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High frequency plant regeneration via somatic embryogenesis in *Podophyllum peltatum* L., an important source of anticancer drug

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***Podophyllum peltatum* (L.) is an important medicinal plant that produces podophyllotoxin with anti-cancer properties. In the present study, we report the establishment of plantlet regeneration of *P. peltatum* via somatic embryogenesis. Somatic embryos differentiated directly from cotyledon explants of zygotic embryos on Murashige and Skoog (MS) basal medium supplemented with 21.6 µM α-naphthaleneacetic acid after 8 weeks of culture. An embryogenic callus developed from cotyledon explants of somatic embryos on MS medium + 6.78 µM 2,4-dichlorophenoxy acetic acid under continuous darkness after 6 weeks of culture. When the embryogenic callus was first grown on MS basal medium + abscisic acid (11.35 µM) for three weeks, followed by sub-culturing onto plain MS basal medium, it led to the development of high frequency somatic embryogenesis. Germination of cotyledonary somatic embryos was noticed on MS basal medium with the addition of 2.89 µM gibberellic acid after two weeks. The germinated embryos grow into plantlets with well-developed roots. Rooted plantlets were acclimatized in soil. The present protocol can be widely used for micropropagation and metabolic engineering.**

Keywords: Anticancer drug, mayapple, medicinal plant, podophyllotoxin, plant regeneration, somatic embryogenesis.

PODOPHYLLUM peltatum (L.) commonly called mayapple, belongs to family Berberidaceae and is native to southern Canada and northeastern USA. It is a perennial and grows in patches with a branched rhizome system. The rhizome contains podophyllotoxin with anticancer properties. The semi-synthetic derivatives of this compound like etoposide, etophos, and teniposide, are used clinically as chemotherapeutic agents for a variety of tumours, including small cell lung carcinoma, testicular cancer and malignant lymphoma[1]. Both *P. peltatum* and *P. emodi* Wall. ex Royle (syn.

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P. hexandrum) have been extensively used for commercial extraction of podophyllotoxin. *P. emodi* Wall. ex Royle (syn. *P. hexandrum*), native to India, is declared an endangered species due to over-exploitation[2].

Propagation of *Podophyllum* plant is difficult because of low seed number, prolonged dormancy, and laborious harvesting procedure[3]. In an effort to develop a sustainable source of podophyllotoxin, *P. peltatum* plants were propagated by vegetative root cuttings[3].

Plant tissue culture technique is an alternative practice for micropropagation and metabolic engineering. In *P. hexandrum*, there are a few reports on *in vitro* cell suspension culture[4] and plant regeneration via somatic embryogenesis[5,6]. However, in *P. peltatum*, only adventitious shoot regeneration from cultured rhizomes, nodes and roots has been reported[7]. Realizing these problems, the present investigation was carried out to establish high frequency plant production via somatic embryogenesis in *P. peltatum*.

Seeds of *P. peltatum* (L.) were purchased from Jelitto Perennial Seeds (Louisville, USA) and stored at 4°C for 1 month. The seeds were surface-sterilized by a 1-min immersion in 70% ethanol, followed by 2% sodium hypochlorite solution for 20 min and then rinsed five times with sterile distilled water. Zygotic embryos were carefully excised from the sterilized seeds and placed on a medium containing MS salts and vitamins, 30 g/l sucrose, solidified with 0.4% gelrite (Duchefa Biocheme, The Netherlands) in petri dishes for 2 weeks.

For somatic embryo induction, cotyledon (ca. 1.0 mm^2) explants detached from germinated zygotic embryos after 2 weeks were inoculated on MS basal medium[8] solidified with 0.8% agar (Duchefa Biocheme), 30 g l^{-1} sucrose supplemented with 0, 5.4, 10.8, 21.6, 43.2 µM NAA respectively. About 30 explants were cultured in each petri dish and this experiment was repeated three times. The frequency of somatic embryo formation was recorded after 8 weeks.

To induce embryogenic callus, cotyledon segments of primary somatic embryos were cultured onto medium consisting of MS basal medium solidified with 0.8% agar and containing different concentrations of 2,4-D (0.0, 2.26, 4.52, 6.78, 9.04 and 11.30 µM) and NAA (0, 5.4, 10.8, 21.6, 43.2 µM), with and without activated charcoal (0.1%) (Sigma, USA). About 10 cotyledon segments (total 200 mg fresh wt) were cultured per petri dish and this experiment was done three times. After 8 weeks the frequency of embryogenic callus induction was recorded.

