

***In vitro* propagation of *Podophyllum peltatum* L. by the cultures of embryos and divided embryos**

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Abstract

Excised embryos and subsequently divided embryos of *Podophyllum peltatum* were cultured on the Murashige and Skoog medium supplemented with different growth regulators, because traditional methods of breaking seed dormancy failed. The growth of excised embryos was stimulated by 1 or 0.1 mg dm⁻³ gibberellic acid (GA₃), 0.1 mg dm⁻³ GA₃ + 0.2 mg dm⁻³ kinetin (kin), or 0.2 mg dm⁻³ kin. GA₃ (1 mg dm⁻³) showed the best effect; after 5 weeks the plantlets had 1.5 - 2 cm long cotyledons, 5 - 6 cm long roots, 88 % of embryos germinated and developed further. The addition of 0.5 mg dm⁻³ zeatin + 0.2 mg dm⁻³ naphthaleneacetic acid (NAA), 0.2 mg dm⁻³ NAA, and 1 mg dm⁻³ kinetin inhibited the growth of embryos. 1 mg dm⁻³ kinetin + 0.1 mg dm⁻³ NAA, 0.1 mg dm⁻³ zeatin and 0.2 mg dm⁻³ 6-benzylaminopurine resulted in a compact appearance of plantlets and a lower germination rate. Divided embryo cultures produced plantlets *via* somatic embryogenesis which occurred only on the 2,4-dichlorophenoxyacetic acid containing media. The maturation of somatic embryos was observed on media without any auxin.

Additional key words: growth regulators (BAP, 2,4-D, GA₃, kin, NAA), seed dormancy, somatic embryogenesis.

Introduction

Podophyllum peltatum L. (*Berberidaceae*) is a medicinal perennial herb commonly found in North American forests from South Canada to South Georgia. In the countries where *Podophyllum peltatum* is cultivated it is propagated vegetatively through 10 cm long rhizome parts. The disadvantage of this method is loss of precious pharmaceutical material. Plant regeneration from callus (while the callus tissue was initiated by incubating leaf and stem segments) has already been reported

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Abbreviations: BAP - 6-benzylaminopurine; 2,4D - 2,4-dichlorophenoxyacetic acid; GA₃ - gibberellic acid; IAA - indole-3-acetic acid; kin - kinetin; MS - Murashige and Skoog medium; NAA - naphthaleneacetic acid; SDS - sodium dodecyl sulphate.

by Fuji (1991). Generative propagation of *Podophyllum* is not easy. The seed dormancy period of the species is very long and almost impossible to break using traditional methods (Badhwar and Sharma 1963, Rush and Russel 1976). However, Barykina (1971) and Selivanova-Grodkova (1973) reported 90 % germination rate under laboratory conditions when the seeds were fresh. We re-examined the germination rate of *Podophyllum peltatum* seeds and the influence of scarification, stratification and GA₃ treatment on it. As in our studies positive results were not obtained, we isolated embryos and germinated them *in vitro*. The isolated embryo cultures of *Podophyllum hexandrum* were established by Amurugam and Bhojwani (1990), but their aim was to initiate somatic embryogenesis and not to find the best media for excised embryo development. To increase the multiplication rate we also tried to regenerate plants from embryos divided into three sections. The main purpose of this study was to find the best method for propagation of *Podophyllum peltatum* through isolated embryos and embryo parts in order to overcome the difficulties in the generative propagation of the species.

Materials and methods

Observation of plant growth and development: In the years 1991 - 1993 the experiment was carried out in the experimental fields of Warsaw Agricultural University at Ursynów and in Warsaw University Botanical Garden. Plants were obtained from 10 cm long rhizome parts. They were grown in soil under field conditions. 40 plants taken at random were observed at 3 to 4-d intervals. A phase of 20 - 50 % of fully developed flowers was considered as the beginning of flowering, and that of 80 % of flowers with fallen petals as the end of this period. The percentage of floweres which developed into fruits, and fruits which set seeds was recorded.

Traditional methods of breaking of seed dormancy: The seeds of *Podophyllum peltatum* had been collected from Kraków Botanical Garden and stored for 10 months before the experiment started. After mechanical decoating stratification was carried out at the temperature of 4 °C in moist river sand for 30 d or GA₃ treatment - for 24 h in the 1000 µg g⁻¹ GA₃ solution. The seeds (4 repetitions of 50 seeds) were placed on moist paper in glass pots in the dark at the temperature of 20 °C. The number of germinating seeds was checked at 7-d intervals; the experiment lasted 40 d.

