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In vitro Propagation for Mass Multiplication of *Podophyllum hexandrum*: A High Value Medicinal Herb

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Abstract: A protocol has been developed for *in vitro* shoot proliferation from callus cultures of *Podophyllum hexandrum* Royle. Callus initiation occurs from root segments of established *in vitro* grown seedlings on Gamborg's B5 media (half strength) supplemented with 2,4-D (0.5-1.5 mg L^{-1}) and BA (0.2-1.0 mg L^{-1}). The rhizome of this plant contains several important lignans and most important being podophyllotoxin, main precursor for anticancer drugs teniposide and ertoposide. In addition *Podophyllum hexandrum* has been reported to have radioprotection properties. Ruthless collection has led to the disappearance of this important medicinal plant from many areas of Himalayas. Thus there is need for immediate conservation of this important medicinal plant through tissue culture means. In the present study shoot proliferation occurs from callus cultures cultured on basal MS medium fortified with BA and IAA either alone or in combination (0.5-5.0 mg L^{-1}) each. Regenerated shoots were rooted with high efficiency on MS medium fortified with activated charcoal (0.5-1.0%) and NAA (0.5-2.0 mg L^{-1}). The rooted plantlets were transferred to green house in jiffy pots containing sand, soil and vermiculite in 1:1:1 ratio.

Key words: Podophyllotoxin, *Podophyllum hexandrum*, *in vitro* propagation, medicinal herb

INTRODUCTION

Podophyllum hexandrum Royle (May apple) belonging to the family Berberidaceae is an endangered medicinal plant, which grows in the northern Himalayan region at an altitude of 3500-4000 meters. Podophyllin, namely the raw extract containing active principle, Podophyllotoxin drawn from the rhizome of *Podophyllum hexandrum.* Podophyllotoxin has been used in dermatological infections like as Condylomata ccuminata (Allevi *et al*., 1993). Podophyllotoxin and its chemical derivatives, teniposide and etoposide are widely used as potent chemotherapeutic agents for a variety of tumours including small cell carcinoma, testicular cancer and malignant lymphoma (Syed *et al.,* 1994). In the search for novel, effective and non-toxic radio protectants, number of plant products have been evaluated for protection against lethal dose of radiation including *Podophyllum hexandrum* (Rajesh *et al*., 2005) and it has been found that pre-radiation administration of the extracts of *Podophyllum hexandrum* mitigated variation induced postnatal and physiological alterations and highly effective in control of both planned and unplanned radiation exposure (Goel *et al*., 2002).

Traditionally dried rhizomes of the plant are mixed with liquid and taken as a laxative or to get rid of intestinal worms as a powerful purgative. Powder of the rhizome is also used as a poultice to treat warts and tumorous growth on skin. Resin from rhizome is used to treat cancerous tumors, polyps and granulation in traditional medicines. The Indian *Podophyllum hexandrum* is superior to its American counterpart, *Podophyllum peltatum* in terms of its higher Podophyllotoxin content (4%) in dried roots in comparison to only 0.25% of *Podophyllum peltatum* (Hollithuis, 1988). Thus the plant has become important as the backbone for production of the two chemotherapeutic agents, teniposide and etoposide.

In the recent years, the frequency of *Podophyllum hexandrum* species in nature has declined considerably because the underground parts of this plant species are being indiscriminately collected in large quantities from the wild to meet the ever increasing demand of Pharmaceutical industry. Taking into consideration its present status in this region, it needs immediate attention for conservation, studies of taking appropriate actions for safeguarding this overexploited plant species, its population biology and genetic diversity is important for

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successful development of conservation strategies by improving propagation techniques and encouraging its cultivation. The regeneration of this plant species in nature is by seed and through rhizome segments, but under natural conditions seeds have been found to germinate after remaining dormant for more than a year (Badhwar and Sharma, 1963). In view of the prolonged dormancy of the seeds, the present study was taken to develop an efficient *in vitro* regeneration protocol for rapid and mass scale propagation possibly for recovery of such a valuable medicinal plant species and improving seed germination and seedling establishment to formulate strategies for its conservation in natural habitat.