Embryogenic callus was cultured on hormone-free MS basal medium (solidified with 0.8% agar) or MS medium with 2,4-D (2.26, 4.52, 6.78, 9.04 and 11.30 µM), or MS medium with different concentrations of ABA (3.78, 11.35, 18.9 and 37.8 µM; Sigma) for somatic embryo development. For ABA treatment, embryogenic callus was cultured on medium with ABA for 3 weeks, followed by sub-culturing on hormone-free MS medium for three more weeks. Around 10 embryogenic callus masses (total 200 mg fresh weight) were cultured in each petri dish and this experiment was replicated three times. After 8 weeks the frequency of somatic embryo formation was recorded.

Somatic embryos of cotyledonary origin were isolated and transferred individually to nutrient media with different compositions; MS, MS + ABA (11.35 µM), and MS with GA_3 (2.89 µM) for maturation and germination. The germinated somatic embryos were transferred to half-strength MS medium solidified with 0.27% gelrite (Duchefa Biocheme) in Magenta plastic boxes (Sigma, USA).

Approximately 20 ml of nutrient medium was dispensed into each petri dish (87 × 15 mm). pH of the medium was adjusted to 5.8, which was then autoclaved at 1.06 kg m^{-2} for 15 min. The culture room was maintained at 24 ± 2°C under white fluorescent bulbs 16/8 h (light/dark) photoperiod with a light intensity of 25 µE m^{-2} s^{-1} for zygotic embryo germination, primary somatic embryo, secondary somatic embryo formation and germination of cotyledonary somatic embryos, except embryogenic callus induction which requires dark incubation at 24°C.

Plantlets possessing shoots and roots were removed from the culture tubes, washed in running tap water and transferred to pots containing a mixture of sterilized perlite, peat moss and vermiculite (1 : 1 : 1 v/v/v) (no additional inorganic salt), covered with polyethylene bags and kept in a greenhouse to become autotrophic. After 4 weeks of hardening, their covers were removed.

A completely randomized design was used in all the experiments. The mean and standard error (SE) were calculated. Data were statistically analysed using DMRT. In the same column, significant differences according to LSD at the $P \leq 0.05$ level are indicated by different letters.

Seeds of *Podophyllum* species have a dormancy of about ten months[9]. In our previous work, the decoated seeds containing endosperm in *P. peltatum* also exhibited dormancy when they were cultured on MS medium (data not shown). However, zygotic embryos isolated from endosperm germinated successfully on MS basal medium after two weeks (Figure 1 *a*, *b*). A similar result was also reported in *P. hexandrum*[5].

Isolated cotyledons of zygotic embryos cultured on MS medium with different concentrations of NAA showed direct somatic embryo formation from the surface after 6 weeks of culture. Various stages of somatic embryos could be seen on the cotyledons after 8 weeks of culture (Figure 1 *c*). Induction of somatic embryos was high (12.5 ± 0.3 embryos) on MS basal medium + NAA (21.6 µM; Figure 2). In preliminary experiments, 2,4-D was not found to be effective for somatic embryo induction (data not shown). In *P. hexandrum,* NAA + 6-benzyl amino purine (BA) was more suitable than 2,4-D for somatic embryo induction from cotyledons of zygotic embryos[6].

Direct somatic embryos together with callus clumps detached from cotyledon segments of primary somatic embryos were transferred onto medium containing 2,4-D or NAA. Secondary embryos were sporadically formed all over the surface of the primary embryos, but appeared

mainly on the radical region. 2,4-D treatment was more effective for secondary embryo formation from primary somatic embryos than NAA treatment, which showed poor response (data not shown). According to these results, to induce embryogenic callus, cotyledonary somatic embryos were cultured on MS medium with different concentrations of 2,4-D (0.0, 2.26, 4.52, 6.78, 9.04 and 11.30 µM) and activated charcoal (0.1%) under dark conditions. Frequency of embryogenic callus induction varied with concentration of 2,4-D (Figure 3). 2,4-D (6.78 µM) with activated charcoal (0.1%) showed maximum induction (Figure 3). In *P. hexandrum*, embryogenic callus has been induced from the culture of zygotic embryos on a medium with BA and indole-3-acetic acid (IAA), and proliferation of embryogenic callus was obtained after transfer of the callus masses to medium with 2,4-D (10.0 µM) and BA (1.0 µM)[5].