Isolated embryo cultures: The seeds from Kraków Botanical Garden after 6 months of storage were soaked for 3 d in tap water daily changed. Thereafter they were rinsed in 70 % ethyl alcohol and then immersed for 20 min in 7.5 % sodium hypochlorite solution. After rinsing the seeds 5 times in sterile water, the embryos were excised and sterilised in 0.075 % sodium dodecyl sulphate (SDS) solution with few drops of emulsifier and then rinsed five times in sterile water. The embryos were germinated under aseptic conditions at the temperature of 25 °C and 8-h photoperiod, irradiance

of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (four repetitions of 8 embryos on each type of medium). The basic medium (MS) was supplemented with 0.7 g dm^{-3} agar, 2 g dm^{-3} saccharose and with following growth regulators: GA₃, IAA, NAA, BAP, zeatin, kinetin (Table 1). The pH of the media was adjusted to 5.8. After 5 weeks the plantlets were transplanted to the soil.

Divided embryo cultures: The seeds of *Podophyllum peltatum* were collected from various botanical gardens in Poland. As the previous method of sterilisation turned out not to be sufficiently effective, this time the seeds were soaked for 6 h in tap water, then rinsed in 70 % ethanol, and five times in sterile water. Subsequently the seeds were immersed for 20 min in 0.1 % HgCl₂. After rinsing them again in sterile water (3 times) the embryos were excised and inoculated on the MS medium with 20 g dm^{-3} saccharose, 7 g dm^{-3} agar and different growth regulators in following combinations [mg dm^{-3}]: M₁- 1 GA₃; M₂ - 2 IAA + 0.5 kinetin; M₃ - 1 2,4D + 0.2 kin + 0.1 GA₃; M₄ - 0.5 IAA + 2 kin; M₅ - IAA + 0.5 BAP; M₆ - 5 2,4D + 0.1 - 2-isopentenyladenine; M₇ - 0.5 BAP + 0.1 IAA; M₈ - 2 IAA + 0.1 BAP; M₉ - 2 NAA + 0.1 kin. The pH of the media was adjusted to 5.8. The cultures were kept at 23 °C and 16-h photoperiod. After one week, embryos bigger than 4 mm were divided crosswise in order to obtain 3 parts: 2 cotyledonary ones and 1 radicle part. The explants were inoculated on the same media on which the embryos had been kept before being divided. 5 weeks later they were transferred to media containing [mg dm^{-3}]: M₁₀ - 0.2 2,4D + 1 kin; M₁₁ - 1 2,4D + 1 BAP. After 5 more weeks the differentiating calli were inoculated to Gamborg (G₆₁) medium (except M₈/M₁₁) while the undifferentiating calli were still kept on M₁₀ and M₁₁ (Table 2). The experiment lasted 20 weeks. The cultures were kept at 23 °C and 16-h photoperiod.

Results and discussion

Plant growth and development: The vegetation season of *Podophyllum peltatum* under Polish climate lasted for about 145 d. On the 7 April 1991 white buds appeared over the soil surface. The intensive growth of stems, leaf petioles and leaf blades ended when the flowering period began (24 April). Flowering lasted 13 d. First fully developed fruits appeared after next 34 d (10 June). The last ripe fruits fell down after 71 d (20 August) and the top parts of plants started getting brown, necrotic and finally died. During whole season 28 % of flowers developed into fruits, and 40 % of fruits set seeds. No diseases or pests were observed on the plants. The rhizomes remained undamaged by winter frost, even though no cover was used. However, all three treatments (stratification, scarification and GA₃) failed to cause germination even though seeds had been stored for 10 month. The microscope observations showed normally developed embryos. It is suggested that growth inhibitors present in the endosperm may be the reason for failure of germination.

Isolated embryo cultures: Inoculating excised embryos on sterile media resulted in initiation of their growth. After one week of culture, the embryos were bigger and

green on all kinds of media. The addition of growth regulators significantly modified their development during next 4 weeks. GA₃ showed the best effect on the growth and development of the embryos. The solution of 0.1 mg dm⁻³ GA₃ caused better growth compared to the control medium (MS). 1 mg dm⁻³ of GA₃ gave the best result: 31 % of embryos germinated and developed healthy looking plantlets. 0.2 mg dm⁻³ kin slightly stimulated the growth of embryos; however, 1 mg dm⁻³ of kin resulted in smaller seedlings than those on the control medium. The addition of 0.2 mg dm⁻³ IAA and 0.2 mg dm⁻³ IAA + 0.05 mg dm⁻³ zeatin significantly inhibited the growth, finally leading to necrosis. The cultures on the MS supplemented with 1 mg dm⁻³ kinetin + 0.1 mg dm⁻³ NAA, had relatively big cotyledons, but very short radicles. Therefore, the MS with 1 mg dm⁻³ of GA₃ could be the routine medium for generative propagation of *Podophyllum peltatum* through the outlined procedure.

Table 1. Effect of different growth regulators added to MS medium on the development of isolated embryos of *Podophyllum peltatum* after 1 and 5 weeks of culture.

