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In vitro Rooting of Podophyllum Hexandrum and Transplanting Technique*

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ABSTRACT

Podophyllum hexandrum Royle is an important medicinal plant that produces podophyllotoxin with anti-cancer properties. In China, it is used as a traditional Chinese medicine. Recent years, unplanned exploiting and collection have led to the disappearance of this species in China and India. Effective methods such as tissue culture should be adopted to conserve it to abtain a large scale. It was an crucial process for the final success of tissue culture that seedlings with roots and true leaves in flasks were transferred to soil in field. A protocol has been development for *in vitro* rooting and hardening and *en vitro* transplant of *Podophyllum hexandrum* Royle. Roots were efficiently established on WPM media supplemented with IAA 1.5 mg·L^{-1} and NAA 0.5 mg·L^{-1}. Before transferred to soil containing turfysoil and perlite (2:1), rooted plants were exposed to air for 5d for adaptation. Two periods were good for transplanting this plantlets.

Keywords: Podophyllum Hexandrum; Podophyllotoxin; *in Vitro* Rooting; Hardening; Transplanting

1. Introduction

Podophyllum hexandrum Royle belonging to the family Berberidaceae is an important medicinal plants of the podophyllotoxin. It grows in the northern Himalayas and the adjacent range. In China, it is named *Sinopodophyllum emodi* (Wall.) Ying[1], which distributes in the western area Tibet, Yunnan, Qinghai, Gansu province[2]. Using of dried roots and rhizomes as a drug for detoxification, rheumatism and relieving pain can be found in traditional chinese medicine. In Tibet, it is used to treat patients who suffer from gynecological inflammation[3]. Podophyllotoxin being the main component from the herb is used as a precursor for the chemical synthesis of antineoplastic drugs. Etoposide, etopophose and teniposide, these podophyllotoxin derivatives, have been successfully utilized in treatment of a variety of cancers[4]. A novel of series of derivatives of podophyllotoxin showed strong antiHIV-1 activities[5].

In rescent years, the commercial source of podophyllotoxin is wild collected from the alpine Himalayas in India and the west regions of China. Wild harvesting of these plants and limited ability to reprodution have depleted the natural population to such a degree that the plant has become an endangered species[6]. It was at the beginning of the last century that *P. hexandrum* were cultivated for the first time in India's Himalayan region[7]. And in China it began in the 1980s. Unfortunately cultivation has neither been carried out scientifically nor attempted on a large scale.

Plant tissue culture seems to be an effective alternative in recovery of some rare and endangered medicinal plants. Although some studies related to *in vitro* propagation techniques for mass shoot multiplication about this plant have been reported[8~10], it is desirable to apply methods for enhancing rooting, hardening and transplant for field trials for large scale restoration. This protocol seems to be the first time to systematically study on hardening technique of tissue cultured seedlings of *Sinopodophyllum emodi* (Wall.) Ying after *in vitro* rooting. These strategies could be adopted to motivate farmers to cultivate such improtant medicinal plants for its conservation.

2. Materials and Methods

Material used: The present study was conducted at Institute of Biology, Gansu Academy of Sciences, Lanzhou in the year 2010. The mature seeds of *Sinopodophyllum emodi* were collected from Xinglong Mountain, in Lanzhou. A sequence of procedures, pretreatment, seed germination, shoot induction and proliferation under *in vitro* condition were taken. The shoot separated by cutting the multiple shoots were used for development of roots. The basal medium that consists of woody plant medium(WPM) supplemented with a combination of growth regulators, vitamins and sucrose 3% with a pH adjuste to 5.8 was used to obtain optimal results.

***In vitro* rooting:** The shoots excised were transferred to WPM medium with 3% sucrose, 6.5% agar and various combinations of growth regulators, NAA (0.5mg·L^{-1}), IBA (0.5~2.0mg·L^{-1}) and IAA (0.5~2.0mg·L^{-1}) for rooting. The pH of medium was adjusted to 5.8. The medium was dispensed in 100ml glass culture vessels which were sterilized by autoclaving at 121℃ for 20min. Cultures were maintained under continuous light conditions (10h) with an illumination of 1500~

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2000Lux and 14h dark. Each treatment was repeated ten times at the same time and four explants were used for each treatment. After 30days, mean number of roots per explant and rooting rate were recorded.

Seedling adaptation: The flasks were removed parafilms, when the proliferated shoots were rooted on WPM medium containing growth regulators. Cultures were maintained under rooting conditons for 0d, 2d, 5d, 10d to adapt to the change. The rooted plantlets of *P. hexandrum* were transferred to jiffy pots in greenhouse. After 30d, transplanting survival percentage were recorded.

