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Research Article

Production of lignans in callus culture of *Podophyllum hexandrum*

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Abstract

Purpose: *Podophyllum hexandrum Royle, a source of highly valued podophyllotoxin has been subjected to heavy collection from the wild. The ever-increasing demand of podophyllum is mainly due to two semi synthetic derivatives of podophyllotoxin that is etoposide and teniposide, which are used in the treatment of various types of cancer. The anti cancer lignan derivative podophyllotoxin in Podophyllum hexandrum is biosynthesized at very low quantities in intact plant, so the biotechnological production of podophyllotoxin has been considered essential.*

Method: *The aseptically germinated embryos of Podophyllum hexandrum were developed on solid nutrient agar slab. For the growth of callus culture, Murashigae and Skoog media (MS media)) with various concentrations of BAP, NAA and GA_3 adjusted to pH 5.8 was used. Podophyllotoxin content in the alcoholic extract of calli and plant root was analysed by HPLC and HPTLC and was also compared with cultivated Podophllum hexandrum root extracts.*

Result: *A fully defined MS medium supplemented with Naphthalene acetic acid and 6-benzylaminopurine (BAP) were effective for both initiation and sustained growth of callus tissue. The relative proportion of callus was markedly influenced by presence of plant growth regulators. The amount of Podophyllotoxin obtained from callus was 0.78 and 0.79 percent as characterized by HPLC and HPTLC respectively.*

Conclusion: *The study revealed that callus culture may be a fruitful tool for the production of Podophyllotoxin resin, an anticancer entity.*

Keywords: *Podophyllum hexandrum, Tissue culture, Podophyllotoxin, HPTLC, HPLC.*

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INTRODUCTION

Podophyllum hexandrum Royle (Berberidaceae) also known as the Indian podophyllum is a perennial herb, growing on the lower slopes of the Himalayas in scrub and forest from Afghanistan eastwards to central China[1,2,3,4]. The rhizomes of *Podophyllum hexandrum* are known to contain several lignans which are dimerisation product of phenylpropanoid pathway intermediates linked by central carbons of their side chain[5,6,7]. The lignans occurring in Podophyllum posses anti-tumor properties. *Podophyllum* being the most active cytotoxic herb contains 4.3% Podophyllotoxin on a dry weight basis. Its insecticidal and phytotoxic activities are also reported[7]. However these lignans are also toxic for the treatment of neoplastic disease in humans. Nevertheless, Podophyllotoxin is used as starting compound for the chemical synthesis of etoposide and teniposide; both being applied successfully as antitumor agents[8,9]. Their cytotoxic action is based on inhibition of topoisomerase II, while Podophyllotoxin acts as an inhibitor of the microtubule assembly. These semi synthetic analogues are indicated for small lung cell cancer, testicular cancer, neuroblastoma, hepatoma and other tumor diseases[10].

The limited availability of *Podophyllum hexandrum* plant due to its long juvenile phase and poor fruit setting ability as well as the time consuming collection of the plants results in shortage of Podophyllum resin. Moreover, because of the non-optimal yield after extraction, Podophyllotoxin is an expensive starting compound for the chemical synthesis of its derivatives. Therefore, the biotechnological production of Podophyllotoxin using plant cell culture derived from *Podophyllum hexandrum* may be an attractive alternative.

Podophyllotoxin content are prone to changes due to environment factors, type of fertilizers applied and stage of harvest. These changes could be controlled by *in-vitro* culture of *Podophyllum hexandrum* for the synthesis of lignan Podophyllotoxin. Hence in the present study experiments were carried out to investigate the production and level of Podophyllotoxin in the callus culture of *Podophyllum hexandrum*.

Optimization for establishment of static culture of *Podophyllum hexandrum* and its regeneration were standardized. Presence of Podophyllotoxin in callus culture was detected by HPLC and HPTLC. This study demonstrates the potentiality of static culture in production of Podophyllotoxin.

MATERIALS AND METHODS

Mature fruits of *Podophyllum hexandrum* Royle were collected during the month of October from G.B Pant Institute of Himalayan Environment and Development, Kosi- Katarmal (Almora) and High Altitude Plant Physiology Research Center, Srinagar (Uttaranchal INDIA). Murashigae and Skoog medium from (PT 0010 without agar and PT 0011 with 8% agar) were purchased from Hi-Media (Mumbai, India). Naphthalene acetic acid (NAA) and Indole acetic acid (IAA) were procured from Sigma Laboratories (Mumbai India). All other chemicals used were of analytical grade and used without further modification.

