Chapter

# Propagation of *Podophyllum hexandrum* Royale to Enhance Production of Podophyllotoxin

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## Abstract

Also known as the Himalayan mayapple, *Podophyllum hexandrum* is a succulent erect herb, glabrous, up to 30 cm tall with creeping long knotty rhizome. The plant produces podophyllotoxin, an anticancer metabolite, and hence can also be used for the treatment of cancer. The roots have also reported anticancer lignans, including podophyllotoxin and berberine. The root is harvested in the autumn and either dried for later use or the resin is extracted. Due to the plant has been overexploited due to medicinal properties and is now enlisted as an endangered species. Therefore, there is a need to grow this plant at a greater scale so as to utilize its medicinal potential to the fullest. Unsuccessful attempts have been made to grow the pant in vitro. Hence, this problem needs to be countered and methods to increase the metabolite production by the plants are also needed in order to maximize the utilization of its medicinal properties. This review focuses on providing solutions to the researchers to develop new techniques to grow the plant in vitro as well as ex situ and also gives an insight on the various methods that have been proved fruitful for increasing the production of podophyllotoxin in *P. hexandrum*.

**Keywords:** *Podophyllum hexandrum*, anticancer, podophyllotoxin, in vitro propagation, ex situ propagation

## 1. Introduction

The Himalayan range in India is recognized as a mega hotspot for biodiversity [1]. **Table 1** gives a list of the different types of plants found in this region, which helps to understand the importance of the Himalayan region in terms of endemism of plant species [2]. Of these floral collections, 1748 species have been identified to be of medicinal importance both traditionally and also as modern phytotherpeutics [3]. These 1748 species have been utilized for various purposes (**Table 2**) [3, 4], 121 of which have been categorized to be rare-endangered species [5]. Besides using these local floras for their own survival in form of food, medicine, agricultural tools, and fodder, the local communities also trade these endemic plant species and their traditional knowledge for generating income [3]. According to a survey conducted for the prioritization of medicinal plants by the All India Trade Survey, the demand of these plants for their medicinal value has increased by 50% while their availability has noted a decrease of 25% [6].

| S. No. | Flora | No. of species | Percentage of endemism (%) |
|---|---|---|---|
| 01 | Angiosperms | 8000 | 40 |
| 02 | Gymnosperms | 44 | 16 |
| 03 | Pteridophyte | 600 | 25 |
| 04 | Bryophytes | 1737 | 33 |
| 05 | Lichen | 1159 | 11 |
| 06 | Fungi | 6900 | 27 |

*The table also gives the percentage of different floras that are endemic to that area, thereby suggesting the importance of the Indian Himalayan region in terms of biodiversity.*

**Table 1.**
*List of different types of flora inhabiting the Indian Himalayan region.*

| S. No. | No. of species | Use |
|---|---|---|
| 01 | 1748 | Medicinal plants |
| 02 | 675 | Edible wild plants |
| 03 | 118 | Essential oils yielding medicinal plants |
| 04 | 279 | Species used as fodder |
| 05 | 155 | Species worshiped as sacred plants |
| 06 | 121 | Rare and endangered species |

**Table 2.**
*The number of different species found in the Himalayan region has diverse uses which cater to the needs of both the traditional local communities and the industrially significant medicinal market.*

The term *Podophyllum* is derived from an ancient Greek word meaning foot leaf. It is also known as mayapple since the fruit ripens in the months of spring. The genus *Podophyllum* is globally represented by three species, the *P. hexandrum*, *P. peltatum*, and *P. sikkimensis*. The *P. hexandrum*, which grows in the Himalayan regions of Asian subcontinent, is commonly known as the Indian mayapple. *P. peltatum* is found distributed in regions of Atlantic North America and is popularly called as the American mayapple [7]. *P. hexandrum* is found to grow normally in the sub-Himalayan regions of India, Pakistan, Bhutan, Afghanistan, China, and Taiwan. In India, it largely covers the states from Jammu and Kashmir to Uttarakhand and some parts of Northeastern India due to its specific environmental requirements. **Table 3** gives a state-wise distribution of this plant species in India [8].

*Podophyllum* is a green herbaceous plant belonging to the family of Berberidacea of the order Ranunculales. Indian *Podophyllum* is a native of the Himalayan region and grows favorably in the temperate and subalpine regions having well-drained, humus-rich soil conditions. In Ayurvedic terminology, it is referred to as Vanyakarkati. The plant has a perennial rhizome and a succulent stem. The stem has a pair of leaves drooping down from the petiole like umbrellas. The stem grows to a height of approximately 30 cm before the complete development of the leaves. The leaves are generally spotted with a width of nearly 25 cm. The flower is pinkish white in color and appears in May. The fruit ripening occurs in August or September and the fruit is bright orange in appearance with a size comparable to that of a lemon.

Several studies have been conducted to identify the best possible methods of propagation of *P. hexandrum*. According to one such study, the propagation of the

| S. No. | State-wise distribution |
|---|---|
| 01 | Ladakh—Zanskar and Suru valley |
| 02 | Jammu and Kashmir—Kashmir region |
| 03 | Himachal Pradesh—Lahaul, Spiti, Kangra, Chamba, and Kinnaur |
| 04 | Uttarakhand—Kumaun and Garhwal region (2000–4000 m above MSL) |
| 05 | Sikkim |
| 06 | Arunachal Pradesh |

**Table 3.**
*State-wise distribution of* P. hexandrum *in India.*

seeds of the plant in an off-site environment under controlled conditions allowed the seedlings to grow a year faster as compared to that when grown in field. The search is on to identify and establish such effective ex situ methods of propagation as these along with several other modes of propagation as in vitro and in vivo methods can help conserve the genetic diversity of the plant besides providing a substantial number of transplants to go back into the wild to combat the vulnerability of overharvesting without compromising on its industrial demand [9]. There is very less literature available showing the growth of *Podophyllum hexandrum* using micro-propagation techniques.

