

In vitro germplasm conservation of *Podophyllum peltatum* L. under slow growth conditions

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Abstract Germplasm conservation of *Podophyllum peltatum* L. was attempted by using synthetic seed technology and media supplemented with osmotic agents. Excised buds from *in vitro* cultures were encapsulated in calcium alginate beads and cultured on different substrates then stored at 5, 10, and 25°C for up to 8 mo. Survival and vigor in re-growth were the parameters used to evaluate the germplasm storage conditions. Vigor in re-growth was measured by number of buds induced after storage, which was achieved on a substrate containing water solidified with 1% w/v agar under 10°C. *In vitro* storage of shoot cultures was also evaluated by supplementing osmotic agents, mannitol, or sorbitol to the media. Such treatment had a negative impact on post-storage re-growth (at 25°C), even though the inclusion of 2% w/v sorbitol and mannitol each to the media increased plantlet survival during 10°C storage treatment. A deleterious effect was noticed among cultures in re-growth when higher concentrations of these supplements were added to the media. Genetic stability was assessed following 8 mo of storage using a PCR-based multilocus DNA fingerprinting technique, amplified fragment-length polymorphism. No differences in the DNA fragment patterns were observed using eight primer combinations in stored clones. However, a polymorphic band was noticed in the accession that served as explant source, suggesting that the mutation has occurred

prior to this study perhaps during the 9 yr of *in vitro* cultivation.

Keywords Germplasm · Lignan · Mayapple · Micropropagation · *Podophyllum* · Podophyllotoxin

Introduction

Podophyllum species are the major source of aryltetralin lignans and among those, podophyllotoxin is the compound of commercial interest since it is the starting material for the semi-synthesis of the anti-cancer pharmaceuticals etoposide, teniposide and etopophos. These chemotherapeutic drugs have been used for the treatment of lung and testicular cancers as well as certain leukemias (Canel et al. 2001). In addition to the anti-cancer drugs, podophyllotoxin is the precursor of a new derivative CPH 82, a compound in clinical trials for the treatment of rheumatoid arthritis in Europe (Svensson and Pettersson 2003). Preparations of podophyllotoxin are commercially available in drugstores for dermatological treatment of genital warts. Despite such medicinal importance, the commercial source of podophyllotoxin, *Podophyllum emodi* Wall (syn. *P. hexandrum* Royle), is wild collected from the alpine Himalayas in India. The destructive harvest of these plants led to the introduction of *P. emodi* in the Convention on International Trade in Endangered Species of Wild Fauna and Flora list as an endangered species.

In a search for an alternative source of podophyllotoxin, we have examined *Podophyllum peltatum* L., the American mayapple, for podophyllotoxin production. Our findings have shown that podophyllotoxin content of leaves of *P. peltatum* are equal to the *P. emodi* rhizomes (Moraes et al. 2000; Canel et al. 2001). Furthermore, the podophyllotoxin

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profile in leaves is a stable genetic trait and has not changed over time (Moraes et al. 2002), indicating that selection and cultivation of high-yielding plants is possible and can become an effective mean of production rather than harvesting wild populations. In fact, our field results have confirmed that cultivation of *P. peltatum* is possible (Cushman et al. 2004; Maqbool et al. 2004), and for maximum yields, plantings of high-yielding podophyllotoxin chemotypes will be advantageous. We are seeking a rational way to preserve the high-yielding accessions of *P. peltatum* by *in vitro* conservation, most importantly to conserve the stored genetic material with maximum vigor, disease free and without somaclonal variation.

Conservation of plant germplasm by *in vitro* technology has been done using slow growth procedures or cryopreservation (Withers 1986). Slow growth is usually achieved by reducing the culture temperature, by modifying culture media with supplements of osmotic agents, growth inhibitors, or by removing growth promoters (Dodds and Roberts 1995). The present study aims to develop an efficient conservation protocol to store high-yielding *P. peltatum* chemotypes at low temperature using synthetic seed technology and different osmotic agents. To ensure that *in vitro* procedures will indeed conserve micropropagated *P. peltatum*, culture in storage will be assayed by amplified fragment-length polymorphism (AFLP) procedure to determine if any mutation has changed the accession's genetic makeup.