Seeds and Seedlings

Seeds were separated from pulp, washed under running tap water for 20 min dried under shade and stored at 4˚C until used. Seeds were cut with a scalpel blade, removing a section of the seed coat with two incisions around the seed hilum region, and then maintained in dark, sterile conditions on moist filter paper at 27˚C until emergence of the radical. Germinated seeds were placed on solid nutrient agar slabs (full strength MS medium, 0.8% agar, pH 5.8) in sterile culture tubes and transferred to growth room conditions, with diffuse light day/night regime (16/8 hr) as well as in the dark.

Callus initiation and growth

Aseptically germinated seed embryos were washed with double distilled sterile water and their surfaces were disinfected with aqueous solution of sodium hypochlorite for 8 min followed by four washings in sterile double distilled water under aseptic conditions so as to remove traces of sodium hypochlorite. The explants (3-4 mm) were carefully excised and

Table 1: Effect of different supplement on segment of excised embryo of ***Podophyllum hexandrum***

Treatment of supplement (μM)				Types of response		
S/No.	NAA	BAP	GA_3	Callus	Colour	Nature
1	0	1.5	0	+ +	Brownish	Compact
2	0	2.0	0	+	Brownish	Compact
3	1.5	0	0	++	Brownish	Compact
4	2.0	0	0	+	Brownish	Compact
5	0	0	0.5	+	Brownish	Compact
6	0.5	2.5	0	+++	Greenish	More Friable
7	1.0	2.0	0	+++	Greenish	More Friable
8	1.5	1.5	0	++	Greenish yellow	Less Friable
9	2.0	1.0	0	++	Greenish yellow	Less Friable
10	1.0	0	0.5	+++	Greenish	More Friable
11	1.5	0	0.5	+++	Greenish	More Friable
12	0.5	0	0.5	+ +	Greenish yellow	Less Friable

Key: (+) = Poor callusing; (+ +) = Average callusing; (+ + +) = Good callusing; (-) = No response.

Table 2: HPLC data of podophyllotoxin extracted from root and callus of *Podophyllum hexandrum*

Sample	RT	Area (m.V/s)	Content of Podophyllotoxin (%)
Standard	6.940	2150.2213	-
Root extract	6.890	120.12	5.46
Callus extract	6.80	17.10	0.78

transferred into MS media with various concentrations of BAP (0.5-2.5μM), NAA (0.5-5μM) and GA_3 (0.5μM-1μM). The medium was adjusted to pH 5.8. The cultures were maintained at 25±2°C in 16 hr. light and 8 hr. dark cycle and were transferred to fresh MS medium after a period of four weeks.

Lignan extraction

Obtained calli and plant root were extracted with ethanol. The lignan extract was redissolved in

Table 3: HPTLC data of podophyllotoxin extracted from root and callus of *Podophyllum hexandrum*

Sample	R_f	Area (m.V/s)	Content of Podophyllotoxin (%)
Standard	0.51	8630.5	-
Root extract	0.51	2415.4	5.48
Callus extract	0.51	352.2	0.79