Use of bioreactors for the growth and production of podophyllotoxin has not yet been evaluated properly owing to the lack of genetic data about the plant's genes involved in metabolite production. The technical know-how about the factors affecting biosynthetic pathway and podophyllotoxin production has not been explored and no significant literature is available. This review focuses on the optimization of culture conditions for the propagation of *Podophyllum hexandrum* and obtains conditions for the enhanced production of podophyllotoxin (**Figure 1**).

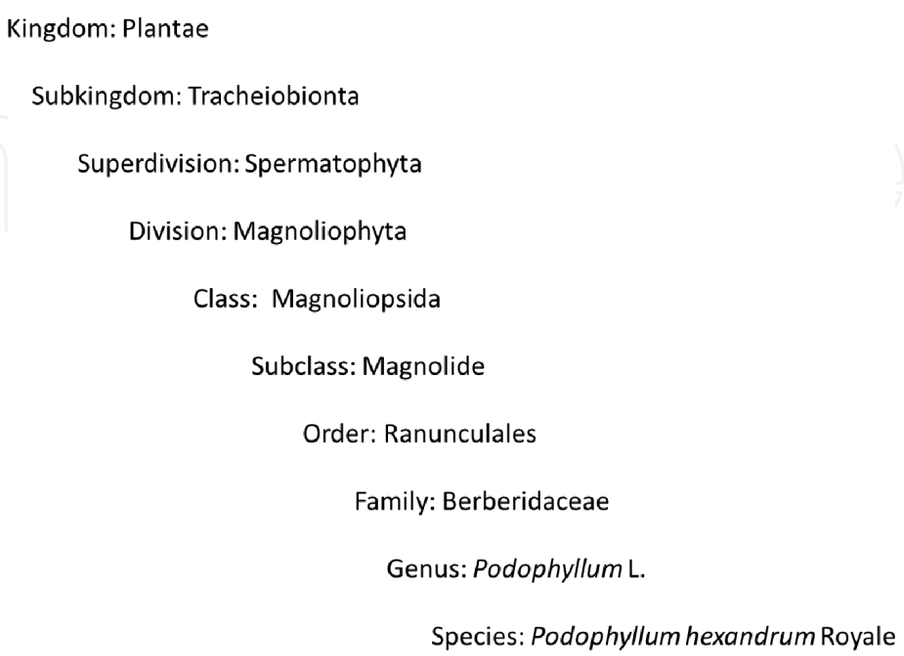



**Figure 1.**
*Taxonomic classification of* P. hexandrum.

## 2. Podophyllotoxin—resin from *Podophyllum hexandrum*

*Podophyllum hexandrum* has been extensively studied primarily for its medicinal properties that are contained in its resin extract, podophyllotoxin. It occurs extensively in the roots and the rhizome of the plant species. The content of podophyllotoxin is also dependent on the growth conditions of the plant, including the environmental factors as soil pH, rainfall, temperature, humidity, etc. [10]. This resin can be extracted from both the species of the plant, *P. hexandrum* (Indian) and *P. peltatum* (American), although it has been well established that the yield of the Indian plant is greater than that of its American counterpart. A number of different components were later isolated from the podophyllin that was isolated both from the American and Indian species [11].

α- peltatin
β- peltatin
} Isolated from *P.peltatum*

4'- demethylpodophyllotoxin
Picropodophyllin-β-D-glucoside
} Isolated from *P.hexandrum*

### 2.1 Phytochemical profile of podophyllotoxin

Podophyllotoxin is a member of the aryltetralin lignans family according to its chemical structure. It is a product of phenylpropane units which are coupled together by β-carbons in their side chain. **Table 4** gives a list of the compounds characterized from both the *Podophyllum* resin as well as *Podophyllum* species [12]. **Figure 2** depicts the chemical structure of the podophyllotoxin in both 2D and 3D formats [13].

Chemical formula of podophyllotoxin: $C_{22}H_{22}O_8$.

Molecular weight of podophyllotoxin: 414.4 g/mol.

A cycle of seven precursors is involved in the production of this resin naturally [14]. Podophyllotoxin is the most active naturally occurring cytotoxic product, hence it is used as a principle ingredient in the preparation of its semisynthetic derivatives that function as cytostatics and are therefore used in the treatment of several types of cancer. The major anticancer drugs obtained from this toxin are etoposide and teniposide. **Figures 3** and **4** give the chemical structure of etoposide [15] and teniposide [16]. Podophyllotoxin inhibits the assembly of the microtubule, thereby inhibiting the process of cell division. It is also reported to have certain antiviral activities by interfering with certain vital viral processes [17].

The quantity of the resin collected is variable with the season and site of collection. The maximum yield of toxin can be obtained in May, when the plant is to flower and decreases in near November 7%, when the plant is in fruiting stage. Also, the yield obtained is higher from the young rhizomes. As the rhizomes mature, the amount of podophyllotoxin accumulation decreases. Assessing the difficulties in the stating and execution of an appropriate methodology for obtaining higher yields of this toxin, besides increasing the numbers of this species in the wild, the present review aims to study and analyze some of the various methods that have been performed to achieve these objectives.