Materials and Methods

Plant material. *In vitro* propagated *P. peltatum* plantlets were produced according to a protocol described by Moraes-Cerdeira et al. (1998). Terminal buds were inoculated in 1994. Since then, these cultures have been maintained on Murashige and Skoog (MS) media without the presence of growth regulators with frequent subcultures at 2-mo intervals (Murashige and Skoog 1962). Axillary buds were excised by cutting the shoot axis just above and below the nodes. Bud culture consisted of 4-mm-long stem segments each containing one node with an axillary bud. These explants were used for further encapsulation.

Slow growth treatments. *Osmotic agent supplements into media.* The effects of osmotic agents on the survival and re-growth of *P. peltatum in vitro* cultures were investigated using MS media supplemented with mannitol and sorbitol at concentrations varying between 2% *w/v* and 4% *w/v* and a combination of mannitol and sorbitol (2% *w/v*) with 3% *w/v* sucrose and 0.8% *w/v* agar. Buds were dissected from aseptically grown cultures and inoculated into slow growth

media to increase subculture intervals. Twenty milliliters of the MS medium and osmotic agents solidified with 0.8% *w/v* agar was dispensed in test tubes (25×150 mm) covered with magenta caps and autoclaved at 121°C. Tubes were sealed with parafilm to reduce desiccation. Cultures were maintained for 4–8 mo into growth chambers under a 16-h photoperiod with fluorescent light and a photon flux of approximately 52 $\mu\text{mol s}^{-1}\text{m}^{-2}$ at 25°C.

Low temperature and bud encapsulation. Buds were mixed with 5% *w/v* sodium alginate (Sigma, St. Louis, MO) solution prepared in MS liquid medium supplemented with 3% *w/v* sucrose and dropped individually into 50 mM complexing solution of calcium chloride in a flask placed on a magnetic stirrer. Both alginate gel matrix and complexing agent were sterilized by autoclaving at 121°C for 20 min. The beads (0.5–0.7 cm in diameter) containing the entrapped buds were left in calcium chloride solution for 30 min for complexation. They were collected and rinsed three times for 5 min in sterile water to remove traces of calcium chloride. The encapsulated buds were then transferred to following substrates: water, water plus 2% sucrose, and MS medium with 3% sucrose. These substrates were solidified with 1% agar. In addition, cotton was also used as support containing MS media.

Non-encapsulated buds were also cultured on MS media containing 3% (*w/v*) sucrose, 0.8% (*w/v*) agar, and submitted to low temperature storage. Thus, encapsulated buds and non-encapsulated bud cultures were stored for 4 and 8 mo at 5, 10, and 25°C (16-h photoperiod under fluorescent light with a photon flux of approximately 52 $\mu\text{mol s}^{-1}\text{m}^{-2}$). After 4 and 8 mo storage, these buds were transferred to multiplication MS media containing 3% *w/v* sucrose and 0.8% *w/v* agar supplemented with 2.2 μM of benzyladenine per liter. Survival and vigor during re-growth were evaluated. Plantlet conversion is defined as the percentage of buds that were able to convert into vigorous healthy plantlet. Six weeks later, the converted plantlets were counted and potted in soil and transferred to greenhouse for acclimatization.

Re-growth and establishment of plantlets. Non-encapsulated cultures and encapsulated buds were monitored during storage for survival and subsequently transferred to shoot multiplication medium under standard culture room conditions at 25°C. The number of new buds and shoots induced on multiplication media was counted 30 d after transference. Data collected reflect the rate of plant conversion from storage buds to proliferating buds, shoots, and plantlets. Proliferated shoots were rooted on MS medium containing 2.4 μM NAA. Six- to eight-week-old plantlets with well-developed roots were potted in soil and transferred to greenhouse for acclimatization.

Acclimatization. The plantlets were potted in Miracle-Gro® potting soil in planting tray kits, with eight cells and a plastic dome, and a based tray (Wal-mart Stores, Inc.) for acclimatization in the greenhouse. The trays were incubated at 25°C, with a relative humidity of 80% under a mist system with 10-min watering cycle every 20 min for a period of 10 h.

Assessment of genome stability. Data analysis. All experiments were repeated at least three times with six replicates. The data were submitted to statistical analyses by analysis of variance followed by the Tukey test with the level of significance set at 5% using SAS version 9.1 (SAS Institute, Cary, NC).

