.2 ab	15.0 ± 2.10 abcd
3.0 + 2.0 + 0.2	73 ± 3.8 abc	14.1 ± 1.80 bcde
3.0 + 2.0 + 0.5	78 ± 4.3 ab	13.1 ± 1.92 bcde
3.0 + 2.0 + 1.0	76 ± 5.2 ab	16.0 ± 2.36 abc

Growth regulators were added into MS medium. Experiments were repeated three times, and each experiment consisted of 20 replicates. Data are recorded ± standard errors of the mean values. Duncan's Multiple Range Test (SSR) at $P < 0.05$ level was calculated. Means within a column followed by the same letters (a–i) are not significantly different from each other

The embryogenic callus was then transferred onto the plant regeneration media in tubes (each clump about 0.5 cm in diameter). Some regenerated plants from each tube were later transferred into tubes containing hormone free MS medium for further growth. After four weeks, the regenerated plantlets were separated, and the agar was washed gently and thoroughly from the roots. The plantlets were then transferred to plastic pots (upper diameter 6 cm × length 8 cm) containing a soil mixture of pearlstone (1∶1) (Zhongxin Perlite Insulation Material Factory, China). These shoots were hardened and grown under the same conditions mentioned

in "Culture media and incubation conditions" section. Initially, they were covered with plastic foil to maintain high humidity (90%). After ten days, the humidity was reduced by slowly removing the foil to harden the plants. Pots were watered regularly, kept in shade for 15 d, and then transferred to green house.

Statistical analysis

Percentage responses for callus formation were recorded after seven weeks of culture. Percentage frequencies for somatic embryo induction from embryogenic callus clumps and the number of somatic embryos per callus clump (250 ± 25) mg were determined after six weeks of subculture. For each of the experiment described above, each treatment consisted of 20 replicates and each experiment was repeated three times. The experimental design was completely randomised (CRD). The data were analyzed using analysis of variance (ANOVA) for CRD design. SSR ($P < 0.05$) was used to compare the means. Standard errors of the means were calculated.

Results and discussion

Effect of the seed coat

Seeds of *H. pedunculosum* exhibit dormancy, an adaptation strategy to overcome the harsh climatic conditions prevailing at higher altitudes. Chemical treatments can be used to germinate dormant seeds. For example, Zheng *et al* (2005) demonstrated that both mechanical and sulfuric acid scarification were effective in increasing the seed germination rate of triploid watermelon. Sulfuric acid is known to reduce the thickness and hardness of the seed coat. In our study, however, no germination of *H. pedunculosum* was observed when intact sterilized seeds were placed on medium, even if the seeds were treated with sulfuric acid. We found that *H. pedunculosum* seeds germinated when the seed coats were removed following treatment with sulfuric acid. The hard seed coat is one of the limiting factors that inhibits germination. After seeds were sowed, germination began after one week for all treatment groups, and maximum germination (37%) was recorded after four weeks in seeds treated with shucking. The germination percentage observed was very low, and was similar to what has been reported for *H. pedunculosum* by Li *et al* (2002).

Induction of callus

Excised embryos were found to germinate within 7–8 d of inoculation on half-strength MS medium. The cotyledons were small (about 1 cm × 0.4 cm) and white coloured at the time of embryo culture. The cotyledons showed rapid growth, increasing in size by 2–3-fold, and they turned green after 10 d in culture. After 2 weeks, the growth of the cotyledons was complete, and the apical bud started sprouting. At this stage the cotyledons were separated from the embryo and planted on MS medium supplemented with various levels (*viz.* 0.1, 0.2, 0.5, and 1.0 mg/L) of 2,4-D alone or in combination with small quantities of 6-benzylamino-purine (BA, 0.5, 1.0, and 2.0 mg/L) to induce callus growth (Table 2). Basal MS medium (MS0), without plant growth regulators, was used as a control.