### 2.2 Medicinal value of podophyllotoxin

The rhizome of the plant contains a resin, known generally and commercially as Indian *Podophyllum* Resin, which can be processed to extract podophyllotoxin or podophyllin, a neurotoxin. Podophyllotoxin is the major lignan present in the resin

| Compound | |
|---|---|
| **Lignans** | **Flavinoids** |
| 1. Podophyllotoxin | 1. Quercetin |
| 2. α-peltatin | 2. Kaempferol |
| 3. β-peltatin | 3. Isorhamnetin |
| 4. 4′-demethylpodophyllotoxin | 4. Quercetin 3-galactoside |
| 5. Desoxypodophyllotoxin | |
| 6. Dehydropodophyllotoxin | |
| 7. Sikkimotoxin | |
| 8. Podophyllotoxin glucoside | |
| 9. Picropodophyllinglucoside | |
| 10. α-peltatinglucoside | |
| 11. β-peltatinglucoside | |
| 12. 4′-demethylpodophyllotoxinglucoside | |

**Table 4.**
*List of different compounds that can be obtained from podophyllotoxin or the Podophyllum species. These compounds can either be lignans or flavinoids chemically.*

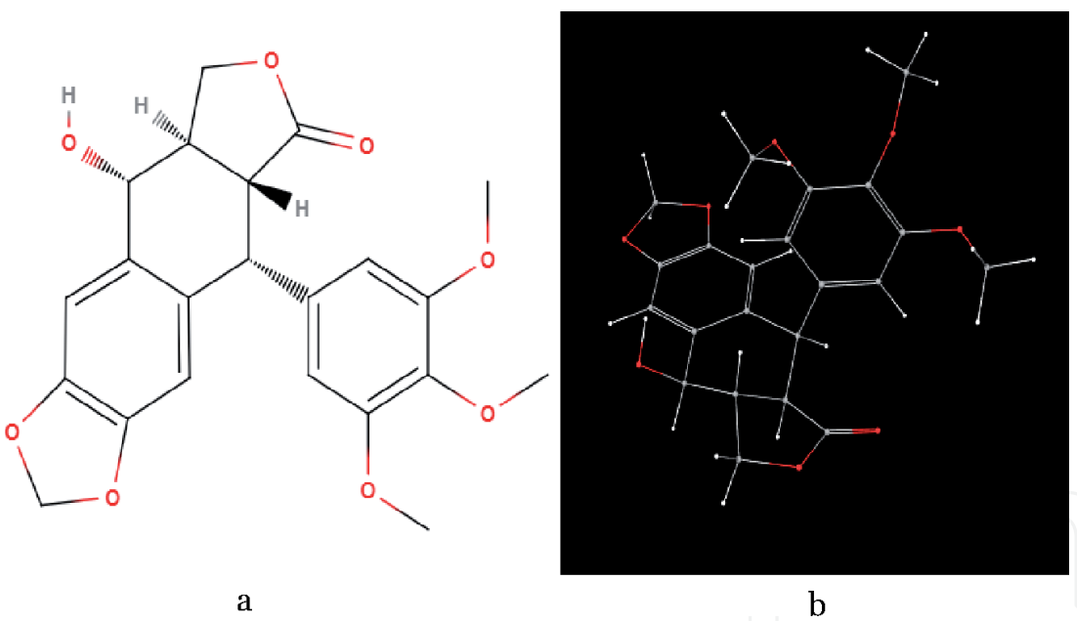
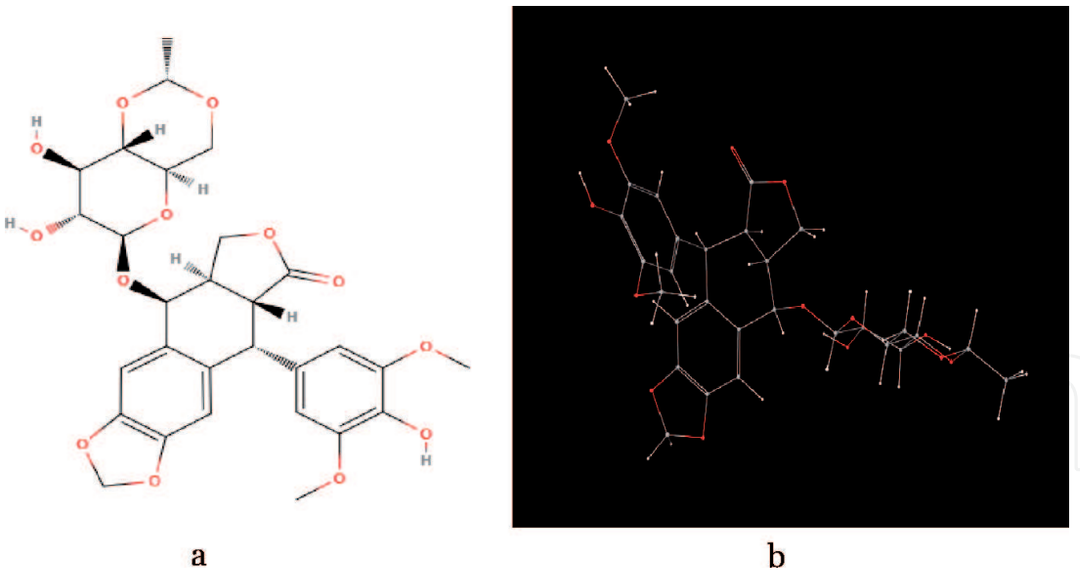