DNA extraction. Total genomic DNA was isolated from leaves, bud cultures, encapsulated buds, and terminal bud of a maternal stock plant that was grown in the greenhouse located at the University of Mississippi. Freeze-dried leaves and buds were ground into fine powder, and the DNA was isolated by CTAB method according to the protocol described by Ferreira and Grattapaglia 1996.

AFLP assay. Genomic DNA (200 ng) was digested by two restriction enzymes (*EcoRI/MseI*) at 37°C for 3 h using a thermocycler model PTC™-100 (MJ Research Inc., Waltham, MA). The digested fragments were ligated with *EcoRI* and *MseI* adapter sequences in a 10.0- μ l reaction volume at 23°C for 3 h.

Fragments were pre-amplified using *EcoRI* and *MseI* primers with one selective nucleotide (*EcoRI*-A/*MseI*-A). The pre-amplification reaction volume of 10 μ l contained 2.5 μ l of the diluted ligation reaction, 7.2 μ l of the pre-amplification primer mix, 1 \times of PCR buffer, and 0.3 U of Taq DNA polymerase (Promega, Madison, WI). The pre-amplification reactions were performed using one cycle of 5 min at 72°C and 26 cycles, each consisting of a 1-min DNA denaturation step at 94°C, a 1-min annealing step at 56°C, and a 1-min extension step at 72°C. To determine whether the pre-amplification succeeded, products were electrophoresed on a 1% agarose gel in 0.5 \times TBE buffer (0.45 M Tris, 45 M boric acid, 0.01 M EDTA) and visualized by ethidium bromide staining.

For AFLP analysis, the generated pre-amplified PCR products were used as a template for selective amplification using the eight primer combinations: *EcoRI*-ATC/*MseI*-ATC; *EcoRI*-ATT/*MseI*-ATG; *EcoRI*-ATG/*MseI*-AGT; *EcoRI*-AGT/*MseI*-ATT; *EcoRI*-ATC/*MseI*-AGT; *EcoRI*-ATT/*MseI*-AGT; *EcoRI*-AGT/*MseI*-ATC; and *EcoRI*-ATG/*MseI*-ATC.

The selective amplification reaction was carried out in a total volume of 20 μ l containing 1.5 μ l diluted pre-amplification mixture, 1 μ l of *EcoRI* primer (Invitrogen,

Calsbad, CA), 1.2 μ l of *MseI* primer, and 16.3 μ l of mix I (12.5 μ l AFLP-grade water, 2 μ l of 10 \times buffer, 1.2 μ l of MgCl₂, 0.4 μ l dNTPs, and 0.2 U of Taq DNA polymerase [Promega]).

The selective amplification was performed using the following cycle profile: an initial step of 2 min at 94°C, followed by 12 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 1 min, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and a final step of 72°C for 2 min. The amplified product was stored at -20°C.

After the selective amplification, each reaction was mixed with 8 μ l of loading buffer (10 ml formamide, 200 μ l EDTA [0.5 M] pH8.0, 10 mg bromophenol blue, and 10 mg xylene cyanol). The AFLP selective PCR products were denatured at 95°C for 5 min and immediately transferred to ice.

The AFLP fragments were separated and visualized using a 6% polyacrylamide gel on a sequencing cell, Sequi-Gen GT (Bio-Rad, Hercules, CA). The DNA marker used was a 50-bp DNA Step Ladder (Promega). The gel was stained with a silver nitrate kit by Promega following the protocol of Creste et al. (2001).

Results and Discussion

The growth suppression approaches using alginate encapsulation in combination with low temperature in storage and the use of osmotic agents were attempted, and the results in Table 1 shows that supplementing osmotic agents mannitol and sorbitol to MS media at different concentrations was less effective for *in vitro* storage of *P. peltatum* germplasm than the storage at low temperature (10°C). A combination of several factors including contamination, desiccation, and nutrient depletion of the media was detrimental during storage. The addition of osmotic agents sorbitol (2% w/v) and mannitol to each of the media has increased culture survival, however, with deterioration of cultures in terms of quality of growth. Emerged plants had weak shoots with signs of chlorosis (Table 1). A decline in survival and re-growth occurred on culture storage at higher concentrations of osmotic agents (4% w/v mannitol and sorbitol each and a combination of 2% w/v sorbitol and mannitol) used individually or jointly. The use of the osmotic agents was not an efficacious procedure. The losses (low percent of plantlet conversion) were noticed and therefore high standard errors (SE) as compared to the mean occurred on re-growth of cultures; each treatment, however, had six replicates initially (Table 1). These results corroborate with Westcott's (1981) descriptions on the toxic effects of mannitol in *Solanum* germplasm storage.

