

In vitro clonal propagation and stable cryopreservation system for *Platycladus orientalis* via somatic embryogenesis

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Abstract *Platycladus orientalis* is a widespread conifer, which is native in eastern Asia, and has recently attracted much attention due to its ornamental value for landscape and gardens. However, native *P. orientalis* populations have been in decline over the past century. Here, we established an in vitro propagation and cryopreservation system for *P. orientalis* via somatic embryogenesis (SE). Whole megagametophytes with four development stages (Early embryogeny: E1 and late embryogeny: L1, L2, and L3) of zygotic embryos from immature *P. orientalis* cones were used as initial explants and cultured on three different basal media such as initiation medium (IM), Litvay (LV), and Schenk and Hildebrandt (SH). Both the developmental stage of zygotic embryos and kind of basal medium had a significant effect on embryogenesis induction with IM ($P < 0.001$, respectively). The highest frequency of embryogenic callus induction was obtained in megagametophytes with zygotic embryos at L2 stage, which ranged as high as 30%. The maturation medium containing IM basal salts, vitamins and amino acids, 15 g l^{-1} abscisic acid (ABA), 50 g l^{-1} maltose, and 100 g l^{-1} polyethylene glycol 4000 (PEG) was found to be the suitable medium for production of somatic embryos. The frequency of somatic embryo formation from both non-cryopreserved and cryopreserved cell lines was also tested. There were no statistical differences on the production of somatic embryos between non-cryopreserved and cryopreserved cells ($P = 0.523$). Genetic fidelity of the plantlets

regenerated from non-cryopreserved and cryopreserved embryogenic cell lines was assessed by both random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analysis. There was no genetic instability in the regenerated plantlets from cryopreserved embryogenic cell lines. Both the SE protocol and cryopreservation protocols described here have the potential to contribute the conservation and clonal propagation of *P. orientalis* germplasm.

Keywords Cupressaceae · Somatic embryogenesis · Cryostorage · RAPD · ISSR

Abbreviations

ABA	Abscisic acid
AC	Activated carbon
BA	Benzylamino purine
IM	Initiation medium
MIM	Maturation medium
GIM	Germination medium
PGR	Plant growth regulator
2,4-D	2,4-Dichlorophenoxyacetic acid
RAPD	Random amplified polymorphic DNA
ISSR	Inter simple sequence repeat
CP	Cryoprotectant

Introduction

Platycladus orientalis (L.) Franco is a genus of coniferous trees in the cypress family (Cupressaceae), containing only one species, and distributed mostly in the moist coastal regions of the Northern Hemisphere (LePage 2003). This tree is an important species for ornamental usages and widely grown for garden and hedge. In addition, it has recently become valuable due to its utilization of various

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sources. In Asia, it has been used in the traditional medicine for hemorrhoids, hypertension, and also for promoting hair growth (Zhang et al. 2013; Kim et al. 2014). *P. orientalis* oil has attracted much attention because of its antibacterial and antifungal activity. In addition, the use of its essential oil has been increasing in the past few years due to its medicinal purpose. In other respects, the essential oils of *P. orientalis* could be commercialized as natural pesticides and herbicides. Gandhi et al. (2015) reported that often the raw material harvesting for production of useful compounds could be limited by over-harvesting which foster the rapid reduction of population in ecological problems. Natural populations of *P. orientalis* have declined over the past century because of the overexploitation. As a result, natural populations of *P. orientalis* are rare and restricted to some provinces in China (Zhu and Lou 2013).

Plant tissue culture technologies offer important opportunities to expedite clonal propagation of plants. In particular, somatic embryogenesis (SE) has shown promise for improvement of important coniferous species. Since SE in *Picea abies* (Norway spruce) was first reported by Hakman et al. (1985), SE has been known as an effective way for cloning genetically improved trees and has become an important technique for mass propagation of coniferous species in the past few decades. For long-term maintenance of conifer embryogenic tissues, they are generally maintained on solid media or in liquid media. However, the process of repeated subcultures not only possesses the risk of microbial contamination, but also brings genetic changes and loss of embryogenic capacity of the tissue which demands for alternative strategies for successful long-term conservation of coniferous.

Cryopreservation at ultralow temperature ($-196\text{ }^{\circ}\text{C}$) in liquid nitrogen (LN) is one of the promising conservation technology for long-term storage of plant cells and tissues. Cryopreservation of embryogenic tissues also reduces labor and costs to maintain (Nairn 1992), limits somaclonal variation during repeated subculture (Dussert et al. 1992). Moreover, cryopreservation system can avoid undesirable contamination and loss of embryogenic potential during a long-term subcultivation (Engelmann 1992). However, there has not been any study on somatic embryogenesis and cryopreservation system in the genus *Platycladus* until now.

The aims of present study were to develop protocols for clonal propagation of *P. orientalis* via SE and to demonstrate the potential of germplasm conservation via cryopreservation of embryogenic cultures. We tested experiments to determine the effects of the developmental stage of zygotic embryos and basal medium on induction of SE in *P. orientalis*. To compare the difference between pre-freeze of embryogenic tissues and post-thaw regrowth of embryogenic tissues, we also tested the effect of cryopreservation on maturation capacity and germination

frequency of somatic embryos in *P. orientalis*. Additional objective was to evaluate the genetic fidelity of regenerated plantlets from cryopreserved somatic embryos using random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analysis.

Materials and methods

Plant materials

Immature fruits of *P. orientalis* (Fig. 1a) were collected from three trees growing on the Kangwon National University campus (Chuncheon, Gangwon province, Korea; $37^{\circ}52'\text{N}$, $127^{\circ}45'\text{E}$) between June and August in 2016. The stages of zygotic embryo development were observed for each collection date and divided into four development stages (Fig. 2). Fruits were soaked in a 50% solution of Medilox-P (0.01% hypochlorous acid, 0.05% sodium chloride) for 1 min and rinsed with sterilized distilled water. Then, the fruits were dissected (Fig. 1b–d) and immature seeds were extracted (Fig. 1e). The seeds were surface-disinfested with 70% (v/v) ethanol for 2 min and then rinsed three times with sterilized distilled water. Seed coats were removed with tweezers and a surgical knife (Fig. 1f) and the megagametophytes containing zygotic embryos were cultured in vitro on semi-solid media (Fig. 1g).

