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Mass propagation and conservation of *Podophyllum emodi* Wall, an endangered medicinal plant of the Himalaya

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Abstract

The technique of excised embryos was employed to facilitate the propagation of *Podophyllum emodi* derived from seeds collected at the different elevation zones of Himachal Pradesh, India. Seed germination was low, irrespective of stage of seed development or zone of collection. Germination was improved significantly (89.14%) in the partially mature and mature seeds collected from alpine and temperate zones when excised embryos were cultured on basal B5 medium. Leaf emergence and plant establishment in the field was also significantly higher in the plants raised through this technique, despite hypocotyl dormancy. The technique was successfully employed for the production of plants to be reintroduced in large numbers into their habitat in the Great Himalayan National Park.

Keywords: conservation; excised embryo culture; mass propagation; *Podophyllum emodi*

Introduction

Podophyllum emodi Wall (syn. *P. hexandrum* Royale) is an important medicinal perennial species commonly distributed throughout the Himalayas, parts of Afghanistan and south-west China (Fujii, 1991). The roots and rhizomes of *P. emodi* are a rich source of aryltetralin lignans. These lignans have shown several biological activities including anti-cancer, antifungal and immunomodulator properties (Kamil and Dewick, 1986; Foster, 1989; Broomhead and Dewick, 1990; Canel *et al.*, 2000). The major lignan with commercial value found in *P. emodi* is podophyllotoxin, which is the starting material for the semi-synthesis of the anticancer drugs etoposide, teniposide and etopophos. These pharmaceuticals are chemotherapeutic agents for the treatment of lung and testicular cancers as well as for leukaemias. Podophyllotoxin is also the precursor to a new derivative, CPH 82, that is being tested for rheumatoid arthritis in Europe and to other derivatives for the treatment of psoriasis and malaria. Besides *P. emodi*, other species like *P. peltatum* (Jackson and Dewick, 1984; Moraes *et al.*, 2000, 2001a, b; Dayan *et al.*, 2003), *P. pelianthum* (Jackson and Dewick, 1985) and *P. versipelle* (Broomhead and Dewick, 1990) are also important sources of podophyllotoxin.

The demand for podophyllotoxin to produce the anti-cancer drugs coupled with its existing use in traditional systems of medicine has resulted in a ruthless uprooting of the underground parts of the plant, leading to intense collection coupled with the lack of organized cultivation. Consequently, *P. emodi* has been declared an endangered species (Bhadula *et al.*, 1996; Airi *et al.*, 1997). Efficient methods are required for its sustained propagation and organized utilization.

Although podophyllotoxin has been found in 40 different species among different families, *P. emodi* is the commercial source. It is important for India to conserve this

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species and to domesticate it into a medicinal crop for lignan production. Thus, early attempts in the domestication of *P. emodi* were through multiplication of rhizomes (Badhawar and Sharma, 1963), but since rhizomes are also the source of podophyllotoxin, considerable loss is incurred in terms of propagules and harvestable material of economic importance (Sadowska *et al.*, 1997). Seed dormancy has constrained the development of *P. emodi* as a medicinal crop (Badhawar and Sharma, 1963).

Under natural conditions, *P. emodi* generally avoids the harsh winter conditions of its first growing season by developing 'hypocotyl dormancy' (Purohit and Nautiyal, 1986). This mechanism prevents the seed/seedlings from germinating and growing in adverse conditions, especially as the winter in alpine or temperate zones commences shortly after the seed is shed. On the other hand, germination in spring helps the seedling to develop sufficiently to adapt to the harsh conditions of the following winter.

Attempts to use tissue culture for mass propagation of *P. emodi* have been made before. Thus, somatic embryogenesis and multiple shoot induction were achieved by Arumugam and Bhojwani (1990) and Nadeem *et al.* (2000). Field survival and failure in maintaining a diverse gene pool for conservation of the remaining accessions of *P. emodi* are the major constraints. Thus, we are attempting to develop excised embryo protocols for circumventing the problems of mechanical and endosperm dormancy (Collins and Grosser, 1984) during sexual propagation in order to facilitate the propagation of large populations, and to conserve the gene pool of the remaining accessions.