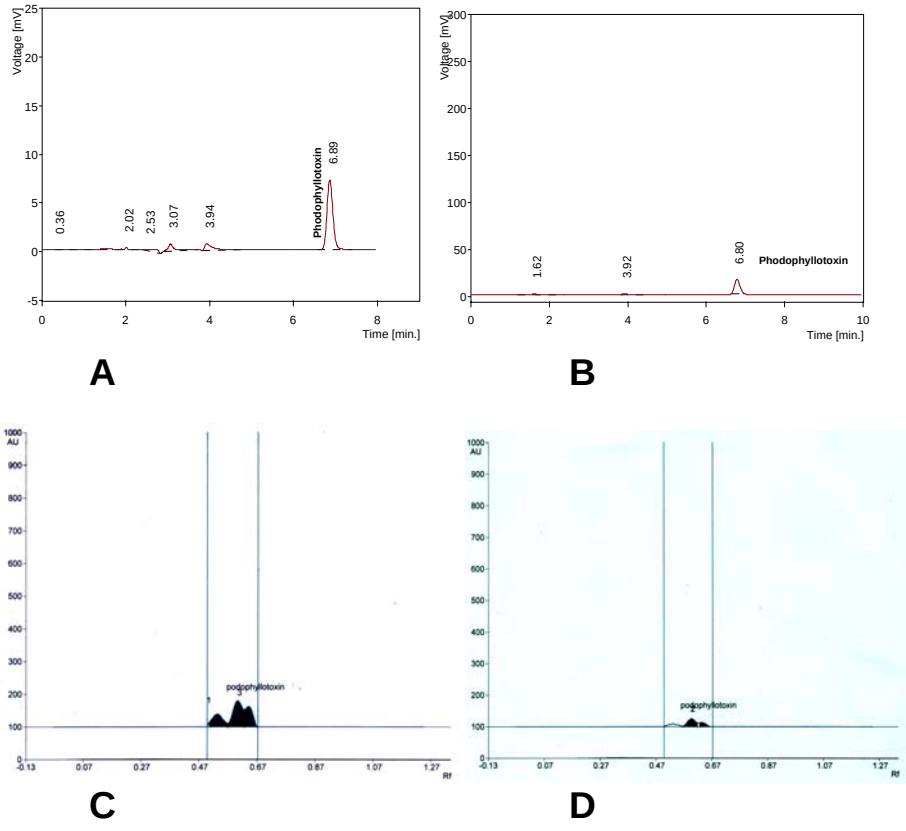

Fig. 1: HPLC chromatogram of Podophyllotoxin from root and callus of *Podophyllum hexandrum* (A&B); HPTLC chromatogram of Podophyllotoxin from root and callus of *Podophyllum hexandrum* (C&D)**.**

analytical grade methanol prior to analysis and analysis of the calli and plant root was carried out to identify and compare the Podophyllotoxin and Resin content in the callus with cultivated *Podophyllum hexandrum* root extracts.

HPLC Analysis

HPLC was carried out using a Column C-18 Bonda pack (250 X 4.6 mm) and solvent system such as Acetonitrile: Water: Methanol: n-

Heptane (30:40: 25: 5) was used with a flow rate of 1.2ml/min with UV detection at 280 nm. Peak areas were assessed by integrator. Retention time for authentic podophyllum lignans were as follows: standard Podophyllotoxin (6.810), root extract (6.890), and for callus extract (6.800).

HPTLC Analysis

Quantitative and qualitative analyses were carried out using the HPTLC method. Callus extracts, and plant extracts were identified using the TLC method. The sample extract was compared with standard Podophyllotoxin. HPTLC was carried out using a silica gel GF254 Precoated plate of 0.2mm thickness as a stationary phase. A Mobile Phase of Acetonitrile: Water (4: 6) was used and UV detection at 210 nm. Peak areas were assessed by integrator.

The extract made from the roots of *Podophyllum hexandrum* contained a mixture of potent pharmacologically active compounds. The major component was the resin, podophyllotoxin. The extract was standardized on the basis of being podophyllotoxin.

RESULTS

In the present study, experiments were carried out on the tissue culture of *Podophyllum hexandrum* (Berberidaceae).Tissue culture study was started from aseptic seed germination, standardization of media, callus initiation and growth study followed by extraction, and estimation of Podophyllotoxin content in the callus cultures and from cultivated roots of the plant.

Seed and Seedlings

For aseptic germination, the seeds were treated with 70% ethanol for 15 seconds followed by 3% sodium hypochlorite for 5 minutes. They were then washed several times with sterile double distilled water to remove excess of sterilants. The result showed that seed germination was within 6 days using the Petri dish method. The sprouted seedling in the immature stage showed better proliferation and growth in MS basal medium with 100 µM solution of gibberrellic acid whereas it remained stagnated if allowed to stay in the cotton bed method for a long time.

HPLC Analysis

The Qualitative tests for the identification of podophyllotoxin and the methods for their quantitative estimation were carried out by using HPLC. Root parts and callus were extracted from *Podophyllum hexandrum* under different conditions and these extracts were analyzed using 1 mg ml^{-1} sample concentration and compared with that of the standard using same concentration (100µM). Callus under different conditions contained podophyllotoxin. The HPLC data are presented in Table 2.

HPTLC Analysis

The qualitative tests for the identification of podophyllotoxin and the methods for their quantitative estimation were carried out using HPTLC. Different parts were extracted from *Podophyllum hexandrum* under different conditions and these extracts were analyzed using 5 mg ml^{-1} sample concentration and compared with that of the standard (98%) using 1mg ml^{-1} concentration (10µl). Callus under different conditions contained podophyllotoxin. The HPLC data are shown in Table 3. The chromatograms obtained by HPLC and HPTLC are shown in Fig. 1.

DISCUSSION

Establishment of a routine protocol for the tissue culture of *Podophyllum hexandrum* proved to be difficult due to erratic seed germination, problems with the sterilization of explants and the poor response of most plants to the culture regimes tested. Sterilization of explants, especially root material from soil-grown plants was unsuccessful[11,12] and germination of seeds under aseptic conditions proved the most satisfactory approach[13]. Germination was dependent on storage conditions and appropriate pre-germination treatment. A successful procedure was developed involving aseptic seed germination, dark condition at 27°C.

For callus initiation, full strength MS (Murashige and Skoog) showed better results[14, 15] and was therefore adopted for further studies. Root

explants from seedlings derived from a single plant were tested on hormone grids. No individual auxin or cytokinin initiated callus formation at the concentrations tested, with the exception of NAA where some response was observed at 0.5 - 5μM after four weeks. High concentrations of BAP (2.5μM) in the presence of NAA enhanced callus initiation. The best response, however, was observed with the addition of GA_3, which reduced callus initiation time to two weeks. Development of a friable callus was particularly associated with BAP and GA_3 and also with NAA in different concentrations.