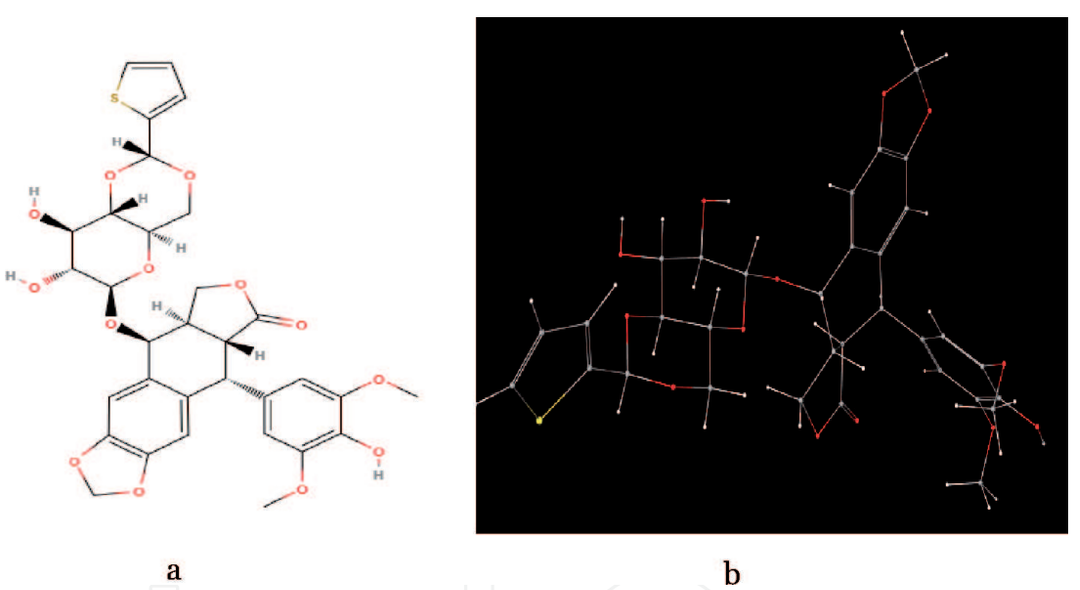

**Figure 2.**
*Chemical structure (a) of the resin podophyllotoxin and its 3D representation in form of wireframe (b).*

and is a dimerized product of the intermediates of the phenylpropanoid pathway. The starting material of etoposide (vepeside), an FDA approved anticancer drug, is podophyllotoxin and has been used to treat testicular cancer as well as lung cancer by inhibiting replication of cancer cells. Podophyllotoxin finds use as a precursor for the semisynthetic topoisomerase inhibitors in the treatment of leukemias, lung and testicular cancers, and dermatological disorders like warts, rheumatoid arthritis, and psoriasis. It also has numerous applications in modern medicine by virtue of its free radical scavenging capacity. An extract of *P. hexandrum* has been shown to provide approximately 80% whole-body radioprotection in mice [17]. Twenty-five percent solution of *Podophyllum* resin is efficacious and a cost-effective treatment with minimal side effects for HIV-related oral hairy leukoplakia, which is a symptom-free lesion



a b

**Figure 3.**
*The chemical structure of anticancer drug etoposide in 2D (a) and 3D (b) forms.*



a b

**Figure 4.**
*Chemical structure of anticancer drug teniposide in 2D (a) and 3D (b) forms.*

[18]. Another in vitro study showed podophyllotoxin as a promising cytotoxin against a set of human cancer cell lines: HL-60, A-549, HeLa, and HCT-8. PTOX was also found to activate proapoptotic endoplasmic reticulum stress signaling pathway [19].

Etoposide, teniposide, and etopophos are the different anticancer drugs derived from podophyllotoxin. These compounds are topoisomerase II inhibitors. Topoisomerase II enzyme is essentially required to cleave the double-stranded DNA and to seal it again after unwinding. It is crucial in the process of DNA replication and repair. Etoposide and other derivatives stabilize the DNA-topoisomerase II complex in a way so that resealing of DNA strands becomes impossible. Cells that are duplicating their DNA in the S phase and preparing for mitosis are very sensitive for this mechanism. The overall effect of these anticancer drugs is the arrest of the cells in late S or early G2 phase of the cell cycle [20–22].

Apart from being an important anticancer compound, podophyllotoxin is also found to possess various other important medicinal properties, some of which include:

Protection against radioactivity: Several researches have confirmed that various extracts of podophyllotoxin including chloroform, methanolic and hydro-alcoholic extracts provided 70–95% protection against radioactivity [23–25].

Antifungal activity against *Aspergillus niger* and *Candida albicans* [26].

The dichloromethane extract of this compound is investigated to possess insecticidal activity [27].

Traditional application: Used as an antihelminthic by Native Americans. In India, the aqueous extracts of the roots have been used as cathartic and also to cure ophthalmia [28].