Materials and methods

Plant material

Red, yellow and green berries of *P. emodi* were collected from two elevations of the temperate (regions below high elevation tree line) and alpine (regions above high elevation tree line) habitats of Chamba district (2200 m asl) and Lauhal and Spiti districts (3200 m asl), Himachal Pradesh, India, during the last week of August and first week of September. Mature red berries were also collected from the alpine zone of Chamba district (3600 m asl), the sub-alpine zone of Lauhal and Spiti districts (3200 m asl), and the temperate zone of Kullu district (2400 m asl).

Berries at three different developmental stages (namely green immature, yellow partially mature and red fully mature) were used in the present study. Seeds separated from pulp were disinfected with 0.1% Bavistin and 0.05% streptomycin sulphate for 15 min and soaked overnight in sterile distilled water at room temperature. The seeds were then subjected to a 1-min dip in 70% alcohol followed by surface sterilization with 0.1% mercuric chloride for 7 min. All traces of mercuric chloride were washed off by four or five rinses in sterile distilled water.

Culture of immature, partially mature and mature excised embryos on different media

The surface-sterilized seeds were dissected with a scalpel blade at the base of the micropylar lobe, and embryos were carefully extruded (Fig. 1a). In order to identify a culture medium for optimal germination of the excised embryos, various media supplemented with 3% sucrose were tested: MS (Murashige and Skoog, 1962), half-strength MS, WPM (Woody Plant Medium; Lloyd and McCown, 1980), half-strength WPM, B5 (Gamborg *et al.*, 1968) and half-strength B5. The influence of 2.5 μM gibberellic acid (GA_3; filter sterilized) on embryo germination was also tested by supplementation to the above media. After germination, the embryos were transferred to their respective media, free of hormones, for further growth.

The pH of the media was adjusted to 5.8 with 1 N KOH and HCl prior to the addition of agar (0.8%) and was sterilized at 121°C for 20 min (McCown and Sellmer, 1987). All cultures were maintained at 25 ± 2°C in darkness for embryo germination, and data were recorded for radicle emergence at 3-day intervals. The total time taken for germination was also recorded. Cultures with fully developed radicles and expanded cotyledons were then transferred for further growth to a 14-h light (52 μmol m^{-2} s^{-1}) alternating with a 10-h dark period.

Hardening

Four-week-old plantlets (6–8 cm long) with well-developed root, hypocotyl and green cotyledonary leaves were transferred to the greenhouse and potted in a potting mix of sand, soil and farmyard manure (3:1:1) for establishment into healthy young plants.

Data were recorded every 15 days for (i) survival percentage of the plants and (ii) true leaf emergence.

Establishment of plantlets in the natural reserve of the Great Himalayan National Park (GHNP)

Each year a total of 600 emblings (plantlets raised from excised embryos) were established, in three blocks with 200 emblings in each block, in the protected area of