Table 2 Effect of different concentrations of 2,4-D alone and in combination with BA for callus induction from cotyledon explants of *H. pedunculosum*

Growth regulators / ($mg \cdot L^{-1}$)	Explant-formed callus / %	Callus color and morphology	Degree of callus response
2,4-D only			
0.1	51 ± 3.1 f	W	+
0.2	59 ± 4.2 e	Cr + S	+ +
0.5	76 ± 2.0 ab	Cr + W + S	+ + +
1.0	67 ± 6.4 cde	W	+ +
2,4-D + BA			
0.5 + 0.5	68 ± 3.5 bcd	PG	+
0.5 + 1.0	79 ± 6.4 a	G	+ + +
0.5 + 2.0	83 ± 3.1 a	G	+ + +
1.0 + 0.5	60 ± 2.0 de	PG	+ +
1.0 + 1.0	75 ± 4.2 abc	PG	+ +
1.0 + 2.0	80 ± 5.3 a	G	+

W: white; Cr: creamy; S: spongy; PG: pale green; G: green. Twenty explants were maintained in each treatment group, and data were recorded for up to 7 weeks of culture. Means within a column followed by the same letters (a–f) are not significantly different by Duncan's multiple range test ($P < 0.05$). $^{+}$:Slight callusing; $^{++}$: More callusing; $^{+++}$: Profuse callusing

Explants cultured on MS medium supplemented with various concentrations of 2,4-D and BA showed rapid growth. After one week of culture on all types of media, tissue swelling was observed on cotyledon explants. Following two weeks of culture, intense cell proliferation on the surfaces of cut edges led to calli formation. Explants cultured on MS0 media did not exhibit any growth and ultimately necrosed after a few days. After four weeks of culture, remarkable callus formation was observed in media supplemented with high concentrations of 2,4-D alone (up to 0.5 mg/L).

Comparing to the media without 2,4-D, these calli were growing faster, delicate, mostly spongy, and white or creamy in color (Table 1). Increasing the 2,4-D concentration did not increase growth further. In fact, 2.0 mg/L 2,4-D inhibited the growth of calli (data not shown). The addition of BA to 2,4-D significantly increased the percentage of explants forming calli. Calli cultured in media supplemented with both 2,4-D and BA were compact and green in color. A high frequency of callus formation was observed at low concentrations of 2,4-D (0.5 mg/L) (Table 1). The highest callus induction rate (83%) was achieved at the end of the sixth week on MS medium supplemented with 0.5 mg/L of 2,4-D and 2.0 mg/L of BA. Our studies, in agreement with Kumar and Murthy (2004), indicate that the combination of 2,4-D and BA is essential for compact green callus induction. On the other hand, Rhimi *et al* (2006) reported callus induction from different explant cultures of *C. melo* using 2,4-D (0.25–1 mg/L) with BA (0.10–0.50 mg/L).

Embryogenic callus induction and somatic embryogenesis

According to a previous report by Kuijpers *et al* (1996), the process of somatic embryogenesis can be divided into different phases, each of which likely has its own specific hormonal requirements. From the results of our experiments, auxin (2,4-D) was the crucial growth regulator, during the earlier culture period, for the induction of calli. However, spontaneous embryo formation did not occur in primary and subcultured calli maintained on the same medium as that used for callus induction. Therefore, the media trials combining various concentrations and combinations of growth regulators were carried out, as shown in Table 2. According to Vieitez *et al* (1992), plant growth regulators, like 2,4-D, are known to play an important role in the induction and maintenance of the embryogenic cultures in most fruit and ornamental tree species. For somatic embryo induction, tissues generally are transferred from high auxin-containing medium to low or auxin-free medium (Bhojwani and Razdan, 1996). Likewise, in *H. pedunculosum*, we observed that the 2,4-D alone, or in combination with BA, was essential for initial establishment for calli in culture. Meanwhile, elimination of 2,4-D, and a corresponding increase of BA concentration, induced somatic embryogenesis (Table 1).