Embryogenic callus in *P. peltatum* was usually yellowish-white and friable (YWF) (Figure 4 *a*). When embryogenic callus was sub-cultured onto MS medium with 2,4-D (6.78 µM) and activated charcoal (0.1%) at 4-week intervals under dark condition, a continuously proliferating callus appeared. However, prolonged culture for more than 8 weeks resulted in the development of somatic embryos (Figure 4 *b*). To investigate suitable conditions for somatic embryo induction, embryogenic calluses were transferred to different concentrations of 2,4-D or ABA. In the somatic embryos induced on medium containing 2,4-D, the earlier stages more normal, but later stages were abnormal, such as fused cotyledon and/or abnormally enlarged embryos without distinct polarity (Figure 4 *d*; data not shown).

A short three-week temporary treatment of embryogenic callus on medium with ABA followed by transference to hormone-free MS medium stimulated the normal development of somatic embryos (Table 1). Maximum development of normal somatic embryos occurred on hormone-free MS basal medium after preculture for 3 weeks on MS medium supplemented with ABA (11.35 µM; Figure 4 *c*). This result indicates that omission of 2,4-D and inclusion of ABA treatment are essential for stimulating normal

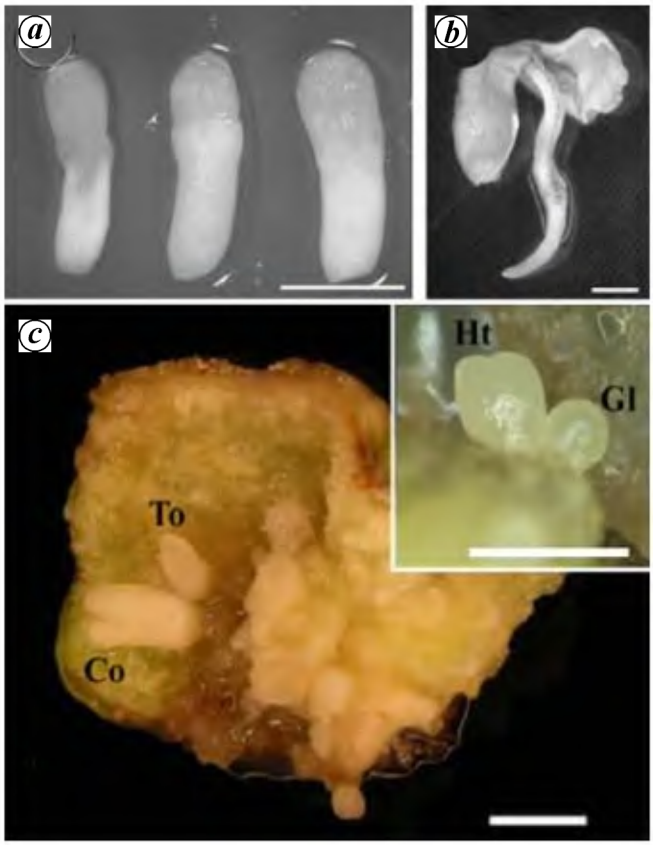

Figure 1. *In vitro* germination of zygotic embryos isolated from *Podophyllum peltatum* seeds. ***a***, Zygotic embryos isolated from endosperms (bar = 1.0 mm). ***b***, Plantlet germinated from a zygotic embryo 2 weeks after culture (bar = 3.0 mm). ***c***, Primary somatic embryos induced from a cotyledon on MS basal medium containing 21.6 µM NAA after 8 weeks of cultures. Gl, Globular stage embryo; Ht, Heart stage embryo; To, Torpedo stage embryo; Co, Cotyledonary stage embryo (all bars = 3.0 mm).

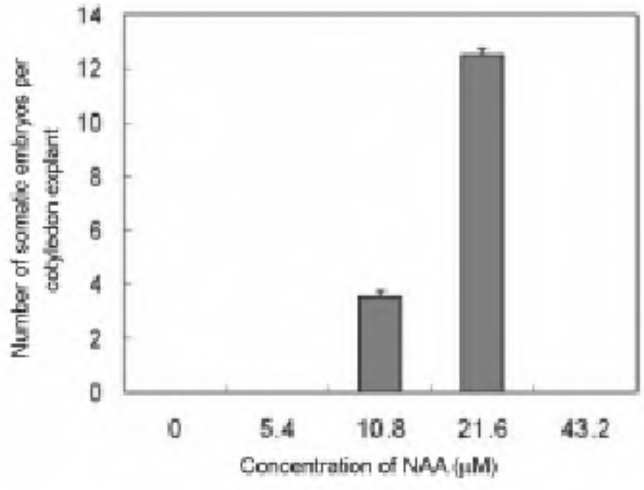

Figure 2. Effect of NAA on somatic embryo induction from cotyledons of *P. peltatum* after 8 weeks. Data represent mean of samples ± SE.