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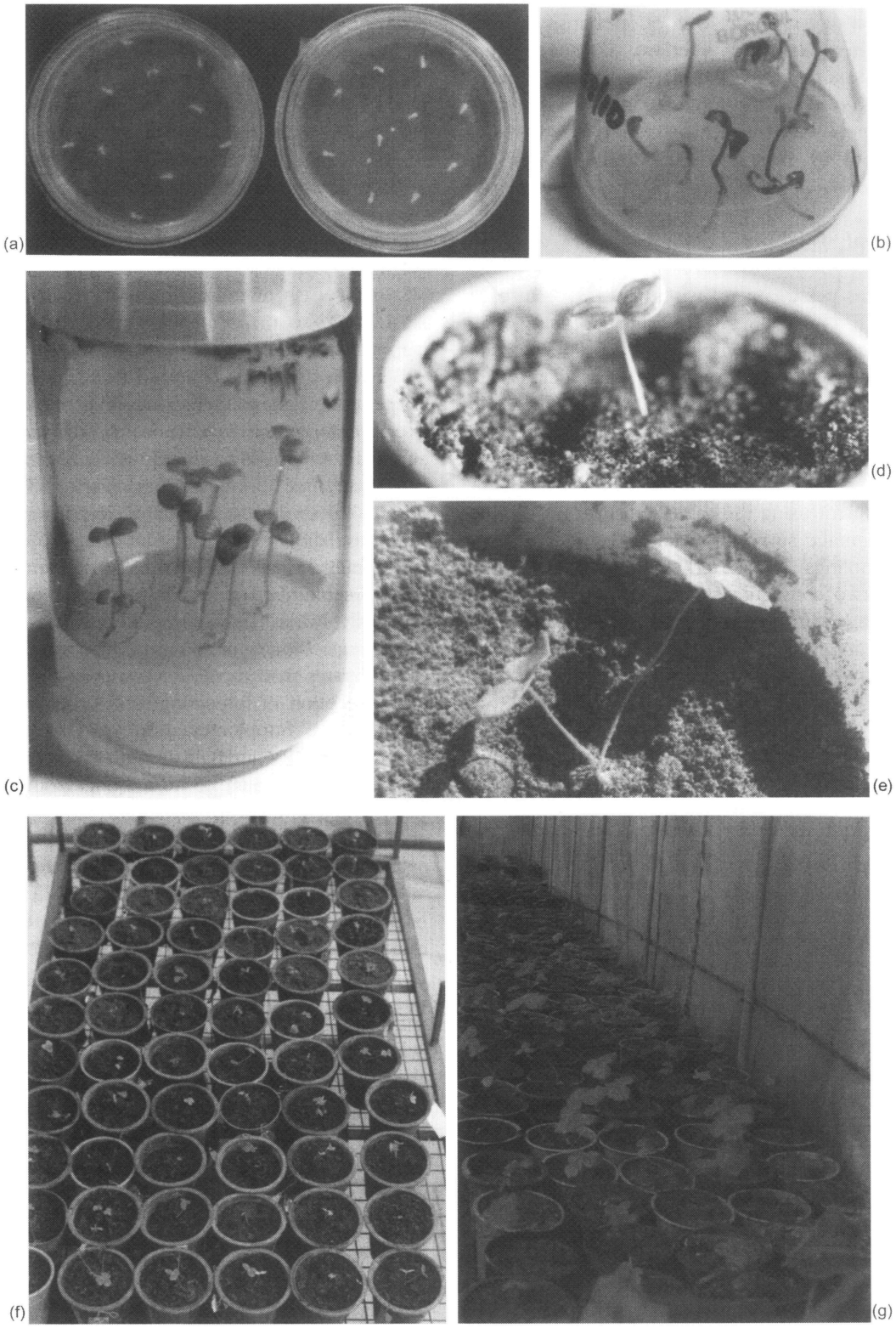

Fig. 1. Excised embryo culture and mass propagation of *Podophyllum emodi*. (a) Excised embryos; (b, c) plantlets from germinated excised embryos; (d) plantlets raised from germinated embryos with two cotyledonary leaves; (e) embling with early emergence of true leaf from the base of the hypocotyl; (f) hardened plantlets raised from germinated excised embryos; (g) 1-year-old plants growing in a polytunnel.

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the GHNP, free of any human interference. Each block was comprised of four rows with 50 emblings in each row. Both inter-plant and inter-row distances were about 45 cm. Data regarding the plantlet survival percentage were collected on the total number of vigorously growing plantlets with true leaves during the two subsequent years (1999–2000 and 2000–2001).