MATERIALS AND METHODS

Material used: The present study was conducted at Regional Research Laboratory (CSIR), Sanatnagar, Srinagar, Kashmir in the year 2003. The mature fruits of *Podophyllum hexandrum* used in the present study were collected from the two different field stations of this laboratory located at different altitudes, Yarikha (Gulmarg) and Veerinag, (Pirpanchal range). Voucher specimen was deposited in the repository of RRL, Srinagar, (voucher No. RRL/PH/Srinagar-2003). The seeds were removed mechanically from the pulp, washed with running tape water for 1-2 h dried under shade and stored at 4°C. The procedure for plant regeneration under *in vitro* condition involves a sequence of different culture conditions for seed germination, callus initiation, shoot proliferation and rooting. The basal medium consists of Murashige and Skoogs (1962) supplemented with a combination of auxins, cytokinins, growth regulators, vitamins and sucrose 3% with a pH adjusted to 5.8 was used in all combinations to obtain optimal results.

Seed germination: The seeds of *Podophyllum hexandrum* stored at 4°C after 2-4 months were used for germination studies. Seeds were first sterilized with mercuric chloride (0.1 %) for 5 min at 25±2°C. For germination studies, seeds were treated with concentrated sulphuric acid, GA_3 and GA_3+BA in combination using different concentrations and then kept in dark at 22±2°C in moist absorbent cotton in sterile Petri dishes.

Effect of light on germination: In all the above combinations, effect of light on germination of *Podophyllum hexandrum* seeds was significantly observed. One set of replicates was placed under continuous light conditions (16-20 h) and 8 h dark. To maintain the moisture content in the Petri plates watering was given after every 3 days. Germination starts after 30 days of sowing in GA_3+BA (4.0 mg L^{-1}) each treated seeds (scarified and kept under dark) and the germination percentage recorded was 64.66% followed by 50.3% in concentrated sulphuric acid treated seeds. In the plates placed under continuous light conditions germination recorded was very poor (15%) during this period. Maximum germination was recorded in experiment containing GA_3+BA (4.0 mg L^{-1}) each after 50 days of sowing of seeds.

Callus initiation: The root segments of *Podophyllum hexandrum* seedlings were transferred to Gamborg's B5(Gamborg *et al*., 1968), half strength media containing 2,4-D (0.5-1.5 mg L^{-1}) and BA (0.2-1.0 mg L^{-1}). The cultures were grown at 28°C under continuous dark conditions for 35-45 days. Callus initiation occurs after 35 days of inoculation of root segments (0.5 cm) taken from *aseptically* germinated seedlings. Seedlings raised from seeds develop long and elongated roots. The roots were cut into small segments of 0.5 cms in length for callus initiation onto Gamborg's B5 media and grown under continuous dark conditions for 35-45 days, then cultures were transferred to fresh media added with growth regulators for multiplication of shoots. The response of root explants on Gamborg's B5 media supplemented with various levels of 2, 4-D and BA are shown in Table 2. It was noted that no callusing was observed in absence of growth regulators. The callus turns dark brown if allowed to grow on the same media for more than 50 days. Under light conditions poor response in callusing was observed.

***In vitro* shoot multiplication:** *In vitro* propagated plantlets of *Podophyllum hexandrum* were produced from callus cultures transferred to a tissue culture medium composed of Murashige and Skoogs (1962) containing 3% sucrose and activated charcoal (1.0%)), 0.8% agar and various combinations of growth regulators, cytokinin, BA (0.5-4.0 mg L^{-1}), auxins; IAA (0.5-4.0 mg L^{-1}). The pH of medium was adjusted to 5.8. The medium was dispensed in 100 ml glass culture vessels which were steam sterilized at 15 psi, 121°C for 20 min. The experiment was repeated more than once and five flasks were used for each treatment. The culture vessels were incubated at 25±2°C in 16 h light with cool fluorescent tubes (Philips TI 40/54). After 6 weeks, number of shoots per explant and mean length of shoots were recorded. The shoots established from callus cultures were transferred to half strength MS medium fortified with 3% sucrose, activated charcoal (1.0%), NAA (0.5-2.0 mg L^{-1}) and IAA (1.0-2.0 mg L^{-1}) for development of roots. The rooted plantlets of *Podophyllum hexandrum* were transferred to jiffy pots for hardening. The shoots were transferred in

rooting media containing MS and various combinations of auxins. The cultures were transferred to fresh medium after 5-7 weeks interval.

Hardening of rooted plants: The rooted plants of *Podophyllum hexandrum* were washed in running tap water and finally transferred to jiffy pots containing sand, soil and vermiculite in the ratio of 1:1:1. Before transferred to soil, plants were exposed to higher humidity and gradually reduced from 90-65% during first 20 days. The potted plants were finally taken to grow under *ex vitro* conditions as shown in Fig. 5.