Culture initiation and maintenance

For initiation of embryogenic cultures, megagametophytes were cut open and zygotic embryos at a pre-cotyledonary stage were cultured on an initiation medium (IM) in $60 \times 15\text{ mm}$ plastic Petri dishes. Major and minor salts of IM were those of half-strength EM medium (Maruyama et al. 2005). Half-strength Litvay (LV) medium (Litvay et al. 1985) and SH medium (Schenk and Hildebrandt 1972) supplemented with $4.5\text{ }\mu\text{M}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and $2.2\text{ }\mu\text{M}$ 6-benzylaminopurine (BA) were also tested for initiation of embryogenic cultures. All media were also supplemented with 10 g l^{-1} sucrose, 0.5 g l^{-1} myo-inositol, 1 g l^{-1} glutamine, and 3 g l^{-1} Phytigel (Sigma). The pH was adjusted to 5.7 before autoclaving at $121\text{ }^{\circ}\text{C}$. Glutamine was filter-sterilized and added to the cooled medium. Ten Petri plates, each with five megagametophytes, were cultured the developmental stage of zygotic embryos in basal media and kept in the dark at $25\text{ }^{\circ}\text{C}$. Embryogenic tissue lines were maintained by transferring to fresh IM supplemented with 30 g l^{-1} sucrose, $2.25\text{ }\mu\text{M}$ (2,4-D), and $1.1\text{ }\mu\text{M}$ BA at every 2 week intervals.

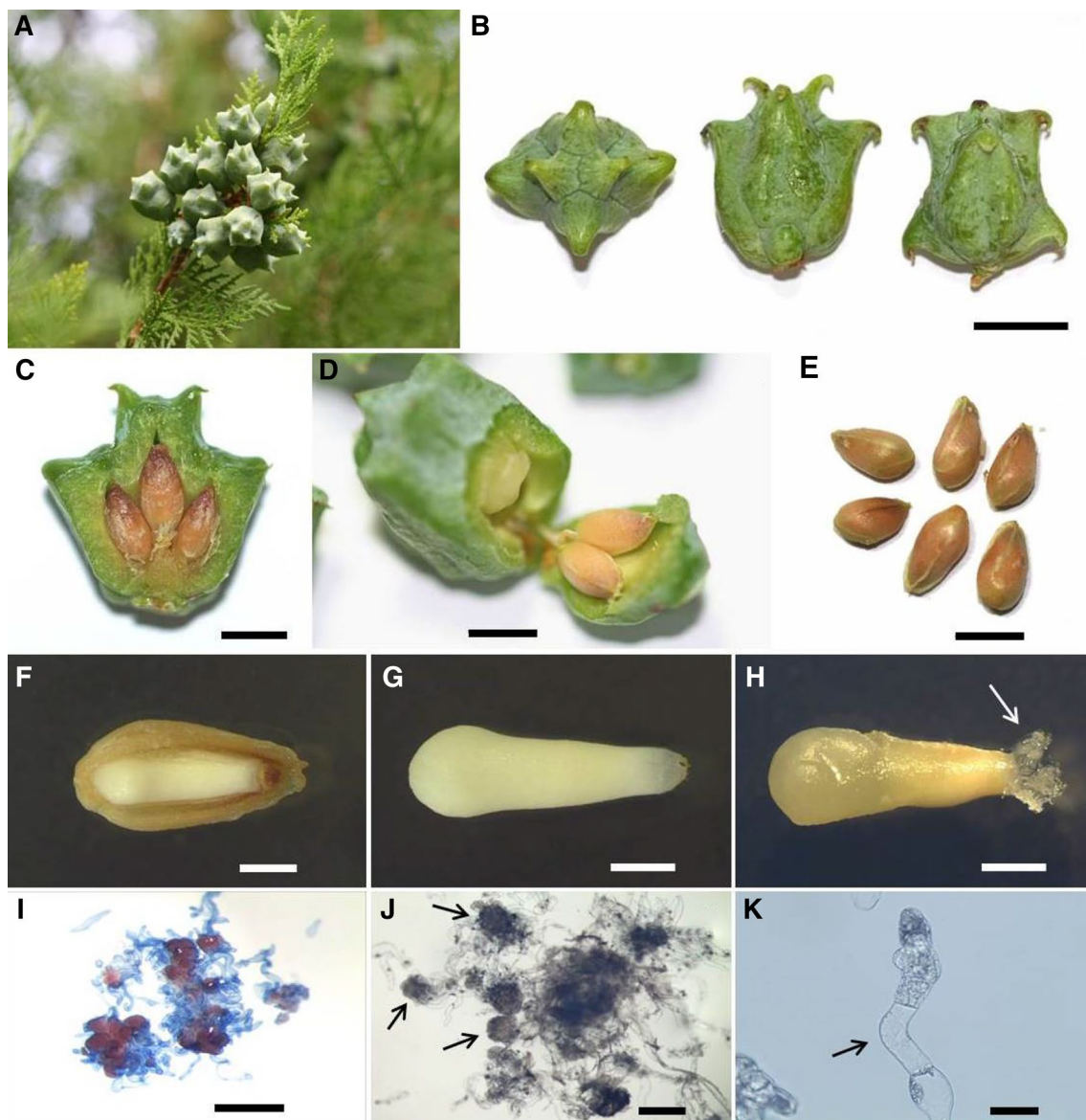


Fig. 1 *P. orientalis* fruits, seeds and megagametophytes. **a** Immature fruits of *P. orientalis*. **b** Three dimensional *P. orientalis* fruits. Bar 1 cm. **c** Longitudinal section of the front of *P. orientalis* fruit. Bar 5 mm. **d** Oblique section of the side of *P. orientalis* fruit. Bar 5 mm. **e** *P. orientalis* seeds from a fruit. Bar 5 mm. **f** *P. orientalis* seed coat with a megagametophyte dissected from a longitudinal section. Bar 1 mm. **g** *P. orientalis* megagametophyte explant. Bar 1 mm.