Statistical analysis

Three replicates per treatment with 10 embryos in each treatment were used. Values were estimated using analysis of variance (ANOVA) employing a completely randomized design. Prior to analysis, the data were transformed using angular transformation (Gomez and Gomez, 1984), and the percentage means were compared against $P \geq 0.05$. Data for true leaf emergence were recorded on three replicates containing 50 emblings each per treatment using the same statistical procedure. Data for percentage survival (mean and standard deviation) of emblings transferred to the GHNP were recorded for two consecutive years on each set of 200 emblings arranged in three replicates.

Results

Effect of different media on the germination of embryos excised from the seeds of two different regions (Chamba and Lahaul and Spiti districts)

While the immature embryos did not germinate at all, the germination rate of partially mature and fully mature embryos on optimal medium, within a week of culture, was as high as 89% (Fig. 1b, c).

A significant difference was observed in the germination response of the excised embryos on the different media tested (Table 1). The highest germination of both alpine and temperate zone embryos was recorded on B5 as well as half-strength B5 media, and there were no significant differences in the percentage germination between years. Although the germination response was significantly lower on either the full- or half-strength WPM or MS media, with the exception of half-strength MS in the case of the alpine embryos, the addition of 2.5 μM GA_3 to these media improved the germination response considerably.

Between the seeds collected from the temperate and alpine zones, there were no significant differences in overall embryo germination on full- and half-strength B5 and half-strength MS media. However, during the hardening phase after 12 weeks of transfer to the greenhouse (Fig. 1d), their development and establishment showed a significant difference (Table 1). Marginal differences in the overall survival of the embling subsequent to hardening were observed between the alpine and temperate zones, especially when they were derived from half- and full-strength B5. The emblings derived from B5 had better survival rates than those derived from the other media, with exception of full-strength WPM in the case of the alpine ones. Supplementation of GA_3 to half- and full-strength WPM and half-strength MS medium improved the survival percentage of the emblings from seeds of both alpine and temperate zones. However, no significant effect of GA_3 supplementation was observed on the survival percentage of the emblings derived from half- and full-strength B5 media.

Table 1. Effect of media composition on the germination, development of emblings (plantlets raised from excised embryos), field survival and third leaf emergence in seeds derived from alpine and temperate zones

		Germination (%) after 1 week		Embling development (%) after 4 weeks		Field survival (%) after 12 weeks	
Medium	Strength	Alpine	Temperate	Alpine	Temperate	Alpine	Temperate
B5	Full	89	89	89	89	75	89
	Full + GA_3	89	89	89	89	89	89
	Half	89	89	89	67	55	67
	Half + GA_3	89	89	89	89	59	64
WPM	Full	7	29	1	1	1	1
	Full + GA_3	72	75	89	89	50	48
	Half	32	30	81	1	27	1
	Half + GA_3	89	89	81	54	58	1
MS	Full	51	7	55	1	75	1
	Full + GA_3	81	81	75	75	36	48
	Half	89	26	30	1	26	1
	Half + GA_3	89	81	75	75	72	89
$P \geq 0.05$		4	2	2	4	2	11

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Germination of immature, partially mature and fully mature embryos on full-strength B5 medium

After 1 week of culture, different germination responses were observed in the embryos at different stages of development. Irrespective of the zone of collection, the immature embryos did not germinate, whereas 89% of the partially and fully mature embryos germinated (Table 2). There was no difference in the germination response between embryos obtained from berries collected from the five different elevations.

True leaf emergence

Prior to the setting of hypocotyl dormancy, i.e. 90 days after field transfer, percentage true leaf emergence in the temperate and alpine emblings was 38% and 54%, respectively (Fig. 2). During the period of winter dormancy comprising 50–60 days, the emergence of the true leaf ceased in the remaining emblings. With the completion of dormancy, the true leaf emerged (Fig. 1e) in 69% and 89% of the temperate and alpine emblings, respectively, 180 days after field transfer. In general, seeds collected above the tree line showed a higher response to true leaf emergence compared to those collected from the temperate zones.