## 2.3 Production of podophyllotoxin

Podophyllotoxin is chemically a member of the lignin group of compounds. Lignans are dimerziation products of two phenylpropane units linked by the β-carbon atom of the side chain [29]. Most of the pathways proposed involve phenolic oxidative coupling of C6-C3 monomers via schikimic acid pathway. Production of optically active lignan dimmers is an enzyme-controlled reaction [30]. A series of compounds of considerable commercial and medicinal interest as clinically useful anticancer drugs are formed by the reductive dimerziation of cinnamic acid or cinnamic alcohols [17]. **Figure 5** gives the biosynthetic pathway for the production of podophyllotoxin in *Podophyllum* species [31].

The synthesis of the derivative compounds as secondary metabolites occurs due to the diversified properties of the ring structures. The pathway for the biosynthesis of podophyllotoxin starts from coniferyl alcohol which is converted into pinoresinol in the presence of an oxidant through a series of reactions that involve the dimerization of a stereospecific reaction intermediate. Complete and conclusive knowledge on this pathway is still not available and research is on to incur more information about the genes and transcription factors that may be involved in the regulation of this pathway.

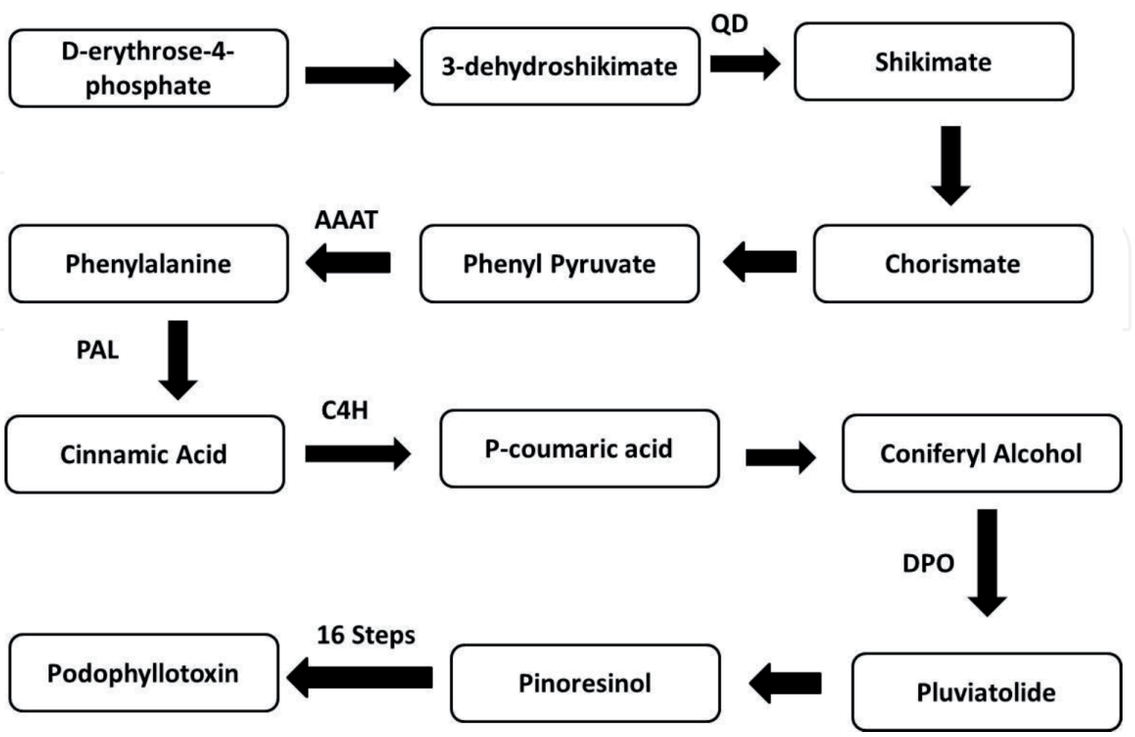



**Figure 5.**
*Biosynthetic pathway for the production of podophyllotoxin (QD: Quinate dehydrogenase; AAAT: Aromatic amino acid transaminase; PAL: Phenylalanine ammonia lyase; C4H: Cinnamate-4-hydroxylase; and DPO: Dirigent protein oxidase) [30, 32, 33].*

### 2.4 Limitations in the propagation of *Podophyllum hexandrum*

Since *Podophyllum hexandrum* is an endangered species and its resin has wide medical applications, there is a necessity to propagate the plant. But the process of propagation of the plant under both natural and laboratory conditions has some strict limitations which restrict the process. One of the major problems for cultivation of this plant is its long juvenile phase and poor fruit setting ability. Also, its seeds take a long period to germinate [34]. The plant has a low capacity of regeneration in natural environment and with the overexploitation of the plant coupled with the accelerated rate of destruction of its natural habitat, it is becoming extremely difficult to revive the plant in the wild.

Since the plant has strict requirement for conditions regarding the growth of the plant in the fields, therefore it is not amenable for cultivation as an agricultural crop, especially in the lowland areas which constitute a major percentage of land in India. Although in vitro approaches for the propagation of this plant with enhanced production of podophyllotoxin have long been studied and tested in various researches, the lack of complete knowledge of the pathway involved in the biosynthesis of podophyllotoxin has made these approaches limited to a small group of growth culture media and supplements which might enhance its production as well as its propagation. Cell and tissue culture techniques, though have shown some hope, are commercially not feasible, and therefore, cannot be used. Several studies are now being conducted on alternative approaches to optimize the culture conditions for the growth of this plant along with enhancement in the yield of podophyllotoxin to find a suitable technique that is both commercially feasible and experimentally reproducible.