Transplanting soil: The rooted plants of *P. hexandrum* were washed in running tap water after adaptation culture and finally transferred to jiffy pots. Various combinations of turfysoil, vermiculite and perlite were used as transplanting soil as follows: turfysoil; turfysoil and vermiculite in the ratio of 1:1; turfysoil and vermiculite in the ratio of 2:1; turfysoil, perlite and vermiculite in the ratio of 1:1:1; turfysoil and perlite in the ratio of 1:1; turfysoil and perlite in the ratio of 2:1. All above of the mediums for transplanting were dry-heat sterilized at 170℃ for 2h. Before transferred to soil, roots of the plants were soaked in the Carbendazin for 1d. After 30d, transplanting survival percentage were recorded.

Transplanting period: Plantlets with well-developed roots were potted in soil and transferred to greenhouse in March and April; May and June; October and November; December in the year 2010 in Lanzhou. Survial were evaluated after 30d.

Maintenance and management: Reduce watering to prevent rhizome rot in the coldest month, January, in Lanzhou. In the same year, the potted plants were transferred to larger pot containing turfysoil. Nutrient solution of 1/2MS (Murashige and Skoogs) were sprayed on the surface of leaves in order to help the growth. In May, the healthily-growing plants of *P. hexandrum* in greenhouse were planted in an moderately shady and open air place, the origin region at the altitude 1700m.

3. Results and Discussion

Roots cannot occurred in medium with a single auxin of IBA (0.5mg·L^{-1}, 1.0mg·L^{-1}, 2.0mg·L^{-1}, 5.0mg·L^{-1}). A low rooting rate and poor plant growth occurred with the addition single auxin of IAA. The two were not efficacious procedures to rooting. For development of roots of *P. hexandrum*, shoots were transferred on WPM medium supplemented with different concentrations of IBA (0.5~2.0mg·L^{-1}) and NAA (0.5mg·L^{-1}), IAA (0.5~2.0mg·L^{-1}) and NAA (0.5mg·L^{-1}) (**Table 1**). Lower concentration of IBA (0.5 mg·L^{-1}) and NAA resulted in rooting , but rooting rate was lower and number of roots of per explant was 1.0. However, when the concentration of IBA was increasing, rooting cannot occurred. The combination of IAA (0.5 mg·L^{-1}) and NAA (0.5mg·L^{-1}) resulted in 0.0 rooting rate. The highest rooting percentage was observed in WPM medium combined of IAA (1.5mg·L^{-1}) and NAA (0.5mg·L^{-1}). The rooting occurred after 20d and reached to maximum value of 60.1% after 30d with a mean length of 0.5 ~ 1cm. The plantlets remain greenish and fast growing. After 45d, the length of roots was 2.0~2.5cm with an average number of 6 ~ 8 (**Figure 1A**).

Before transferred to soil, rooted plantlets were exposed to air under its original rooting conditions for hardening (**Figure 1B**). After only a short period of acclimatization (0d, 2d), plantlets can not immediately adapt to changes. Emergence of quickly wilting resulted in a low the survival percentage under *ex vitro* conditions after transplant (**Table 2**). With a certain range, the more time the plant adapt to changes, the higher survival rate after transferred to jiffy pots. The times decide the closure function of stoma of leaves to prevent excessive water loss. Yet the chance of contaminate and injury to roots due to dry medium will mean an increase after 10d of being kept in the fully opened flasks. So with the proceeding of the acclimatization procedure (5d), plantlets were transplanted in container filled with a mixture of soil. New leaf and roots were developed after 30d.

The plants of *P. hexandrum* in the wild flourish well in rich forest soil often loose and consisting of humus. Consequently, at the beginning of transplant, compact soil with a high water-retaining property was bad for growth, because at this time, roots and rhizomes of plantlets with a weak ability to absorb water for leave transpiration easily became brown to rot. The highest survival was observed in pots containing turfysoil + perlite (2:1) (**Table 3**). New leaves and roots were formed after 25~30d. A larger proportion of perlite, turfysoil + perlite (1:1), was likely to cause nutrient loss for frequent watering. It was therefore not adopted for furthe studies.

Table 1. Effects of different concentrations of combinations on rooting (30d for rooting).

Media combinations/ mg·L^{-1}	Total explants	Rate of rooting/ %±SE	No. of roots per explant±SE
WPM+IBA+NAA (0.5each)	40	26.7±0.42C	1.0±0.38C
WPM+IBA+NAA (1.0+0.5)	40	0.0	0.0
WPM+IBA+NAA (1.5+0.5)	40	0.0	0.0
WPM+IBA+NAA (2.0+0.5)	40	0.0	0.0
WPM+IAA+NAA (0.5each)	40	0.0	0.0
WPM+IAA+NAA (1.0+0.5)	40	29.8±0.51D	1.5±0.24B
WPM+IAA+NAA (1.5+0.5)	40	60.1±0.60A	2.4±0.12A
WPM+IAA+NAA (2.0+0.5)	40	56.3.±0.57B	2.0±0.10A

mg·L^{-1}=Milligram per liter, SE=Standard Error

Figure 1. ***In vitro*** **rooting of** ***Podophyllum hexandrum*** **and transplant. A** ***In vitro*** **rooting, B Seedling adaptation, C Transferred plantlets in jiffy pots, D Transferred plantlets in larger pot, E Transferred plants in the origin region at the altitude 1700m under a shade, F Roots with the average diameter 0.25 cm.**

Table 2. Effects of different adaptation periods on hardening of rooted plantlets.