The transfer of the cotyledon callus from callus induction medium to MS medium with various combinations of BA and NAA maintained the vigorous condition of the callus. After the first subculture, some of the calli, which were grown in media containing the three different combinations of BA and NAA, became compact and globular. Somatic embryogenesis started from the embryogenic callus after three weeks of subculturing in medium containing both BA and NAA. The embryogenic potential of the calli and the mean number of embryos per callus clump showed significant differences ($P < 0.05$), depending on the growth regulators supplemented (Table 2). More embryos regenerated on HNAA medium (1.0 mg/L of BA + 2.0 mg/L of NAA) than on LNAA medium (1.0 mg/L of BA + 0.5 mg/L of NAA). The mean number of embryos per callus on MS + BA (1.0 mg/L) + NAA (2.0 mg/L) was 13.5 ± 3.02, whereas the number of embryos on MS + BA (1.0 mg/L) + NAA (0.5 mg/L) was only 9.9 ± 2.17. Plants growing on the above media types mostly developed from the existing shoot tips (germinated somatic embryos) on the embryogenic callus. However, they showed small shoot growth and numbers, and had shoots of no more than 1 cm in height. The addition of KN to the BA and NAA-containing media significantly increased the frequency of somatic embryogenesis and the number of embryos per callus clump. The maximum percentage of embryogenic calli (83%) was achieved on MS medium supplemented with BA (2.0 mg/L) + NAA (2.0 mg/L) + KN (0.5 mg/L). The highest number of embryos per culture (19.3 ± 2.60) was achieved on the same medium. Root initiation started simultaneously from these well-developed shoots from the third week onwards in media supplemented with NAA (Fig. 1c). The presence of both NAA and KN in this media improved the development of the embryos and allowed whole plantlets to develop in 4–5 weeks (Fig. 1a–c).

Two different somatic embryogenesis processes have been observed in plant systems: (1) Direct embryogenesis (i.e., directly on the explant) and (2) Indirect embryogenesis *via* an intervening callus phase. In *H. pedunculosum,* embryo formation was indirect. The use of exogenous 2,4-D was an effective inducer of the embryogenic process. This auxin-analog herbicide

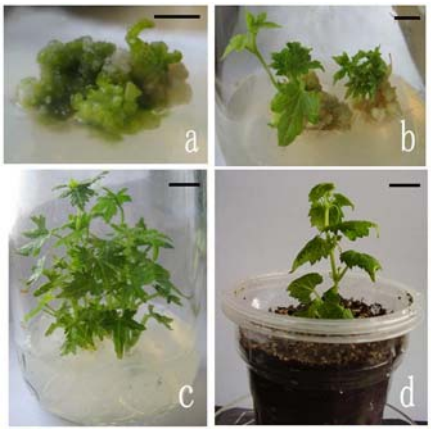

Fig. 1 (a–d) *In vitro* regeneration of plantlets through somatic embryogenesis in *H. pedunculosum*

a: Establishment of embryogenic tissue from the cotyledon callus

b: Development of shoots from embryoids

c: Shoots, roots development and elongation of plantlets

d: An *in vivo* raised *H. pedunculosum* plantlet, two weeks after transfer to plastic cups. New leaves have started emerging

plays a specific signaling role in the initiation of somatic embryogenesis in other plant systems (Dudits, Bogre, and Gyorgyey, 1991; Nomura and Komamine, 1995). However, the continuous presence of 2,4-D blocks further development of the embryo. In the present study, once calli had embryogenically potential cells, other plant growth regulators, such as NAA and KN, easily induced embryos and increased embryo numbers significantly (Table 2). This also provides further evidence that different growth regulators are required at various stages of differentiation in somatic embryogenesis (Pasternak *et al*, 2002).

Plant regeneration

The regenerated plants of *H. pedunculosum* were closely entangled in clusters on the BA + NAA + KT supplemented medium. One cluster of plants from each tube was commonly observed after culturing the embryogenic callus for four weeks. The plants and attached callus were transferred onto MS0 medium. These plants exhibited vigorous growth on MS0 medium and grew shoots up to 5 cm height (Fig. 1C). Root number remained stable when culturing the plants regenerated from 0.1 mg/L BA on MS0 media (LBA), and the MS0 media effectively enhanced root development of plants regenerated from 3 mg/L BA medium (HBA) (data not shown). Using the MS0 protocol, both root number and root lengths were high.

After four weeks of culture on MS0 media the plants filled the culture tubes. Well developed plantlets were separated from the cultures and established in small plastic pots containing a soil mixture of pearlstone (1∶1) (Fig. 1d). Approximately 75 % of the plantlets were established after being transferred onto the soilrite mixer.

Conclusion

In conclusion, this investigation reports an efficient system for the *in vitro* multiplication of *H. pedunculosum,* an endangered medicinal herb of Tibet. As part of a domestication strategy, these plants can be grown and further cultivated even as a potential industrial crop. The application of this protocol can help minimize the pressure on wild populations and contribute to the conservation of this valuable Tibetan herb.

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