## 3. In vitro method of propagation

Techniques of plant tissue culture have long been explored as instruments for the mass production of many overexploited and medicinally important plants as well as secondary metabolites. In vitro plant, cell, and organ cultures have been considered more feasible and amenable as compared to whole plants for the production of secondary metabolites since the plants are cultivated in simple and well-defined media under controlled conditions and they are independent of the natural environment for their growth and survival.

### 3.1 Somatic embryogenesis

The study isolated embryogenic callus from zygotic embryos and placed in 30-ml MS media supplemented with NAA and PVP. They kept the culture in complete darkness in rotary shaker (100 rpm) at 25 ± 2°C. After establishing the optimum strength for the MS basal media, embryogenic calli were cultured on MS media (0.75 strength) supplemented with 3 g/l PVP and varying concentrations of sucrose, glucose, fructose, and mannose. Thirty proliferated somatic embryos were cultured on 0.75 strength of MS basal media supplemented with 3 g/l of PVP and varying concentrations of ABA. The cultures were incubated at 25 ± 2°C for 16-h photoperiod and analyzed after 2 weeks. Matured somatic embryos were transferred to 0.75 strength of MS basal media supplemented with PVP and varying concentrations of GA3. Somatic embryos germinated on GA3 were dried and ground to fine powder and podophyllotoxin was extracted. Quantification of podophyllotoxin using water system with PDA detector at a wavelength of 250 nm was performed. Relative amounts of podophyllotoxin were calculated by comparing the peaks from

the chromatogram [22]. 2, 4-D, and NAA were seen to have profound effect on the callus growth. 1 mg/l of 2, 4-D in combination with 3 g/l of PVP gave the best results for culture establishment. A change in osmotic pressure directly affects the development of embryos. Best results of callus growth were obtained for 0.75 strength of MS basal media. It also had higher podophyllotoxin content. Sucrose was found to be the best carbon source, and 4% sucrose with 0.75 strength of MS basal media gave better results. 1 mg/l ABA concentration showed efficient maturation and plants showed better podophyllotoxin content at this concentration. The study concluded that best suspension cultures may be obtained for 0.75 strength of MS basal media supplemented with 1 mg/l 2, 4-D, and GA3, each with 4% sucrose and incubation at 25 ± 2°C.

### 3.2 Precursor feeding

It has been reported that upon using coniferin as a precursor for the podophyllotoxin, production increased by 12.8 times. The problem with this technique is that coniferin is not commercially available. Therefore, Lin et al. devised a technique of coculturing of *Linum flavum* hairy roots and *Podophyllum hexandrum* cell suspensions in 2003. In this study, *Linum flavum* hairy roots and *P. hexandrum* cell suspensions were used to build a coculture system for the in vitro production of podophyllotoxin. *Agrobacterium rhizogenes* strains, LBA9402 and TR105, were used to initiate hairy roots from seedlings of *L. flavum*. The roots were maintained with liquid MS media supplemented with sucrose of conc. 30 g/l at a pH of 5.9. The roots were incubated in flasks containing 25-ml MS media in a rotary shaker running at 100 rpm in the dark. [35]. *L. flavum* hairy roots were cocultured with *P. hexandrum* cell suspension cultures in a dual 500-ml shaker flask with the bottom side openings linked by a 4–5 cm length of 3 mm silicone tubing. The culture was incubated at 25°C in dark in a rotary shaker running at 100 rpm. *Linum flavum* hairy roots and *P. hexandrum* cell suspensions were cultured separately in two 2-L bioreactors. 5 g FW of 3-week old *L. flavum* root was inoculated directly into 1.8 L of LS medium and the airflow rate was set at 80–100 cm3/min for maintaining DO tension above 85%. 360 ml of 3-week old suspension cultures of *P. hexandrum* were inoculated in LS medium and total volume was made up to 1.8 L and the airflow rate was set at 120–150 cm3/min to maintain the dissolved oxygen tension above 80% air saturation. Medium exchange between the two plants was started 12 days after the inoculation. Cultures were harvested after 29 days and medium samples were analyzed periodically. The results from the study concluded that the dual bioreactor containing the coculturing of the two plants showed a better podophyllotoxin concentration per biomass (mg-1 dry weight) of 0.062 as compared to 0.032 mg-1 dry weight in single reactor. The concentration of coniferin was also found to increase in the reactor containing both the plants.

### 3.3 Production through hairy root cultures

The study showed that strains of *A. rhizogenes* used for embryo transformation in *P. hexandrum* produced transformed calli. HPLC profiling of these transformed calli revealed that the culture contained three times more podophyllotoxin in contrast to controls [36]. In this study, the seeds of *P. hexandrum* were rinsed in Tween 20 and surface sterilized with 0.2% mercuric chloride for 10 min and soaked in water for a day. Dissected embryos were cultured on MS medium and incubated in continuous light at 25 ± 2°C. *Agrobacterium rhizogenes* strains viz. 15,834, Aq, and K599 were grown on nutrient agar at 29°C and cultured in YMB liquid medium for 48 h. Different explants were examined for the induction of hairy root cultures.