h Embryonic tissues produced from the megagametophyte. Bar 1 mm. Arrow indicates the putative embryonic tissues. **i** *P. orientalis* embryogenic cells after double staining; embryonal head cells stained red (acetocarmine) and suspensors stained blue (Evan's blue). Bar 500 μ m. **j** *P. orientalis* embryogenic cells proliferating in liquid medium and the embryonal heads (arrows). Bar 500 μ m. **k** Single proembryo with long suspensor (arrow). Bar 200 μ m

Cryopreservation (freezing, thawing, and recovery)

Cryopreservation steps were used following methods described previously (Ahn et al. 2017). Briefly, five cell lines randomly selected were used for cryopreservation. Embryogenic tissue was transferred into 25 ml of liquid IM in 50 ml flasks and cultured in dark on a rotary shaker at 100 rpm for 1 week. The cultures of embryogenic suspension from each

pretreatment (24 h on liquid IM supplemented with 0.4 M sorbitol) were inoculated into cooled (4 °C) IM with 0.4 M sorbitol and 10% dimethylsulfoxide (DMSO) as cryoprotectant (CP). Then, aliquots of 1.5 ml of embryogenic tissue were pipetted into pre-chilled (4 °C) 2 ml CoolCell® Filler Vial (BioCision, San Diego, California, US) and placed into a pre-chilled (4 °C) CoolCell® 3 ml LX freezing container (BioCision, San Diego, California, US). The container was

Fig. 2 Staging system of *P. orientalis* zygotic embryo development. *E1* early embryogeny, *L1* the first stage of late embryogeny, *L2* the second stage of late embryogeny, *L3* the third stage of late embryogeny. *Bar* 500 μ m



transferred to an ultra-cold freezer ($-70\text{ }^{\circ}\text{C}$) for 24 h. Cryovials were then transferred into LN at $-196\text{ }^{\circ}\text{C}$ for 1 week. All the cryopreserved samples for 1 week were retrieved. Cryovials were thawed for 2–3 min in a water bath ($39\text{ }^{\circ}\text{C}$), and immediately embryogenic tissue was pipetted and spread onto a sterile nylon mesh with 30- μ m pore (Membrane Solutions), over stacked filter paper discs. Then, the nylon mesh with embryogenic tissue was placed onto fresh semisolid IM gelled with 0.8% Plant agar (Duchefa) and incubated in the dark at $24\text{ }^{\circ}\text{C}$. Nylon mesh with embryogenic tissue was transferred to fresh semisolid IM at 3, 24 h, and 7 days, to facilitate dilution of residual DMSO. Embryogenic tissue was transferred directly onto semisolid IM after 1 month and maintained every 2 weeks of subculture intervals. All experiments were performed three times.

Somatic embryos maturation

Five embryogenic culture lines were selected for maturation capacity test. Embryogenic tissues that had been either cryo-stored for 1 week or noncryo-stored were suspended in 25 ml of liquid IM in 50 ml Erlenmeyer flasks and grown for 10 days on gyratory shaker at 100 rpm in the dark. An aliquot of 1.5 ml in liquid IM was pipetted onto disks of nylon mesh (30 μ m pore size) in a Büchner funnel, and then rinsed two times with 20 ml of IM liquid medium without plant growth regulators (PGRs) and subjected to mild vacuum to remove excess liquid medium. Each nylon mesh with embryogenic tissues was placed on maturation medium (MIM) containing IM basal salts, vitamins and amino acids, 15 g l^{-1} abscisic

acid (ABA), 50 g l^{-1} maltose, 100 g l^{-1} polyethylene glycol 4000 (PEG) in $100\times 15\text{ mm}$ plastic Petri dishes. Then, embryogenic tissues were spread onto the filter paper surface with sterile forceps if they were clumped. Totally, 30 Petri plates were used for five lines \times two treatments \times three replications, and cultures were incubated in the dark at $25\text{ }^{\circ}\text{C}$ for 8 weeks. Morphologically well-developed cotyledons were counted and used for germination and conversion experiments.

Germination and plantlet conversion

After 8 weeks of maturation in the presence of ABA, cotyledonary somatic embryos collected from MIM were transferred onto germination medium (GIM) containing IM basal salts, vitamins and amino acids. GIM which includes of 10 g l^{-1} sucrose was solidified with 9 g l^{-1} Bacto agar (Difco) with 3 g l^{-1} activated charcoal (AC). The cultures were kept in the dark at $25\text{ }^{\circ}\text{C}$ for 1 week and then moved in a tissue culture room under cool white fluorescent lights ($40\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$, 16 h photoperiod). Ten germinants were transferred onto GIM in $100\times 40\text{ mm}$ plastic Petri dishes. After 4 weeks, all germinants were transferred in polycarbonate Magenta™ culture vessels (Sigma Chemical Co., St Louis, MO, USA) containing 100 ml of semisolid GIM with 30 g l^{-1} sucrose, 3 g l^{-1} AC, and 9 g l^{-1} Bacto agar, and kept under the conditions described above. Plantlets with well-developed epicotyls and roots were planted in the Plastic Seedling Starter Trays (Jiangsu, China) containing a perlite: peatmoss: vermiculite (1:1:1 v/v); afterward, the trays were

placed in a transparent acrylic box for hardening off at the same tissue culture room. Survival rate of plants was evaluated after 8 weeks.