Hardening of emblings derived from embryos germinated on different media

The effect of different media on the survival percentage of the emblings was observed when they were transferred to pots in the greenhouse (Table 1). Better survival percentages were recorded in the emblings derived from culture on either full- or half-strength B5 media (with or without GA_3) 12 weeks after field transfer. However, the survival percentage of alpine zone emblings derived from GA_3-supplemented full- and half-strength WPM, full-strength MS and GA_3-supplemented half-strength MS was significantly higher.

Table 2. Germination percentage of immature, partially mature and mature embryos on B5 medium from alpine and temperate zones

	% Germination	
Stage of embryo	Alpine	Temperate
Immature (green berries)	1	1
Partially mature (yellow berries)	89	89
Fully mature (red berries)	89	89
$P \geq 0.05$	NS	NS

NS, not significant.

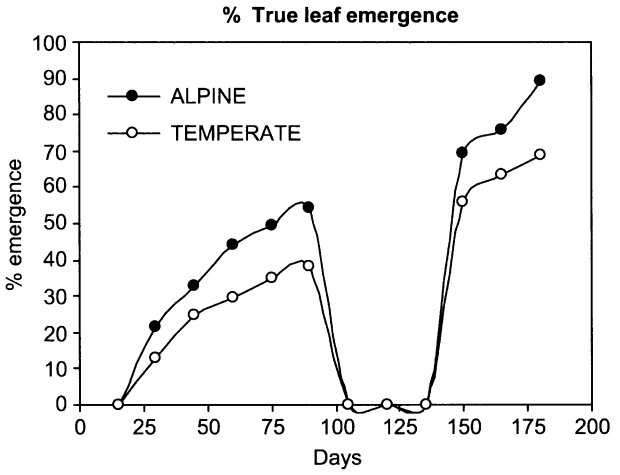

Fig. 2. True leaf emergence in plantlets from seeds of the alpine and temperate zones.

Establishment of emblings in the natural reserve

While 94% of the 566 plantlets with true leaves grew vigorously after 1 year of transfer to the GHNP, 485 plantlets (81% survival) with true leaves showed vigorous growth after the second year (Table 3).

Discussion

An effective protocol for the mass propagation of *P. emodi* plants through embryo culture is described in this paper. This technique offers an important solution to the domestication of the species, which has been a persistent problem using conventional modes of propagation, i.e. sexual and vegetative propagation. Propagation only through vegetative means may result in loss of valuable representative genetic diversity, which may be below the level of species (Mallet, 1996) and may also result from the confinement of the plants to local colonies (Parker, 1989). Sexual reproduction in *Podophyllum* is restricted by a long juvenile phase (Rust and Roth, 1981), poor fruit setting, long dormancy periods of 10–24 months, low and erratic seed germination, and poor

Table 3. Percentage survival of emblings after the first and second years of field transfer to the natural reserve of the Great Himalayan National Park

Year	No. of vigorously growing plantlets with true leaf	% Survival of healthy plantlets[a]
1999–2000	566	90 ± 1.2
2000–2001	485	81 ± 2.8

[a] Values are means ± SD, $N = 600$.

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seedling establishment (Badhawar and Sharma, 1963; Arumugam and Bhojwani, 1990).

The present study confirmed an earlier report (Arumugam and Bhojwani, 1990) that *Podophyllum* seeds exhibit seed coat-imposed dormancy and endosperm dormancy. Existence of such innate dormancy may be the reason for the lack of a reproducible protocol for breaking seed dormancy and inducing a uniformly high germination response (Badhawar and Sharma, 1963). Although frugivores are known to bring about germination of *Podophyllum* after passage of the seeds through their gut (Rust and Roth, 1981), the problem of dormancy still persists in these plants. In the light of all these problems, the technique of excised embryo culture seems to be a promising solution.