Ten- to 15-day old aseptically growing embryos of *P. hexandrum* (in two sets) were wounded and incubated in acetosyringone (25 mM) in combination with lO mM glucose, 5 mM morpholino ethane sulphonic acid (MES), and 150 mM NaCl and were incubated for 20 min in 48-h old cultures of the respective *A. rhizogenes* strains. The embryos were then transferred to MS basal media containing acetosyringone (50 pM) with and without 2, 4-D, BAP and were incubated in two sets, one in light and the other in dark. The growth of transformed and control cultures was monitored after culturing 40 mg of inoculum in 50 ml of medium in 250-ml flasks in triplicates. The cell suspension culture was harvested every 3 days up to the 12th day and every 2 days after 12 days and the increase in weight was recorded. For podophyllotoxin production, tissue (both transformed and control) was air dried, weighed, and powdered. Extraction was done by treating the callus with methanol for 8 h in a soxhlet apparatus at 60°C. The methanolic extract was concentrated in a rotavapor. The residue was dissolved in methanol (AR) prior to analysis. Podophyllotoxin content was analyzed by HPLC. It was concluded from the study that the strains of *A. rhizogenes* used, namely A4 and 15,834, showed fast-growing calli at the site of infection. Hairy root phenotype was not observed despite the addition of acetosyringone alone or in combination with glucose, MES, and NaCl. Approximately, threefold increase in the podophyllotoxin content was observed as compared to control cultures. HPLC analysis indicated a maximum of 0.7% podophyllotoxin in cell suspension cultures derived from callus lines transformed with *Agrobacterium rhizogenes* strains, A4 and 15,834, while the control calli gave a maximum of 0.2% podophyllotoxin only. Highest accumulation of podophyllotoxin in cell suspension cultures was obtained during the stationary phase up to 18 days after which it declined.

### 3.4 Biotransformation

Biotransformation approach of podophyllotoxin production has helped to develop the derivatives of this resin, which have enhanced the anticancer properties coupled with the antimitotic activity of podophyllotoxin. So, Rajesh et al. initiated the *Agrobacterium*-mediated biotransformation of *P. hexandrum* for increased production of podophyllotoxin [35]. Mature seeds of the plant were collected from its natural habitat, washed with running tap water, and then rinsed with 0.1% (v/v) Teepol solution. The seeds were then sterilized with 70% (v/v) ethanol for 1 min followed by 0.1% mercuric chloride for 10 min and were finally rinsed several times with sterile double-distilled water. The seeds were stored in flasks containing 30 ml of sterile double-distilled water for a day on an orbital shaker running at 120 rpm. Three strains of *A. tumefaciens*, LBA 4404, EHA 101, and bEHA 105 containing the pCAMBIA 2301 binar vector having nptII and gusA genes were used. Both these genes are controlled by CaMV 35S promoter and poly (A) terminator. The cocultivated embryogeniccalli were washed and later inoculated in MS basal media supplemented with 150 mg/l kanamycin and 200 mg/l timentin and then were incubated for 6 weeks at 25 ± 2°C under a 16-h photoperiod. The surviving embryogenic calli were separated and subcultured onto fresh selection media. The matured somatic embryos were germinated for 2 weeks on a germination medium (GM) containing 150 mg/l kanamycin at 25 ± 2°C under a 16-h photoperiod. Rooting plantlets were transferred into paper cups containing perlite, peat moss, and vermiculite (1:1:1 v/v/v) and covered with polythene bags to maintain 80% relative humidity. The results from the study showed that timentin was found to exhibit a better efficiency than cefotaxime at all the concentrations tested. Timentin at 200 mg/l inhibited the growth of the three strains of *Agrobacterium* that were tested. Cefotaxime controlled *Agrobacterium* growth at 300 mg/l at which the rate

of somatic embryogenesis was 27.33% (13.66 out of 50 calli responded). Three days of cocultivation proved to be optimal as under these conditions, 65.33% of embryogenic calli (32.66 out of 50) GUS histochemical analysis revealed that the transgene was successfully integrated and expressed in the *P. hexandrum* genome.

## 4. Ex situ method of propagation

Attempts to obtain podophyllotoxin through cell cultures or chemical synthesis techniques are still far from being economically feasible. The objective of the following study was to enhance the root formation and podophyllotoxin production of *P. hexandrum* cultivated in a glasshouse [37]. Two batches of plants grown for different time periods were obtained from two different regions and stored at 7–8°C in the dark to prevent the formation of shoot. They were later cultivated in glasshouse in the peat-perlite soil (2:1 w/w). For every condition and time point, 15 plants were randomly harvested. The root biomass and podophyllotoxin content of the plants of each temperature group were analyzed at the beginning. The plants were harvested for a period of 20 or 40 days, but all the plants were cultivated for a minimum period of 20 days before giving them the methyl jasmonate treatment. Fifteen plants were immediately harvested for baseline control, 30 plants were sprayed with water (control), and 30 plants were sprayed with 5 l of 1.5 mM methyl jasmonate. After 9 days, 15 plants from each group were harvested for analysis. The plants in the treatment group were sprayed again for 3 consecutive days with 5 l of 3 mM methyl jasmonate each day and harvested for analysis the next day. Roots from each plant were collected, rinsed with tap water, and dried for 18 h at 40°C. They were later pooled in groups of three, ground and stored at room temperature in closed containers in the dark. 10 ml of methanol was added to 1 g of the plant material. The sample was vortexed at 2500 rpm and incubated at 65°C in a water bath for 10 min. Then the mixture was centrifuged at 4°C at 2400 g for 10 min. The supernatant was separated and transferred to a fresh tube. This extraction process was performed five times. The podophyllotoxin content was determined by HPLC analysis and the samples were stored at 4°C before analysis. Podophyllotoxin is stable in the refrigerator at 4°C for at least 3 months and at 25°C. The results of the study showed a higher concentration of podophyllotoxin in MeJ-treated plants (30 mg/g) as compared to MeJ-deficient plants (18 mg/g).