RESULTS AND DISCUSSION

The highest mean germination percentage and earliest onset of germination was observed in GA_3+BA pretreated seeds in all plates kept under dark conditions after 40-50 days of sowing. Maximum value of germination (64.66%) was obtained in seeds treated with GA_3 +BA (4.0 mg L^{-1} each) placed under continuous dark condition after 50 days of sowing of seeds (Table I). Different combinations used were 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg L^{-1} GA_3 and BA each. In concentrated sulphuric acid treated seeds germination started 30-40 days after sowing and reaches to maximum value of 50.33% after 60-70 days. Highest value of germination was recorded in GA_3 + BA (4.0 mg L^{-1}) followed by scarification and dark conditions. Scarifications have advanced the time of germination by 3 to 5 weeks. The combined effect of scarification, chemical treatment and dark conditions overcome the dormancy of seeds and advanced germination percentage significantly. For establishment of *Podophyllum hexandrum* cultures, callus cultures obtained from inoculation of root segments of *Podophyllum* seedlings on Gamborg's B5 (half strength) media fortified with 2,4-D (0.5-1.5 mg L^{-1}) and BA (0.2-1.5 mg L^{-1}) was transferred on shoot proliferation media comprising of MS basal medium supplemented with different concentrations of PGR's ;BA (0.5-5.0 mg L^{-1}) and IAA, (0.5- 3.0 mg L^{-1}). Basal medium with BA concentration (0.5 mg L^{-1}), IAA (1.0 mg L^{-1}) added with activated charcoal (1%) showed better results than the other combinations and was therefore adopted for further studies (Table 2). Higher concentrations of IAA or BA (4.0 mg L^{-1}) resulted in wilting of tissues after 10-15 days of inoculation. Media composition for shoot elongation and growth were evaluated (Table 2). The combination of MS supplemented with BA (0.5 mg L^{-1}) and IAA (1.0 mg L^{-1}) added with activated charcoal (1.0%) resulted in optimum growth of plantlets. The optimal growth conditions were obtained by incorporating these chemicals at various concentrations. The various growth regulators tested included IAA (0.5-3.0 mg L^{-1}) and 2,4-D (1.0-2.5 mg L^{-1}), cytokinin 6-BAP (0.5-5.0 mg L^{-1}) and kinetin (0.5-1.0 mg L^{-1}). Maximum number of shoots was formed when the callus was placed on MS medium supplemented

Table 1: Effect of different chemicals on seed germination of *Podophyllum hexandrum* under different conditions

No.	Condition	Chemical treatment conc. mg L^{-1}	Time taken in germination (Days)	Mean germination %±SD
		GA_3+BA (1.0 each)	45-55	20.33±3.78
1.	Dark	GA_3+BA (2.0 each)	60-70	25.33±5.86
		GA_3+BA (4.0 mg L^{-1})	40-50	64.66±7.05
		GA_3+BA (1.0 each)	60-70	07.66±1.52
2.	Light	GA_3+BA (2.0 each)	60-70	10.33±2.70
		GA_3+BA (4.0 mg L^{-1})	55-65	15.66±4.00
3.	Dark	Conc. Sulphuric acid	60-70	15.33±6.02
4.	Light	Conc. Sulphuric acid	60-70	50.33±2.08
5.	Dark	GA_3+BA (4.0) (Unscarified)	80-90	11.33±1.13
6.	Dark	(GA_3+BA (4.0) (Scarified)	55-70	50.00±3.57

mg L^{-1} = Milligram per liter, SD = Standard deviation

Table 2: The effect of different concentrations of 2,4-D and BA on the callus induction from the root segments of *Podophyllum hexandrum* on B5 medium. The cultures were grown in the dark for 45 days. The experiment was repeated more than thrice and results presented are from one of the three replicate experiments with similar results

Media combinations (mg L^{-1})	Total explants	Relative growth of callus
Control	20	-
B5+2, 4-D (0.5)	20	+
B5+2, 4-D (1.0)	20	+
B5+2, 4-D (1.5)	20	+
B5+2, 4-D+BA (0.5+0.2)	20	++
B5+2, 4-D+BA (0.5+0.5)	20	+++
B5+2, 4-D+ BA (0.5+1.0)	20	+++++
B5+2, 4-D+ BA (1.0+1.5)	20	++
B5+2, 4-D+ BA (1.5+0.5)	20	+++
B5 + BA (1.0)	20	++