Two earlier reports in this regard (Arumugam and Bhojwani, 1990; Nadeem *et al.*, 2000) on embryo excision and culture elaborated upon the process of callus induction and somatic embryogenesis, but not on mass establishment of heterogeneous populations in the field. Nadeem *et al.* (2000) largely emphasized shoot multiplication and somatic embryogenesis, but did not deal with mass establishment of seedlings or emblings. The present work, while focusing on the limited utility of *in vitro* cultures via an excised embryo culture technique, emphasizes the importance of mass propagation and conservation of collected germplasm through seeds and not through *in vitro* multiplication.

In the present study, the seeds of both the alpine zone (encompassing life forms that exist above the climatic high-elevation tree line, irrespective of latitude) and temperate zone (with at least 1 month of frost or with 1 or more months with mean temperature of $<18°C$ and with at least 4 months with mean temperature of $>10°C$) (Korner, 2001; Silander, 2001) failed to germinate significantly either under *in vitro* or *in vivo* conditions. However, germination was significantly increased when excised embryos of partially mature and mature seeds collected from various climates were cultured under *in vitro* conditions (Table 2). Therefore, it is evident that the excised embryo cultures have the potential for high and uniform germination and also for consistently high plant establishment and survival. This is not surprising because the employment of excised embryo culture to circumvent the problems of seed germination is well documented in several plant species (Collins and Grosser, 1984). Thus, the germination of excised embryos of *P. emodi* from both alpine and temperate zones was not only higher (i.e. 89% compared to 4% in the case of seeds collected from any elevations/zones), but also rates of survival and plant establishment were better. Since the red berries are generally lost to grazing sheep, it was thought necessary to evolve a strategy whereby green immature berries could be used for propagation, thereby reducing the loss of useful red berries. This was proved to be true as the protocol we have developed allowed large numbers of healthy plants to be regenerated from embryos taken from green berries.

When different media were tested for optimal response, *P. emodi* from both the zones seemed to have specific requirements for germination and overall development. Both full- and half-strength B5 medium evoked the highest germination compared to MS or WPM. Moreover, percentage survival and plant establishment were also high in the plants derived from B5 medium. The relatively poor response of excised embryos on MS and WPM was, however, overcome to a large extent when GA_3 was added to MS or WPM, both in the temperate and alpine seed-derived embryos. The role of GA_3 in overcoming the problems of reserve mobilization during germination is well known (Bewley and Black, 1982). The positive effect of GA_3 in this study is probably due to the activation of α-amylase in the embryos and hence in the mobilization of reserves and, thus, in their germination as in other species (Bewley and Black, 1982).

The preference for B5 by the embryos of *P. emodi* in this study also shows their requirement for specific mineral nutrients. The B5 medium has a higher ratio of NO_3^- to NH_4^+ compared to WPM or MS, wherein the NH_4^+ to NO_3^- is higher (McCown and Sellmer, 1987). The ratio of NO_3^- to NH_4^+ present in the B5 medium is probably important for the mobilization of starch and regulation of the osmoticum by *P. emodi* embryos, as has been reported in other species (Ziegler, 1995). This was further confirmed by the fact that the otherwise poor germination on full- and half-strength WPM and MS could be significantly improved by supplementation of 2.5 μM GA_3, the major factor responsible for mobilization of starch.

The *ex situ* conditions of the present study probably helped in overcoming the environmental barriers of true leaf emergence that are known to exist under *in situ* conditions. Inhibition of true leaf emergence due to hypocotyl dormancy has already been reported by Purohit and Nautiyal (1986).

The present paper proposes a standardized and reproducible protocol for the mass propagation of *in vitro*-raised plants in the field, irrespective of the genotype of the source plant. Besides minimizing the time period for seedling establishment from between 11 months and 4 years (Rust and Roth, 1981; Singh *et al.*, 1999) to only 4 months, we have also been successful in establishing greenhouse-hardened plants derived from embryos under *in situ* conditions. We believe that this work represents the first documented case of successful *en masse* establishment of embryo-derived plants of this endangered species in the Great Himalayan National Park.

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