## 5. Latest advancements in production of podophyllotoxin and growth of *Podophyllum hexandrum*

### 5.1 Role of endophytes in the production of podophyllotoxin

Endophytes are the group of microorganisms that are found to colonize the interior of the plant irrespective of the type of association they have to maintain with the host. Although this group includes a number of species of microbes, it is less explored. They have great potential of application in agriculture, bioremediation, medicine, etc. [38]. A study reported the isolation of an endophytic fungus, *Fusarium solani*, from the roots of *P. hexandrum*, which was found to synthesize podophyllotoxin at a rate of 29.0 μg/g on a dry weight basis [39]. The results were confirmed by HPLC and mass spectroscopy techniques. Upon isolation of the fungal hyphae from the roots of *P. hexandrum*, selection of the fungal species was done by assessing the presence of podophyllotoxin in the three strains that had been isolated, after which the rDNA analysis confirmed the fungus under study to

be *Fusarium solani*. Shake flask experiments were performed, the results of which indicated that maximum biomass production was obtained on the 10th day of growth while the maximum yield in podophyllotoxin was found around the 8th day of growth.

### 5.2 Identification of key transcription factors involved in regulation of biosynthetic pathway for podophyllotoxin production in *P. hexandrum*

Although the exact pathway of biosynthesis of podophyllotoxin is still not known, information about the regulatory components of this pathway is also unavailable. Considering the importance of transcription factors and their role in upgrading the industrial synthesis of podophyllotoxin, the present study worked to identify different transcription factors that might be involved in regulating the pathway of podophyllotoxin biosynthesis [40]. The study worked to identify various classes of transcription factors via the mining of transcriptomes of *Podophyllum* species and validation of these factors by qRT-PCR analysis coupled with the analysis of podophyllotoxin content from the different tissues of *P. hexandrum*. Extensive survey of available literature revealed that four transcription families (TFs), basic leucine zipper (bZIP), myeloblastosis (MYB), WRKY, and basic helix-loop-helix (bHLH) were involved in the regulation of phenylpropanoid pathway in several species of plants. It was therefore hypothesized that these TFs might also be involved in the regulation of biosynthesis of podophyllotoxin in the species of *Podophyllum*. Two distinctive transcripts were identified which encoded for bZIP and MYB TFs in the rhizomes of *P. hexandrum* and which were associated with podophyllotoxin content. Upon quantifying the content of podophyllotoxin and analyzing the comparative expression between the high (2.51%) versus the low (0.59) content of podophyllotoxin accessions, the results showed a 0.04- to ~16-fold increase in the transcripts of the transcription factors, further supporting the involvement of the identified TFs with the content of podophyllotoxin. For *P. hexandrum*, the highest transcript abundance was observed for bZIP (19.60-fold) in the rhizome showing 2.51% of podophyllotoxin as compared to the shoots which showed only 0.01% of the resin. In silico analysis of putative promoter regions of the genes associated with this pathway in other species of plants have shown the presence of certain sequence elements for MYB and WRKY TFs, which suggested their involvement in regulating the production of podophyllotoxin. Abundance of the transcript was evaluated with respect to the transcription families using fragments per kilobase of transcripts per million mapped reads (FPKM) and qRT-PCR-based transcript by in silico techniques. The values of TFs FPKM (fragments per kilobase of transcripts per million mapped reads) ranged between 0.0014–12.01 and 0.014–1162.01 in the transcriptomes correlating with shoots and rhizomes of *P. hexandrum* and *P. peltatum*, respectively. Gene expression pattern was observed through two different platforms and the results were in synchrony with each [31].

## 6. Conclusion

From the study, it can be concluded that growth of *Podophyllum hexandrum* is difficult in vitro, but the plant can be grown successfully by somatic embryogenesis provided that media and other culture conditions are optimized in a stepwise manner. The traditional culturing techniques do not tend to increase the podophyllotoxin production, whereas its production can be increased by coculturing of hairy roots of the plant along with *L. flavum*. This process is called is precursor feeding while molecular techniques such as genetic engineering of the plant with the help of

*Agrobacterium tumefaciens* have also showed an increase in podophyllotoxin production. The other methods to increase metabolite production include the growth of hairy root culture and spraying of methyl jasmonate in the plants under greenhouse conditions. There are certain gaps which have not been taken care of like there is very less literature available showing the growth of *Podophyllum hexandrum* using micropropogation techniques. The genetic data about the plant's genes involved in metabolite production are not easily available. Use of bioreactors for the growth and production of podophyllotoxin has not yet been evaluated properly. There is further in-depth study required to be done on *Podophyllum hexandrum* before we can begin the large-scale podophyllotoxin production from the plant.

The formation of State Medicinal Plant Board ensures increased cultivation and conservation of the medicinally important endangered plants by providing appropriate funding to conserve these species [41]. Although the efforts of agrotechnological innovation and biotechnological processes cannot be undermined, only a few of these endemic plant species are under cultivation majorly by the efforts of local farmers and NGOs [3].

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## Conflict of interest

The authors declare that they have no conflict of interest whatsoever.

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