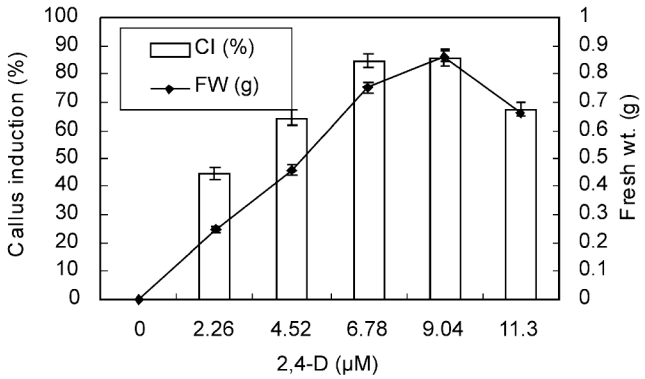

Figure 3. Effect of 2,4-D concentration on embryogenic callus induction from cotyledon explants of *P. peltatum* cultured on MS medium for six weeks. Per cent callus induction (CI) is calculated based on the number of explants producing embryogenic callus to the total number of explants cultured per petri dish. FW, Fresh weight of callus in grams.

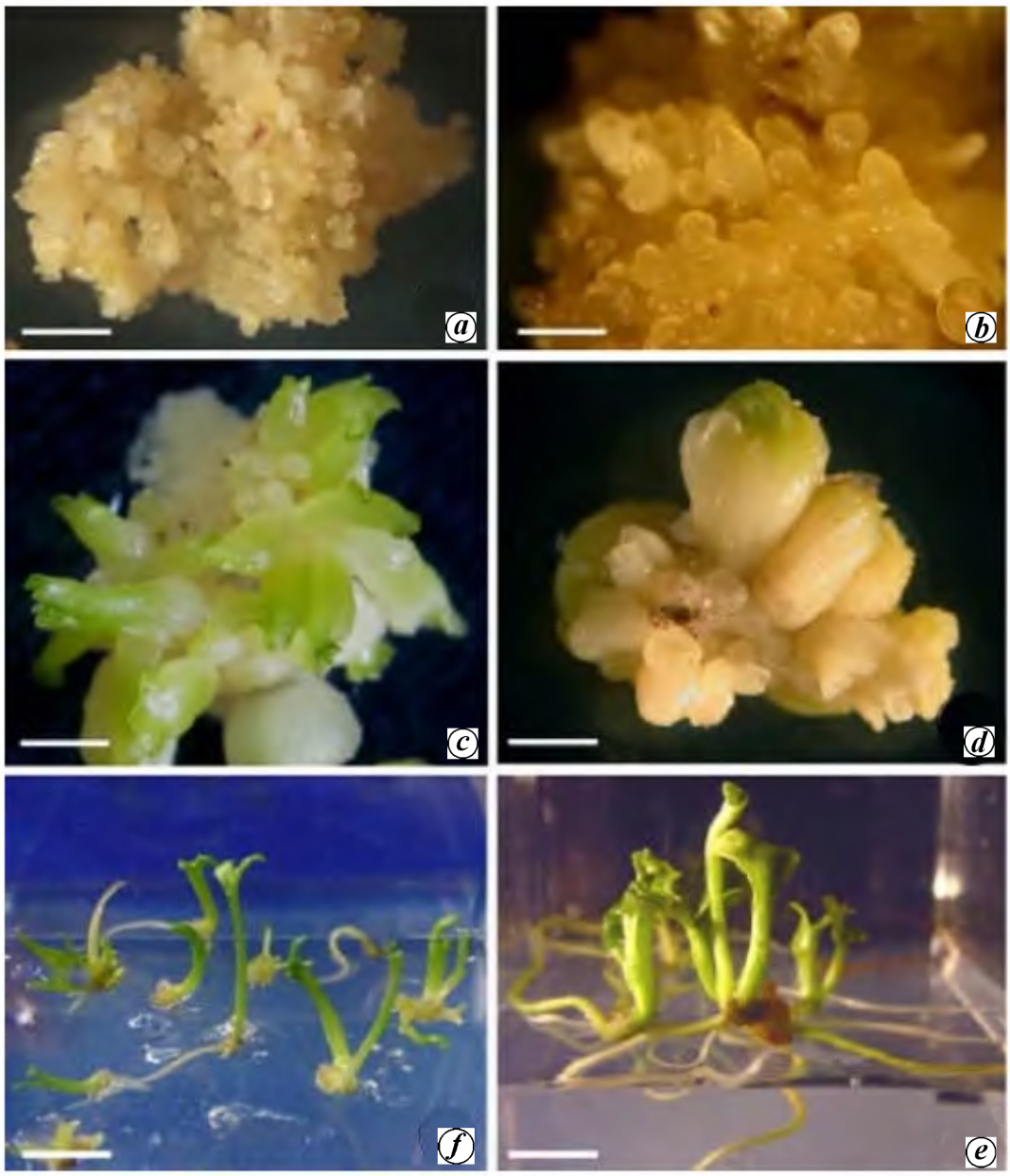

Figure 4. Plant regeneration via somatic embryogenesis from cotyledon explants of *P. peltatum*. ***a***, Embryogenic callus maintained on MS medium supplemented with 2,4-D (6.78 µM) and activated charcoal (0.1%) after 4 weeks of subculture (bar = 1.0 mm). ***b***, Development of somatic embryos on hormone-free MS basal medium after 3 weeks of pre-culture on MS medium supplemented with ABA (11.35 µM) (bar = 0.7 mm). ***c***, Development of normal somatic embryos on hormone-free MS basal medium (bar = 2.0 mm). ***d***, Abnormal somatic embryos formed on MS medium supplemented with 6.78 µM 2,4-D after 8 weeks culture (bar = 2.0 mm). ***e***, Germination of somatic embryos on medium with GA_3 (2.89 µM) after two weeks culture (bar = 3.0 mm). ***f***, Plantlets with leaves and roots grown on hormone-free 1/2 MS medium (bar = 5.0 mm).

embryo development in *Podophyllum*. Inclusion of ABA stimulated or increased the number of somatic embryos in embryogenic genotypes of *Dactylis glomerata*[10]. In *P. hexandrum*, the early globular embryos developed on medium containing 2,4-D were transferred to a medium with NAA for further development of somatic embryos[5].

When cotyledonary somatic embryos were transferred to hormone-free MS medium, 46% turned green and continued their development. Germination of somatic embryos was enhanced on MS medium supplemented with GA_3 (2.89 µM). The percentage frequency of germination of cotyledonary somatic embryos was 76 (Table 2, Figure 4*e*). The germinated embryos were grown into plantlets after being transferred to Magenta boxes containing 1/2 MS medium (Figure 4*f*) and successfully acclimatized in soil mixture (sterilized perlite, peat moss and vermiculite (1 : 1 : 1