Genetic stability test

To identify the effect of cryopreservation on genetic variation of *P. orientalis*, RAPD and ISSR analysis were conducted using the University of British Columbia, Canada (UBC) primers (Table 1). Genomic DNA extraction and PCR analysis were conducted using the procedure of Ahn et al. (2011). Briefly, fresh leaves of regenerated plantlets from non-cryopreserved and cryopreserved cell lines were ground into fine powder in liquid nitrogen using mortar and pestle. Genomic DNA was extracted using a Genomic DNA Extraction Kit [Mini] (Real Biotech Corporation, Banqiao city, Taipei County, Taiwan). Cycling conditions for RAPD were 94 °C for 7 min; followed by 45 cycles of denaturation at 94 °C for 1 min, annealing temperature at 35 °C for 1 min, polymerization at 72 °C for 2 min and final extension at 72 °C for 10 min. For ISSR, the first heating temperature was at 94 °C for 5 min; followed by 45 reaction cycles of 30 s at 94 °C, 30 s at 52 °C, 60 s at 72 °C and a final 10 min at 72 °C. The RAPD and ISSR reaction samples were loaded on to 1.5% agarose gel in 0.5×TBE buffer and detected by ethidium bromide staining under UV-lights (GelDoc-It^{TS2} Imaging System).

Statistical analysis

Normality of the residuals was checked with Shapiro–Wilk normality test (Shapiro and Wilk 1965). The significance of the effect of basal medium on induction of SE in *P. orientalis* and the effect of the cryopreservation on somatic embryo production per plate were examined by analysis of variance (ANOVA) with statistical package ‘R (version 3.4.0)’ in ‘R Studio (version 1.0.143)’. Percentage data were transformed by arcsine square-root transformation before analysis. Significant differences between means were identified using the Tukey’s test (Tukey 1953) at a significance level of 5%.

Results and discussion

Initiation of embryogenic tissue

Cultures were initiated from three mother trees of *P. orientalis* during June and August 2016. Embryogenic cultures produced from megagametophyte explants (Fig. 1h) were transferred to fresh semisolid IM for proliferation of embryogenic tissue (Fig. 1i). We observed proliferating embryogenic cells in liquid medium under a multiphoton confocal laser scanning microscope system (LSM510; Carl Zeiss Jena, Germany) (Fig. 1j, k). The frequency of cultured megagametophytes with zygotic embryos was used to determine the optimal zygotic embryo stage and initiation medium. A high initiation frequency appeared to occur between L1 and L2 stage and developmental stage of zygotic embryos had a significant effect ($P < 0.001$, $F = 16.356$, $df = 3$) on the induction of embryogenesis (Fig. 3). Analysis of variance results indicated that culture medium had a significant effect on embryogenesis induction ($P < 0.001$, $F = 50.189$, $df = 2$) (Fig. 4). Various basal media were used for initiation of embryogenic cultures in other species of Cupressaceae (Table 2). In

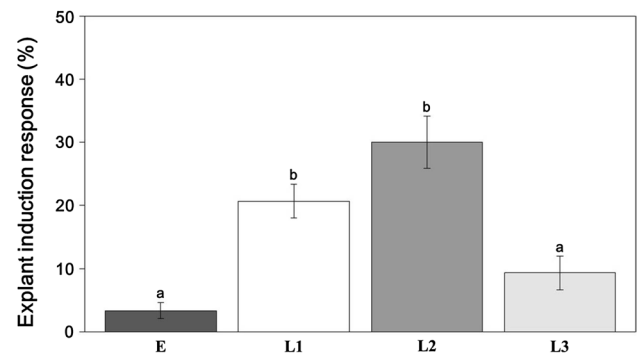


Fig. 3 Effect of the four embryo developmental stages on embryogenic tissue induction from the culture of *P. orientalis* megagametophyte. *E1* early embryogeny, *L1* first late embryogeny, *L2* second late embryogeny, *L3* third late embryogeny. Standard errors bars are shown. Bars marked with different letters indicate significant differences among the developmental stages of zygotic embryos according to the Tukey’s Test at $P \leq 0.05$. Analyses for initiation are based on arcsine transformed data

Table 1 Primers used and polymorphisms shown by them in RAPD and ISSR analysis of *P. orientalis*

RAPD primer	Sequence (5'-3')	ISSR primer	Sequence (5'-3')
UBC 515	GGG GGC CTC A	UBC 809	AGA GAG AGA GAG AGA GC
UBC 540	CGG ACC GCG T	UBC 811	GAG AGA GAG AGA GAG AC
UBC 548	GTA CAT GGG C	UBC 818	CAC ACA CAC ACA CAC AG
UBC 594	AGG AGC TGG C	UBC 820	GTG TGT GTG TGT GTG TC
		UBC 827	ACA CAC ACA CAC ACA CG
		UBC 829	TGT GTG TGT GTG TGT GC

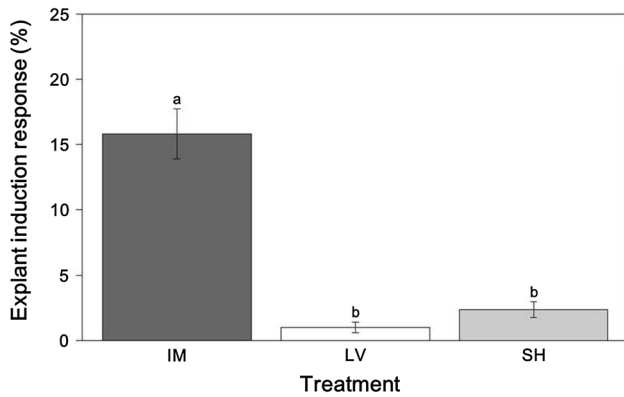


Fig. 4 Effect of culture medium on embryogenic tissue induction from *P. orientalis* megagametophyte. Standard errors bars are shown. Bars marked with different letters indicate significant differences among the culture media according to the Tukey's Test at $P \leq 0.05$. Analyses for initiation are based on arcsine transformed data

particular, EM basal medium was used for induction of SE in *Chamaecyparis pisifera* (Maruyama et al. 2002), *C. obtusa* (Maruyama et al. 2005) and *C. thyoides* (Ahn et al. 2017). Ahn et al. (2017) reported that the highest embryogenesis induction rate was obtained in media completely lacking PGRs. Furthermore, they mentioned that *C. thyoides* cultures on medium without PGRs grew faster than those on medium supplemented with $9 \mu\text{M}$ 2,4-D and $4.4 \mu\text{M}$ BA. Our results also showed that the highest

embryogenesis induction rate was with hormone-free IM medium although there was no statistically significant difference (data not shown). Given our results and those of previous studies, the hormone requirement for SE induction in *P. orientalis* may be similar to *Chamaecyparis* species.