The research in synthetic seed technology has been prolific, although with few applications on *in vitro* storage.

Table 1. Effect of various *in vitro* storage conditions on non-encapsulated shoots of *P. peltatum*

Storage conditions	Re-growth ^z ±SE after storage 4mo	Survival (%)	Re-growth ^z ±SE after storage 8mo	Survival (%)
Low temperature				
5°C	1.7±1.0 c	83	1.3±1.0 c	50
10°C	11.3±1.2 a	100	17.5±1.9 a	100 ^y
25°C	3.3±0.8 bc	70	2.8±1.5 c	60
Osmotic agents (25°C)				
2% Sorbitol	5.8±0.5 b	100	9.5±0.4 b	100 ^y
4% Sorbitol	2.7±2.1 bc	67	3.0±2.0 c	67
2% Mannitol	2.3±0.7 c	100	2.2±1.5 c	100
4% Mannitol	2.7±0.5 bc	33	2.5±2.4 c	50
2% Sorbitol+2% mannitol	3.0±2.1 bc	33	2.5±1.9 c	50

Data represent the mean of three replicates with six explants for each treatment. Means followed by the same *letter* do not differ statistically at $p=0.05$ according the Tukey test

SE standard error

^z Re-growth was measured in percentage of cultures that presented new buds in 30 d after the removal of the storage condition

^y Cultures that produced more than ten new buds per explant

Brodelius et al. (1982) have demonstrated that alginate beads reduce respiration and consequently the growth of encapsulated tissues. Thus, Maruyama et al. (1997) have successfully stored somatic embryos by taking advantage of synthetic seed technology in addition to shoot tips protocols of three species; *Cedrela odorata* L., *Guazuma crinita* Mart., and *Jacaranda mimosaeifolia* Don during 6–12-mo storage at 12–25°C, followed by their acclimatization in the

greenhouse. The *P. peltatum* plantlet's conversion from alginate-encapsulated buds maintained under various conditions and for different periods of storage was possible (Table 2). Growth was suppressed and/or minimal in all encapsulated buds stored on water/agar substrate/support at the tested temperatures with maximum plantlet conversion after storage. Growth suppression had positively reduced the labor during culture maintenance in our tissue culture

Table 2. The effect of shoot encapsulation on *in vitro* storage conditions of *P. peltatum* explants

Media and support	Storage temp.	Re-growth ^z ±SE after storage 4mo	Plantlet ^y conversion (%)	Re-growth ^z ±SE after storage 8mo	Plantlet ^y conversion (%)
Water + agar	5°C	13.0±1.2 a	100	11.2±1.7 ab	100
	10°C	15.8±2.4 a	100	15.5±1.9 a	100
	25°C	7.5±1.0 c	100	5.0±1.4 cd	100
2% Sucrose + agar	5°C	8.0±2.4 bc	100	5.0±3.0 cd	83
	10°C	12.0±1.4 ab	100	11.7±1.0 ab	100
	25°C	5.1±1.5 cde	100	3.7±3.1 cd	100
MS + agar	5°C	4.0±2.0 cde	83	3.3±2.8 cd	67
	10°C	6.5±3.4 c	83	7.3±4.0 bc	67
	25°C	1.7±1.4 de	67	1.5±1.5 d	50
MS + cotton fibers	5°C	2.0±1.7 de	67	1.8±1.4 d	67
	10°C	5.0±3.1 cd	83	4.0±3.2 cd	67
	25°C	1.2±1.3 e	50	0.8±1.3 d	33

Data represent the mean of three replicates with six explants for each treatment. Means followed by same *letter* do not differ statistically at $p=0.05$ according the Tukey test

SE standard error

^z Re-growth of encapsulated shoots is based on new shoots per explant induced in 30 d after the removal of the storage condition

^y Plant conversion measures the number of encapsulated shoots that produced new buds in a 30-d period after storage



Figure 1. *In vitro* germplasm storage of *P. peltatum*. *A* Encapsulated axillary buds on water/agar, *B* sucrose/agar, *C* MS salts/agar, *D* MS salts/cotton wool, *E* shoot multiplication at 10°C after storage for 8 mo, *F* acclimatization in greenhouse. Bars represent 1.0 cm (*A*, *B*, *C*, *D*, and *E*) and 12.5 cm (*F*).

laboratory and also promoted uniformity of growth among the converted plantlets.