Table 1. Effect of pre-culture on embryogenic callus on MS medium with ABA for three weeks, followed by sub-culturing on hormone-free MS basal medium on somatic embryo production

	Number of somatic embryos per embryogenic callus		
ABA (μM)	Normal somatic embryos	Abnormal somatic embryos	Total
0	9.3 ± 0.6^{b}	4.4 ± 0.3^{a}	13.7 ± 0.9^{b}
3.78	13.6 ± 0.8^{ab}	1.3 ± 0.3^{b}	14.9 ± 0.1^{ab}
11.35	15.3 ± 0.8^{a}	–	15.3 ± 0.8^{a}
18.9	8.3 ± 0.8^{bc}	–	8.3 ± 0.8^{c}
37.8	–	–	–

Data represent mean values ± SE from three independent experiments. Data were statistically analysed using DMRT. In the same column, significant differences according to LSD at the $P \leq 0.05$ level are indicated by different letters.

Table 2. Effect of plant growth regulators on germination of somatic embryos in *P. peltatum*

Composition of medium	Germination (%)	Time taken for germination (weeks)
MS control	46 ± 2.5^{b}	3
MS + GA_3 (2.89 μM)	76 ± 2.1^{a}	2
MS + ABA (11.35 μM)	–	–

Data represent mean values ± SE from three independent experiments. Data were statistically analysed using DMRT. In the same column, significant differences according to LSD at the $P \leq 0.05$ level are indicated by different letters.

v/v/v)) with 25% survivability. GA_3 was effective in promoting maturation and early germination of embryos, followed by half-strength hormone-free MS medium.

In conclusion, the present investigation has shown that high frequency plant production can be accomplished via somatic embryogenesis in *P. peltatum*. Several genes related with the synthesis of podophyllotoxin have been isolated and purified from *P. peltatum*[11]. It is hoped that, the genetic transformation protocol will create new opportunities to produce metabolically engineered *P. peltatum* plants using the method outlined in this communication.

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