Maturation capacity

Since the late 1980s, when the first report on cryopreservation of conifer embryogenic cultures was published, cryopreservation protocols have been widely applied for storage of germplasm. In particular, cryopreservation has been used to avoid the loss of embryogenic potential, undesirable genetic changes, and time-consuming regular subcultures during long-term in vitro culture maintenance (Salaj et al. 2012). This is the first report of which we are aware on cryopreservation of a member of the genus *Platycladus* via SE system. Following retrieval from cryostorage, cryo-stored samples were able to resume growth and they became fully re-established within 3 months (Fig. 5). Embryogenic tissues from the non-cryopreserved and cryopreserved cell lines were tested for their maturation capacity to produce somatic embryos. We were also able to obtain somatic embryos from each cell line. During the maturation period (8 weeks), we could observe various developing stages of somatic embryos (Fig. 6a, b). In general, many factors such as the cell type, cell size, the composition of vitrification

Table 2 Literature survey on the use of initiation medium in the cypress family (Cupressaceae)

Plant species	Explants	Medium	The highest induction frequency (%)	References
<i>Cupressus sempervirens</i>	IZE	AE ^a , DCR ^b	8%	Lambardi et al. (1995, 1998) Sallandrouze et al. (1999)
<i>Chamaecyparis pisifera</i>	IS	LP ^c , MS ^d , EM ^e , SM ^f , MSG ^g	33.3%	Maruyama et al. (2002)
<i>C. obtusa</i>	MG	SM	48%	Taniguchi et al. (2004)
<i>C. obtusa</i>	MG	modified EM	17.2%	Maruyama et al. (2005)
<i>C. thyoides</i>	MG	modified EM	27%	Ahn et al. (2017)
<i>Juniperus communis</i>	MG	LP	50%	Helmersson and von Arnold (2009)
<i>Cupressus sempervirens</i>	MG	DCR	6.7%	Barberini et al. (2016)
<i>Cunninghamia lanceolata</i>	IZE MG	DCR	13.86%	Hu et al. (2017)

IZE immature zygotic embryos, IS immature seeds, MG megagametophytes

^avon Arnold and Eriksson (1981)

^bGupta and Durzan (1985)

^cQuoirin and Lepoivre (1977)

^dMurashige and Skoog (1962)

^eMaruyama et al. (2000)

^fSmith (1996)

^gModified from MS medium

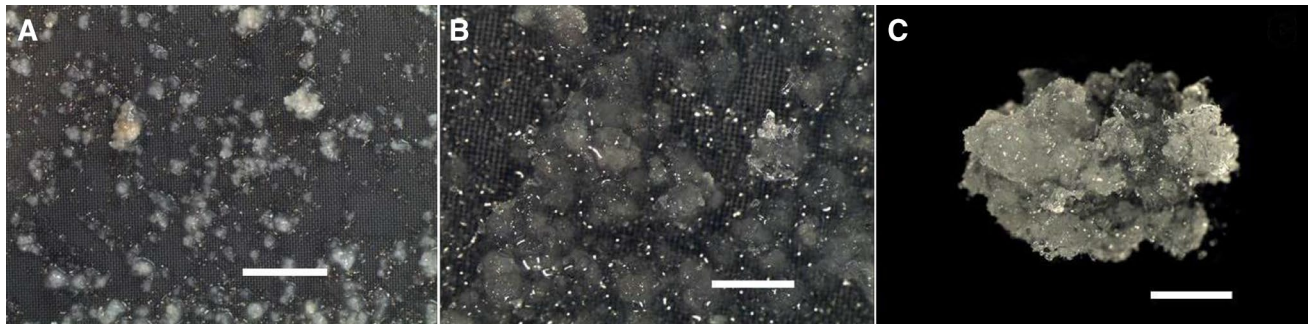


Fig. 5 Re-growth of *P. orientalis* embryogenic tissue following cryostorage. **a** One week following recovery from liquid nitrogen. *Bar* 2 mm. **b** Two weeks following recovery from liquid nitrogen. *Bar* 1 mm. **c** Four weeks following recovery from liquid nitrogen. *Bar* 2 mm