Adaptation period/ d	Total plants	Survival/ %±SE
Control (0)	30	20.4±0.56C
2	30	54.7±0.65B
5	30	89.3±0.34A
10	30	90.2±0.41A

SE=Standard Error

Table 3. Effects of different transplanting soils on hardening of rooted plantlets.

Transplanting soil	Total plants	Survival/%±SE
turfysoil	30	71.3±0.43C
turfysoil+vermiculite (1:1)	30	54.6±0.61D
turfysoil+vermiculite (2:1)	30	50.0±0.57D
turfysoil+perlite+vermiculite (1:1:1)	30	77.5±0.56B
turfysoil+perlite (1:1)	30	95.0±0.23A
turfysoil+perlite (2:1)	30	98.2±0.20A

SE=Standard Error

Microclimate in greenhouses was so complex that conditions of illumination, temperature, humidity, greenhouse ventilation, etc. affect all activities of plants. Temperature and humidity as a major factor plays an important role in growth of *P. hexandrum*. It were not extremely suitable for transplanting this plant in the coldest and hottest months of winter and summer in Lanzhou. Four different periods were chosen in which the potted plants were taken to grow in the greenhouse . The survival are shown in **Table 4**.

Weather was getting cold during October and November in Lanzhou. The minimum and maximum temperature appeared at night and sunny noon in the greenhouse with an average temperature of 23℃, and humidity of 69 ~ 98%. The highest survival was obtained in this period. So it was suitable for the plant of *P. hexandrum* to grow. Leaves became thicker and dark green after 20d with new leaves. The period of March and April also was good for transplanting plantlets. Lower temperate and higher humidity are the main reasons for rot of roots and rhizomes in December. Wilting and the lowest survial was observed during May and June due to the hotter temperate.

In the coldest month, leaves of the plantlets wither. Simultaneously, the parts under ground go dormant. Once the weather become warming the next year, shoots unearthed. The results available shows that seedlings from tissue culture taken to grow under *ex vitro* conditions have the same characteristics of dormancy as the wild one. The potted plants were transferred to larger pot containing turfysoil to grow rapidly (**Figure1D**). In May when the rainfall was frequent relatively, plants were planted in the origin region at the altitude 1700m under a shade (**Figure 1E**). Larger leaves, deep green, flourished, more leaves (2~3) were observed. After two months, the number of roots was 10~12 with the average diameter 0.25cm (**Figure 1F**). Transplanting was very much successful.

4. Conclusions

The endangered plant of *P. hexandrum* is inherently slow growing. At present the rate of propagation of *P. hexandrum* in nature is far less than the rate of its exploration. Previous studies were mostly carried out for *in vitro* productiong of podophyllotoxin in cell culture of *P. hexandrum*. Recent studies were also carried out for *in vitro* propagation for mass multiplication of *P. hexandrum*. Our study focus in enhancing rooting, hardening and transplanting of tissue cultured seedlings. Roots has been developed in WPM medium supplemented with IAA 1.5 $mg \cdot L^{-1}$ and NAA 0.5 $mg \cdot L^{-1}$ as optimum for effective regeneration of roots. Before transferred to soil containing turfysoil and perlite (2:1), rooted plants were exposed to air for 5d. Two periods were good for transplanting plantlets. The ultimate success of this plantlet production undoubtedly depended on the survival and vigorous growth during and after transplantation.

Table 4. Effects of four transplanting periods on hardening of rooted plants.

Transplanting period/m	Total explants	Minimum temperature/℃	Maximum temperature/℃	Minimum humidity/%	Maximum Humidity/%	Survival/ %±SE
Mar. - Apr.	30	10.5	27.3	60.0	98.3	90.2±0.78B
May. - Jun.	30	15.5	46.8	38.5	98.9	38.4±0.55D
Oct. - Nov.	30	10.0	36.0	69.0	98.2	98.1±0.67A
Dec.	30	4.0	19.3	88.6	98.8	56.7±0.60C

The data of Minimum temperature, Maximum temperature, Minimum humidity and Maximum Humidity represent the mean of the period. SE=Standard Error.

So far, new seeds can hardly be collected in Gansu Province for the decrease of wild population of this species. *In vitro* propagation provides an opportunity to abtain seedings of *P. Hexandrum*. This was the first report of enhancing rooting, hardening and transplanting of seedlings from propagation systematically. It can be valuable for large-scale restoration of this plant.

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