Mark denotes no response, + mark denotes magnitude of response; n=5 and 4 explants/flask, mg L^{-1}= milligrams per liter

Table 3: Shoot regeneration from callus cultures of *Podophyllum hexandrum* cultured on MS media supplemented with different concentrations of PGR's. The experiment was repeated more than thrice and results presented are from one of the three replicate experiments with similar results

Media combinations (mg L^{-1})	No. of shoots per explant±SD	Mean length of shoots±SD
Control	0.0	0.0
MS+BA+IAA (0.5 each)	3.00±0.52	3.5±1.26
MS+BA+IAA+ (1.0+0.5)	3.46±0.36	4.1±0.02
MS +BA+IAA+AC (0.5+1.0)	4.26±0.74	3.4±0.40
MS +BA+IAA (2.0+1.0)	3.80±0.34	5.2±0.79
MS +BA+IAA (3.0+2.0)	2.33±0.22	4.3±0.61
MS+BA+IAA (4.0+2.5)	2.53±0.41	3.2±0.28
MS+BA+IAA (4.0+3.0)	2.80±0.28	4.0±0.10
MS+Kn+IAA (1.0 each)	3.20±0.43	3.1±0.37

n = 4 explants per flask, Observations were taken 6 weeks after inoculation. mg L^{-1} = milligram per liter, SD = Standard Deviation

Table 4 Rooting response in established seedlings of *Podophyllum hexandrum* after inoculation on MS medium supplemented with different concentrations of auxins (observation recorded after 35-45 days)

Media combinations supplemented with auxin (mg L^{-1})	Mean No of roots±SD	Rooting in days	Mean length of roots (cm)±SD
Control	0	0	0
MS ½	1 9±0 30	16-20	1 9±0 83
MS+NAA (0 5)	1 3±0 41	15-17	2 7±0 55
MS+NAA (1 0)	2 5±0 44	20-23	3 2±0 40
MS+NAA+IAA (0 5+1 0)	2 9±0 50	14-18	2 3±0 26
MS+NAA+IAA (1 0+0 5)	1 8±0 24	10-15	2 0±0 43

* The data is based on 5 replicate cultures, while the experiment was repeated thrice, mg L^{-1} = milligram per liter, SD = Standard Deviation

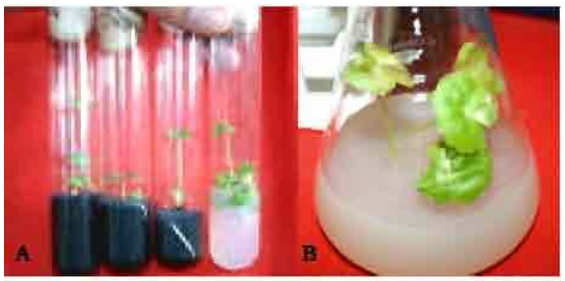

Fig. 1: Established shoots from callus cultures on MS medium added with BA, IAA and activated charcoal

Fig. 2: Rooted plantlets developed on MS medium fortified with different concentration of auxins

with BA(0.5 mg L^{-1}) and IAA (1.0 mg L^{-1}) as shown in Fig. 1-5. The cultures remain greenish on medium supplemented with activated charcoal (1.0%). Basal medium with MS fortified with NAA (0.5 mg L^{-1}) and IAA (1.0 mg L^{-1}) resulted in 60% rooting of shoots and numbers of roots per shoot were 2-6.

Development of a suitable and efficient regeneration protocol of *Podophyllum hexandrum* proved to be difficult due to erratic seed germination problems associated with the sterilization of explants and poor response of most explants in different media combinations

Fig. 3: Rooted plantlet of *P. hexandrum* in green house conditions for hardening