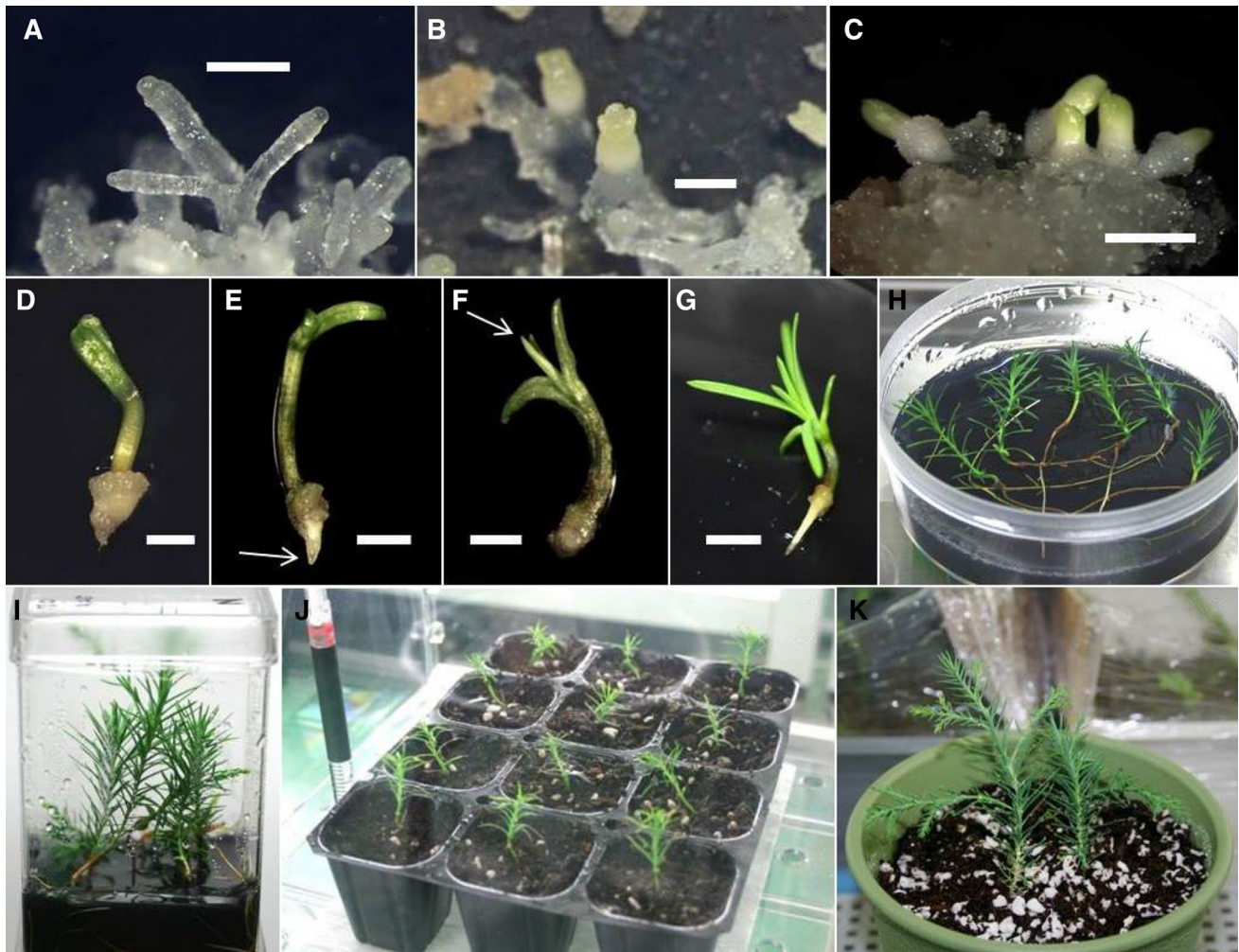


Fig. 6 SE and plant regeneration in *P. orientalis*. **a** Pre-cotyledonary *P. orientalis* somatic embryos following 2 weeks on EM maturation medium. *Bar* 1 mm. **b** Mature somatic embryo with well-developed cotyledons following 8 weeks on EM maturation medium. *Bar* 1 mm. **c** Somatic embryo following 1 week on EM germination medium. *Bar* 2 mm. **d** Germinating somatic embryo following 1 week on EM germination medium in the dark and 1 week in the light. *Bar* 2 mm. **e** Germinating somatic embryos following 2 weeks on EM germination medium in the light, with first true needles emerging (*arrowhead*). **f**

Germinating somatic embryos following 2 weeks on EM germination medium in the light, with expanded true taproot (*arrowhead*). **g** Germinated somatic embryos with expanded true needles and elongated taproot **h** Somatic seedlings following 8 weeks on EM germination medium in the light, with elongated roots and leaves. **i** Somatic seedlings on EM germination medium in a Magenta™ vessel. **j** Somatic seedlings following transfer to potting mix in a transparent acrylic box. *Black tape on a pipette on the left* 5 cm. **k** Somatic seedlings growing in greenhouse

Table 3 The mean number of somatic embryos from either before or after cryopreservation in *P. orientalis*

Embryogenic lines	Non-cryopreserved cell lines	Cryopreserved cell lines
PO-1	25.33 ± 5.04 a	19.00 ± 2.08 ab
PO-2	10.66 ± 3.17 bc	8.00 ± 2.08 bc
PO-3	32.00 ± 6.24 a	28.33 ± 6.76 a
PO-4	21.00 ± 5.50 ab	24.33 ± 2.33 a
PO-5	5.66 ± 2.33 c	6.00 ± 4.58 bc

Different letters within columns indicate significant differences between non-cryopreserved cell lines and cryopreserved cell lines at $P \leq 0.05$ within that same group using Tukey's Test for multiple variances (\pm standard error)

medium, and CPs affect successful cryopreservation of cells (Bomal and Tremblay 2000). In conifer culture, particularly, genotypic influence (variation among cell lines) on producing somatic embryos has been shown from previous study (Klimaszewska et al. 2009). Our result also showed that there was a significant difference among the cell lines ($P < 0.001$, $F = 10.728$, $df = 4$). In addition, structure and developmental stages of somatic embryos in *Pinus nigra* were differently observed cell line (genotype) dependently (Salaj et al. 2012). In non-cryopreserved cell lines, the number of somatic embryos of PO-3 was the highest (32 ± 6.24) while PO-5 showed the lowest number of somatic embryos (5.66 ± 2.33) (Table 3). In the case of cryopreserved cell lines, PO-3 and PO-5 produced highest number (28 ± 6.76) and the lowest number of somatic embryos (6 ± 4.58), respectively (Table 3). These results are in concordance with previous study that somatic embryo maturation is highly variable from line to line (Ramarosandratana et al. 2001). Generally, cryopreservation changes either the cell morphology or the maturation capacity of the cell, but we could not find any difference between the non-cryopreserved and cryopreserved cell lines. Furthermore, the number of somatic embryos was not significantly different among cell lines ($P = 0.523$, $F = 0.424$, $df = 1$). According to our results, the cryopreservation of *P. orientalis* embryogenic cells may not be related to the maturation capacity to yield somatic embryos, but genotypic influence may be highly relevant.