Growth regulator [mg dm ⁻³]	1 week growing embryos [%]	appearance of explants in comparison with control	5 weeks embryo developed in plantlets [%]	appearance of plantlets in comparison with control
Control	34 (25 - 37.5)	embrya enlarged, green cotyledons	9 (0 - 12.5)	~ 1.5 cm long, normally developed cotyledons, ~ 4 cm long radicles
0.2 kin	22 (12.5 - 25)	slightly bigger	6 (0 - 12.5)	similar
0.2 kin + 0.1 GA ₃	22 (12.5 - 37.5)	slightly bigger	6 (0 - 12.5)	similar
0.1 GA ₃	44 (37.5 - 50)	significantly bigger	16	slightly longer cotyledons and radicles
1 GA ₃	34 (25 - 37.5)	the biggest embrya, dark green cotyledons	31 (25 - 37.5)	1.5 - 2 cm long, thick cotyledons, 5 - 6 cm long radicles
1 kin + 0.1 NAA	44 (37.5 - 62.5)	thick embrya, cotyledons smaller, radicles shorter	9 (0 - 25)	compact plantlets, cotyledons slightly bigger, radicles thicker but shorter (1 - 1.5 cm)
0.1 zeatin	13 (0 - 25)	thick embrya, cotyledons smaller, radicles shorter	3 (0 - 12.5)	compact plantlets, cotyledons slightly bigger, radicles thicker but shorter (1 - 1.5 cm)
0.2 BA	34 (25 - 37.5)	thick embrya, cotyledons smaller, radicles shorter	6 (0 - 12.5)	compact plantlets, cotyledons slightly bigger, radicles thicker but shorter (1 - 1.5 cm)
0.2 IAA	9 (0 - 25)	significantly smaller	0	necrosis of explants
0.2 IAA + 0.05 zeatin	44 (37.5 - 50)	significantly smaller	0	necrosis of explants
1 kin	9 (0 - 25)	significantly smaller	0	necrosis of explants

Divided embryo cultures: In our experiment only the following sequence of media resulted in differentiation of calli: M₃/M₁₀, M₃/M₁₁, M₈/M₁₁, M₈/M₃/M₁₀ (Table 2). Plantlets were obtained from the divided embryos via somatic embryogenesis. The somatic embryos appeared only on 2,4D containing media (M₁₀ and M₁₁). The maturation, however, did not proceed, unless the embryo bearing callus was transferred to a medium without the auxin (Table 2). Somatic embryos inoculated to G₆₁ medium developed a radicle and little curved cotyledons (during 3 weeks). The plantlets kept on G₆₁ developed the first leaf in next 3 - 5 weeks. Amurugam and Bhojwani (1990) have also reported the lack of maturation of *P. hexandrum* somatic embryos on media containing 2,4D. The same has been observed by Lang and Kohlenbach (1975) in *Macleya cordata*, Sondhal and Sharp (1977) in *Coffea arabica*, Dos Danto *et al.* (1980) in *Medicago sativa* and Gleddie *et al.* (1991) in *Solanum melongena*. In all the mentioned cases, the embryos inoculated to media with a mild auxin (NAA) continued their development.

Table 2. The effect of various sequences of media on the development of the divided embryos of *Podophyllum peltatum*. Growth and development was evaluated after 5 weeks on 1st medium, next 5 weeks on the 2nd medium and after 10 weeks on the 3rd medium. For media composition see Materials and methods.

1 st medium 5 weeks		2 nd medium 5 weeks		3 rd medium 10 weeks	
M ₁	no growth, no callus, necrosis	-	-	-	-
M ₂	compact, green calli	M ₁₀	necrosis	-	-
M ₃	compact green calli	M ₁₀	beginning of embryogenesis	G ₆₁	maturation of somatic embryos - plantlets
		M ₁₁	beginning of embryogenesis		maturation of somatic embryos - plantlets
M ₄	green, but no calli	M ₁₁	calli, but decayed	-	-
M ₅	light green, hydrated calli	M ₁₀	further growth, but no differentiation	M ₁₁	undifferentiated light, green, hydrated calli
M ₆	compact green calli	M ₁₁	necrosis	-	-
M ₇	contaminated	-	-	-	-
M ₈	green but no calli	M ₁₁	beginning of embryogenesis	M ₁₁	somatic embryos, no maturation, necrosis
		M ₃ /M ₁₀ *	light green calli, beginning of embryogenesis	G ₆₁	maturation of somatic embryos - plantlets
M ₉	green but no calli	M ₁₀	green compact calli, no differentiation	M ₁₁	undifferentiated brownish, hydrated calli

* - explants kept on M₃ for 1 week before passage to M₁₀.

The outlined procedure takes of course much more time than the isolated embryo cultures, but significantly increases the generative multiplication rate of *Podophyllum peltatum*, species which sets a very limited number of seeds.

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