Fig. 4: Field grown plants of *Podophyllum hexadrum* in Gene bank of RRL-Sringer

Fig. 5: Callus formation from root segments cultured on Gamborg's B5 medium fortified with, 2, 4-D and BA

tested. Under natural conditions germination of seeds takes from few months to one year (Badhwar and Sharma, 1963) and germination of seeds under aseptic conditions proved to be very much satisfactory approach. The evidence so far available shows that time dependent seed dormancy do not play any role in erratic and poor

germination of seeds. Seeds were removed from ripe mature pods under aseptic conditions, stored in dark under moist conditions at 4°C followed by scarification resulted in early germination. The influence of various chemicals on seed germination is known in the literature, however after harvesting, GA_3+BA (4.0 mg L^{-1} each) treated seeds resulted to have double the germination percentage and advanced the germination significantly. Other treatments, like GA_3 alone were ineffective and without scarification reduced germination percentage has been recorded. However, GA_3 treated berries of *Podophyllum hexandrum* were found to result in double the germination percentage and advanced germination time by about 3 weeks (Nautiyal *et al*., 1987). Time of germination was advanced by treatment of seeds with GA_3+BA and concentrated sulphuric acid for 3 min followed by repeated washing with sterilized distilled water. The total germination percentage was greatly improved by GA_3+BA treatment and sulphuric acid followed by scarification and kept in dark. The effect of dark conditions and scarification was found significantly on germination of *Podophyllum* seeds (Choudary *et al*., 1963). The principle source of erratic seed germination seems to be the hard seed coat that is main limiting factor in radical emergence and prevents water uptake. As reported in other studies modification of seed coat enhanced seed germination (Hsiao, 1979) and germination percentage was enhanced markedly by following similar strategy for modification of hard seed coat of *Podophyllum hexandrum* seeds through chemical treatments.

Initiation of callus cultures from root segments was very much successful. Callus cultures were subsequently transferred on MS basal media fortified with BA (0.5-5.0 mg L^{-1}) and IAA (1.0-3.0 mg L^{-1}) added with activated charcoal (1.0%) and gives rise to maximum 6 shoots/explant. The shoots were rooted in MS media with various combinations of auxins, NAA (0.5-2.0 mg L^{-1}) and IAA (0.5-2.0 mg L^{-1}) (Table 4). Rooted plantlets were transplanted in jiffy pots filled with a mixture of sand: soil and vermiculite in the ratio of 1:1:1 for field transfer. The pots have been covered over with a plastic bag to maintain a relative high humidity and placed in hardening unit at 25±2 °C under 16 h photoperiod.

Although many authors have reported *in vitro* propagation of *P. hexandrum* for multiple shoot multiplication earlier, (Arumugam and Bhojwani, 1990) and (Nadeem *et al*., 2000) from zygotic embryos. Silva *et al.* (1998) reported plant regeneration from root explants of *P. hexandrum* in liquid media; however the present study seems to be the first report of *in vitro* propagation of this species on solidified Murashige and Skoogs basal medium via callus formation with promising results.

CONCLUSIONS

In vitro propagation is used to speed up regeneration. The success of the system lies in the development of suitable regeneration procedure of such important and difficult to propagate elite species. This can be achieved by improving decontamination strategies and determining the effects of various culture conditions, requirements of plant growth in order to develop optimum conditions for plant growth both in *vitro* and *in vivo* conditions. *In vitro* techniques seem to be an effective alternative in recovery of some rare and endangered medicinal and aromatic plant species. The suitability of this regeneration protocol for genetic transformation will be investigated for biochemical analysis.

This study indicates that *Podophyllum hexandrum* populations in northwest Himalayan regions are genetically highly diverse. Because of the intense collection of this plant from the wild and lack of organized cultivation, *Podophyllum hexandrum* has been declared as a threatened species. At present the rate of propagation of *Podophyllum hexandrum* in nature is far less than the rate of its exploration. This species or at least a large part of its genetic diversity may be lost in the near future owing to its importance and consequent exploitation as a medicinal plant, if appropriate measures are not taken for its conservation. Since single or even few plants will not represent the whole genetic diversity in *Podophyllum hexandrum* and to avoid genetic erosion it is desirable to apply methods for enhancing seed germination and adopt tissue culture techniques for large scale multiplication, hardening, and acclimatization for field trials for large scale restoration. The present study has been taken along these lines. Strategies should be adopted to motivate farmers to cultivate such important medicinal plants. They should be encouraged through economic packages to adopt strategies for their conservation. They should be made aware of the importance of such important medicinal plants. For maintenance of genetic diversity, plant material/seeds should be taken from different locations to ensure this. *In vitro* propagation provides an opportunity to achieve genetic variability through Somaclonal variation and such plantlets can be exploited at genetic level to produce some desirable characters such as higher concentration of podophyllotoxin.

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