Somatic embryo germination and plantlet conversion

A total of 300 somatic embryos were transferred onto plates of GIM (Fig. 6c). Over the course of 4 weeks, greening of embryos followed by hypocotyl, epicotyl and radicle elongation resulted in plantlets with a taproot and primary leaves (Fig. 6d–g). As shown in Fig. 7, both non-cryopreserved and cryopreserved cell lines were not significantly different in the germination percentage ($P = 0.596$, $F = 0.291$, $df = 1$). Moreover, there was no significant difference in the

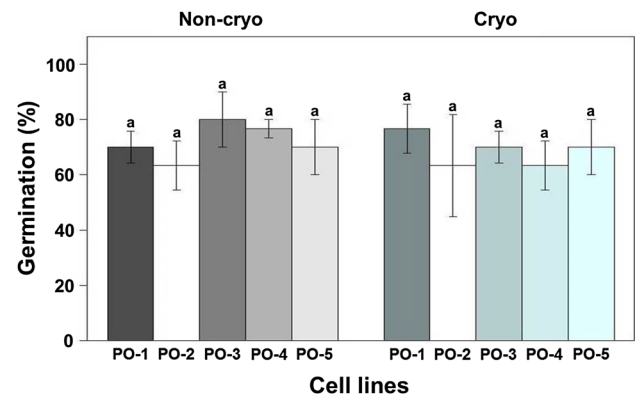


Fig. 7 Comparison of germination percentage of *P. orientalis* somatic embryos between non-cryopreserved cell lines and cryopreserved cell lines. Standard errors bars are shown. Bars marked with same letters indicate no significant differences among the cell lines according to the Tukey's Test at $P \leq 0.05$

germination percentage among the cell lines ($P = 0.793$, $F = 0.419$, $df = 4$) and also no interaction between the treatment and cell line ($P = 0.842$, $F = 0.349$, $df = 4$). The overall germination rate was 71.9%, varying from 63.3 to 80% among the non-cryopreserved cell lines and the overall germination rate was 68.6%, varying from 63.3 to 76.6% among the cryopreserved cell lines (Fig. 7). According to Hirano et al. (2005), cryopreservation did not decrease the germination rate of immature seeds of *Bletilla striata* by vitrification. From our results, we speculated that the cryopreservation of *P. orientalis* embryogenic cells may not be related to the germination capacity of somatic embryos. In general, the better the capacity for embryo production and the quality of the somatic embryo, the higher the germination/conversion rate of the embryos in conifer somatic embryogenesis. In *Pinus sylvestris* culture (Aronen et al. 2009), slim-type embryos showed better conversion rates into plantlets. We also observed similar pattern (data not shown). Most *P. orientalis* germinants showed exceedingly long root elongation (Fig. 6h). After 8 weeks of culture on GIM, the germinants with true leaves and elongated taproots were transferred to Magenta™ vessels for 8 weeks (Fig. 6i) and finally transferred to pots containing soil for acclimatization after 6 weeks (Fig. 6j). More than 80% of *P. orientalis* plantlets successfully acclimated and continued growing in the greenhouse (Fig. 6k). The overall survival rate of plantlets was approximately 60%. No visible differences were observed in development and morphology of plantlets between non-cryopreserved and cryopreserved cell lines.

RAPD and ISSR analysis

For successful cryopreservation, one of the most essential agents is not only to consider the choice of the CP but also