Optimum growth response was achieved with combined GA_3 and NAA treatment, which appeared independent of GA_3 concentration in the range tested (0.5 - 1.0μM), but dependent on the NAA concentration (0.5-1.0μM). A similar high response was observed in the presence of BAP concentration (0.5-1.0μM). Higher concentrations of BAP (up to 2.5μM) were found to facilitate growth when NAA levels were low, but retarded growth when NAA levels were high. Callus growth was more effectively supported on a MS medium with full strength, rather than at half strength MS medium[16]. The effect of different growth regulators on callus culture has been summarized in Table 1. Quantitative estimation of podophyllotoxin was carried out using HPLC and qualitative estimation of podophyllotoxin was carried out using HPTLC which revealed the presence of presence of podophyllotoxin in callus culture.

CONCLUSION

Podophyllum hexandrum has been considered a rare and threatened species, large scale removal of its underground parts still continues at rates well over natural regeneration. Therefore special attention needs to be given for its propagation and conservation. The use of seeds/plant materials from different population would help to ensure the high Podophyllotoxin content by tissue culture techniques. The combined and sustained effort with seed germination, *in vitro* culture and estimation is required to reduce the pressure on natural population. This study was successful in standardizing the media for tissue culture, aseptic germination, callus initiation, extraction and estimation of podophyllotoxin from Callus and cultivated *Podophyllum hexandrum* extract.

REFERENCES

1. *Airi S, Rawal RS, Dhar U Purohit AN. Population studies on Podophyllum hexandrum Royle- A dwindling, medicinal plant of the Himalaya. Plant Genet. Resour. News. 1997; 110: 29-34*
2. *Gupta R, Sethi KL. Conservation of medicinal plant resources in Himalayan region. In: Jain SK. Mehra KL. (ed.). Conservation of tropical plant resources, B.S.I. Howrah, India. 1983; pp 101-107.*
3. *Giri A, Narasu ML. Production of Podophyllotoxin from Podophyllum hexandrum: A potential natural product for clinically useful anticancer drugs. Cytotechnol. 2000; 34:17–26.*
4. *Choudhary DK, Kaul BL, Khan S. Cultivation and conservation of Podophyllum hexandrum- An overview. J. Med. Aro. Plant Sci. 1998; 20:1071–1073*
5. *Kamil MW, Dewick PM. Biosynthesis of lignans alfa and beta-peltetin. Phytochem. 1986a: 25(9):2089-2092.*
6. *Kamil MW, Dewick PM. Biosynthetic relationship of aryltetralin lactone lignans to dibenzylbutyrolactone lignans. Phytochem. 1986b; 25:2093-2102.*
7. *Jackson DE, Dewick PM. Aryltetralin lignans from Podophyllum hexandrum and Podophyllum peltatum. Phytochem. 1984; 23(5):1147-1152.*
8. *Canel C, Moraes RM, Dayan FE, Ferreira D. Molecules of interest 'Podophyllotoxin'. Phytochem. 2000; 54:15–20.*
9. *Jackson DE, Dewick PM. Biosynthesis of Podophyllum lignans-II. Interconversion Aryltetralin lignans in Podophyllum hexandrum. Phytochem. 1984; 23(5):1037-1042.*
10. *Goel HC, Prasad J, Sharma A, Singh B. Anti tumour and radio protective action of Podophyllum hexandrum. Indian J. Exp. Biol. 1998; 36(6):583-587.*
11. *Badhwar RL, Sharma BK. A note on germination of Podophyllum seeds. Ind. For.1963; 89:445-447.*
12. *Semwal JK, Purohit AN, Gaur RD. Seed germination in some Himalayan alpine plants. Seed Res. 1983; 11(1): 42-46.*
13. *Nadeem M, Palni LMS, Purohit AN, Pandey H, Nandi SK. Propagation and conservation of Podophyllum hexandrum Royle: an important medicinal herb. Biol. Cons. 2000; 92:121-129.*
14. *Kadkade PG. Formation of Podophyllotoxin in Podophyllum peltatum tissue cultures. Naturwissenschaften. 1981; 68:481-482.*
15. *Kadkade PG. Growth and podophyllotoxin production in callus tissue of Podophyllum peltatum. Plant Sci. Lett. 1982; 25:107-115.*
16. *Chattopadhyay S, Srivastava AK, Bhojwani SS, Bisaria VS. Development of suspension culture of Podophyllum hexandrum for the production of podophyllotoxin. Biotechnol. Lett. 2001; 23:2063-2069.*