No signs of shoot or root growth was noticed during the 8-mo storage. Adding sucrose to the media has prevented

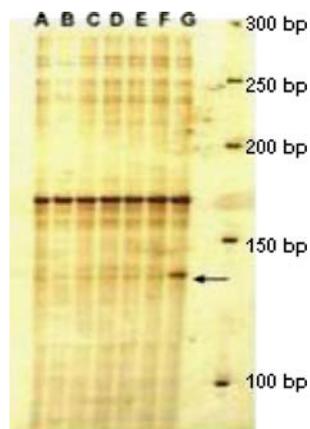


Figure 2. Part of an autoradiogram of AFLP fragments based on primer pair Eco+ATC/Mse+AGT, showing genetic profile of *P. peltatum* bud cultures. (*A*, *B*) Encapsulated axillary buds on water/agar at 5°C and re-growth after 8 mo in storage, respectively. (*C*, *D*) Encapsulated axillary buds on water/agar at 10°C and re-growth after 8 mo in storage, respectively. (*E*, *F*) Encapsulated axillary buds on water/agar at 25°C and re-growth after 8 mo in storage, respectively. *G* Mother plant that served as explant donor.

dehydration in storage but did not improve germplasm shelf-life, while frequency of plantlet conversion was higher on buds stored on 2% w/v sucrose/agar support with roots breaking their alginate capsule during storage (Fig. 1). Replacing agar with cotton support negatively affected the plantlet conversion in re-growth. At 2 mo in storage at different temperatures, buds have grown out of their capsules showing well-developed roots and by the fourth month, some vessels lost water and the cultures showed signs of dehydration, and eventually some died. Independent of the four tested substrate/supports using encapsulated buds or not (non-encapsulated buds), cultures incubated at 10°C survived remarkably better than any other temperature with a 10-fold vigorous shoot proliferation (Fig. 1).

All *P. peltatum* cultures in storage were able to form roots during re-growth and successfully acclimatized in soil under a mist system. After low temperature storage, plantlets improved their survival during acclimatization and more vigorous growth in field plantings was observed. Similar reports have documented post-storage beneficial effects in apricot by Koubouris and Vasilakakis (2006).

Germplasm preservation by *in vitro* technology should reduce mutations to an extremely rare event (Liu et al. 2004). Thus, AFLP assay was used to evaluate the genomic stability of *P. peltatum* cultures during *in vitro* multiplica-

tion and storage. No genetic change was detected among the plantlets regenerated after 8 mo in storage under different conditions (Fig. 2A–F). Nonetheless, among 291 monomorphic bands, one polymorphic band was found in the plant collected from the wild. This polymorphic band had approximately 140 bp size (Fig. 2G). Since the micropropagated *P. peltatum* cultures have been maintained from the last 9 yr with subculture frequency every 2 mo, somaclonal mutation might have happened at multiplication phase during maintenance rather than in storage. With similar variations depending on the number of subcultures, the tissues had undergone mutations and have been reported by other researchers (Webster and Jones 1989; Hirochika et al. 1996; Chaturvedi et al. 2001; Arce-Montoya et al. 2007). Also, there is a possibility that the stress imposed on explants may have resulted in this point mutation. According to Phillips et al. (1994), there is a programmed loss of cellular control in the explants resulting in chromosomal rearrangements, DNA methylation, mutations in coding sequences, and changes in copy number of repeated DNA arrays. However, it is possible that in collecting the samples for DNA analysis at the same site, plant from a nearby colony was collected instead of the mother stock. In Moraes et al. (2005), samples from this site revealed the presence of two highly distinct chemotypes. Future work is needed on isolation, sequencing, and homology to known sequences that are stored in genebanks to clarify the meaning of this fragment difference.

Our studies provide an effective protocol for storage of *P. peltatum* under slow growth conditions. Using synthetic seed technology and a low incubation temperature of 10°C, *P. peltatum* germplasm can be stored effectively for 8 mo without subcultures, alleviating maintenance labor in the laboratory. The study also demonstrates that genetic markers are an important tool in detecting genomic stability during the process of shoot multiplication and in storage.

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