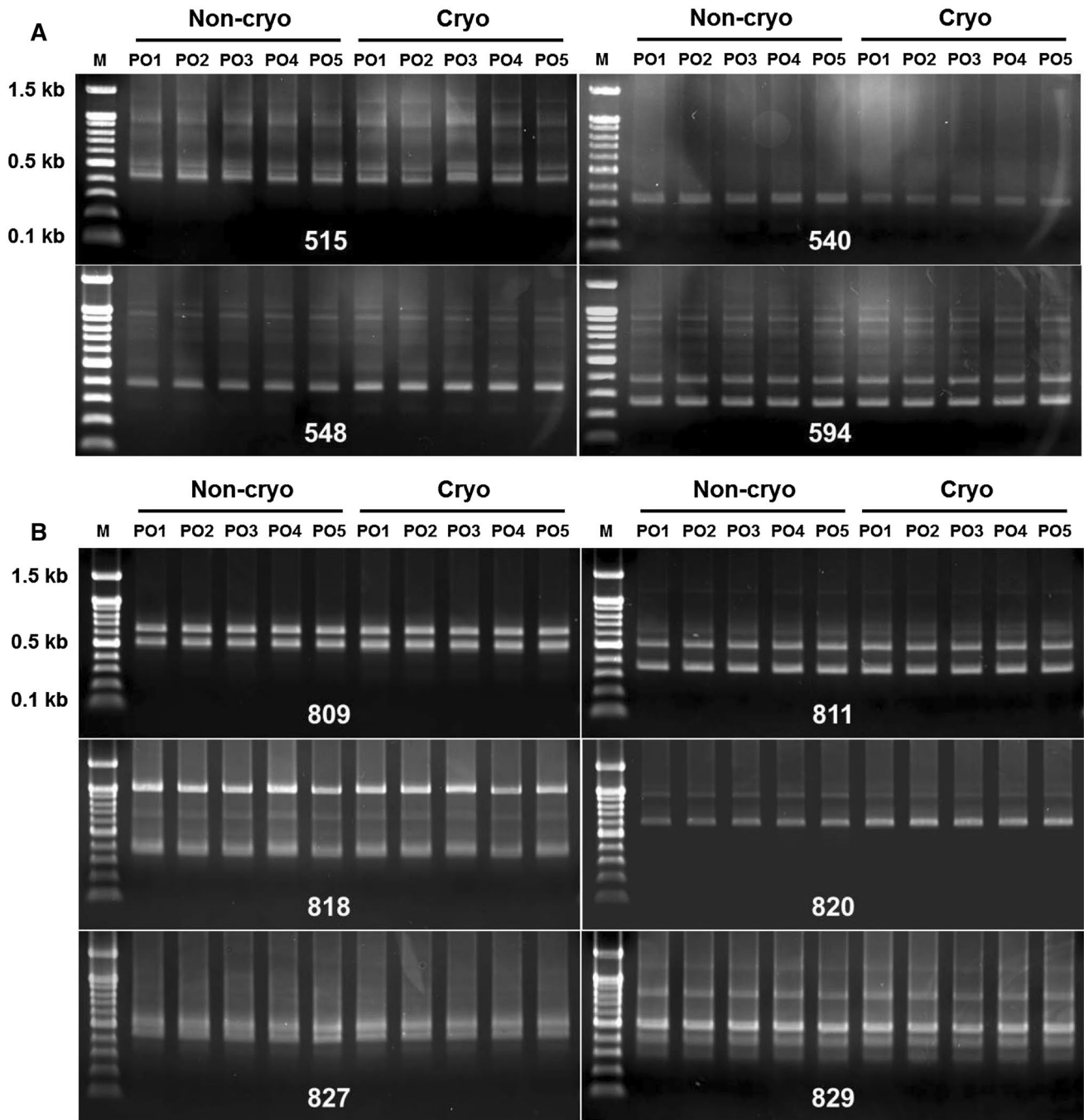


Fig. 8 Profile of PCR products obtained from **a** RAPD and **b** ISSR analysis of cryopreserved and non-cryopreserved cell lines of *P. orientalis* using the 10 different primers. Lane *M* 0.1 kb DNA ladder,

Lane 2–6 non-cryopreserved cell-lines, Lane 7–11 cryopreserved cell-lines. *Non-cryo* non-cryopreserved cell lines, *Cryo* cryopreserved cell lines, *M* molecular marker

to determine optimum concentration of CP. In particular, DMSO is also known to be toxic for some cells and cause genetic variation in plant cells (Arakawa et al. 1990; Panis and Lambardi 2005). We tested the genetic fidelity of the samples from both cryopreserved and non-cryopreserved cell line 5 by using RPAD and ISSR analysis. Totally, ten UBC primers for RAPD and ISSR analysis were used. Four

primers were selected for RAPD analysis based on the reproducibility and banding pattern (Fig. 8a). The size of the fragments was from 100 to 1500 bp. A total of 14 bands were generated from four RAPD primers. No RAPD fragment pattern variation was detected. Eight primers were selected for ISSR analysis based on the reproducibility and banding pattern (Fig. 8b). The size of the fragments was also

from 100 to 1500 bp. A total of 17 bands were generated from four ISSR primers and there was also no ISSR fragment pattern variation. These results are similar to those of a previous study that detected using a single DNA fingerprinting probe in *Picea glauca engelmanni* complex (Cyr et al. 1994). In case of *Picea glauca* (De Verno et al. 1999), however, somaclonal variation was observed in some in vitro embryogenic cell lines by using RAPD analysis. Aronen et al. (1999) also reported considerable genetic variation in embryogenic cultures of open-pollinated *Abies cephalonica* treated with DMSO before freezing in LN. On the other hand, our results showed that no reproducible variation of the RAPD and ISSR profiles was detected within both non-cryopreserved and cryopreserved cell lines. Molecular markers provide a direct measure of genetic variation. In particular, RAPD analysis is simple and rapid method. It is useful to analyze genetic stability of cryopreserved material. ISSR analysis is also a useful and inexpensive method to detect genetic variation (Debnath 2009). RAPD and ISSR analysis are sometimes criticized due to lack of reproducibility compared to other molecular tools. However, we performed more than three times for each experiment and the results constantly revealed high reproducible polymorphic bands. These results proved that the frequency of genetic variation from our cryopreservation system was low. In *Pinus pinaster* (Marum et al. 2009), genetic instability in was detected in an embryogenic cell lines as soon as 6 months of culture under proliferation conditions. Marum et al. (2009) concluded that embryogenic tissues should be cryopreserved as soon as possible after the establishment of embryogenic cell lines because genetic variation may arise during the proliferation stage. No genetic variation from our study may be resulted with more prompt cryopreservation after initiation. When comparing the profiles derived prior and after long-term cryopreservation of *Abies cephalonica* embryogenic cell lines, the genetic fidelity analyses with RAPD markers revealed some changes (Krajňáková et al. 2011). If possible, therefore, further study may need to consider long-term cryopreserved cells for genetic stability in *P. orientalis*.

Conclusions

In the *P. orientalis* embryogenic cultures both the developmental stage of zygotic embryos and basal medium had a significant effect on embryogenic tissue induction. There was no difference in maturation capacity to form somatic embryos between non-cryopreserved and cryopreserved cell lines. We optimized successful cryopreservation system of embryogenic tissue in *P. orientalis* without any loss of embryogenic potential. Neither the maturation capacity of embryogenic cell lines nor germination rates of somatic embryos were negatively affected by cryopreservation. In

addition, there was no genetic variation between non-cryopreserved and cryopreserved embryogenic cell lines from RAPD and ISSR analysis. This clonal propagation and stable cryopreservation system for *P. orientalis* could aid greatly with germplasm conservation and restoration efforts for the species. This is the first report of a plant regeneration and cryopreservation system for the genus *Platyclusus* via SE.

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Author contributions YEC designed all experiments and wrote drafts of the manuscript. CHA conducted the experiments and data analysis, took photos and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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