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Somatic Embryogenesis of Rare Stewartia Species and Elite Cultivars©[a]

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ABSTRACT

The methods of somatic embryogenesis proved successful for five Asian and two North American Stewartia species. Immature embryos were used as explants and cultured on gelled WPM basal medium with and without PGRs (Lloyd & McCown, 1980). Within 3-4 weeks embryogenic callus was produced from the immature embryo explants and these contained tiny developing somatic embryos. The embryogenic callus was propagated via liquid culture and plated on maturation media (WPM basal nutrient) devoid of PGRs for production and development of somatic embryos. Following the maturation process the embryos were placed on germination medium which was the same as maturation medium but supplemented with 0.25% activated charcoal. After 3-6 weeks, the somatic embryos were germinated and continued to develop into somatic seedlings and were transferred to soil and acclimated to the greenhouse and natural environment.

Keywords: Liquid culture, somatic embryos, woody plant basal medium (WPM),

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INTRODUCTION

Stewartia is a genus of flowering shrubs and trees in the family Theaceae. Stewartia species are native to eastern Asia, with the exception of two species, *S. ovata* and *S. malacodendron*, which are indigenous to southeastern North America. All species have large and showy flowers that are 3-11 cm in diameter, usually with 5 white petals (Figures 1a, 1b). The barks of the trees are very distinctive in that the colors range from smooth orange to yellow-brown and flakes. Despite having outstanding ornamental value and features, Stewartias are not readily available for landscaping in the horticultural trade due to difficulty with mass propagation (Struve & Lagrimini, 1999; Nair & Zhang, 2010). *S. pseudocamellia* (Japanese Stewartia) has been propagated *in vitro* from single node explants (McGuigan et al. 1997), but axillary shots were not produced at a level applicable for commercial production. In addition to the commercial propagation of Stewartia, there is a need for developing propagation techniques for the conservation of North American species that are rare (*S. ovata*) or endangered (*S. malacodendron*). Preservation of these species through seed is limited by difficulty with collection, seed yield and viability, and germination requirements. The objective of this research was to examine somatic embryogenesis as a means to mass propagate Asian and North American Stewartia species and to provide material for cryopreservation of valuable Stewartia germplasm.

MATERIALS AND METHODS

Explant sources

Immature fruit collected in August 2015 from cultivars *S. malacodendron* 'Delmarva', *S. ovata'* Red Rose', and *S. ovata* 'Royal Purple' were obtained from The Polly Hill Arboretum (Tisbury, MA). Immature fruit of *S. pseudocamellia* var. *koreana*, a cold tolerant cultivar, was provided by the University of Maine in August 2016. Seed capsules of *S. sinensis*, *S. koreana*, *S.*

monadelpha, and *S. pseudocamellia* were obtained from plants of a private collector in August 2015 and 2016. Wild material of *S. malacodendron* was collected from two sites in Georgia and one in Alabama and wild material of *S. ovata* was collected from one site each in North Carolina and Georgia every August from 2012-2016.

Culture initiation

The disinfestation and culture initiation procedures for immature fruit of the Stewartia species employed the methods outlined for somatic embryogenesis of American chestnut (Merkle et al., 1991). Under sterile conditions and using a stereomicroscope, the immature fruit was dissected to retrieve the immature seeds containing the immature zygotic embryos, which were excised from the seed and placed on a semisolid nutrient medium with or without plant growth regulators (PGRs). The basal nutrient medium was Woody Plant Medium (WPM) (Lloyd & McCown, 1980) supplemented with 3% sucrose, 500 mg/L glutamine, and 3 g/L Gelrite, set at a pH of 5.65. The PGR treatments tested included picloram at a concentration ranging from 0.05 mg/L to 10 mg/L; indolebutyric acid (IBA) at concentrations of 0.1 and 1.0 mg/L; 2, 4-dichlorophenoxyacetic acid (2,4-D) at three concentrations, 1, 2, and 4 mg/L; 0.1 mg/L IBA in combination with 0.5 or 1.0 mg/L 6-benzylaminopurine (BAP); 0.1 mg/L IBA in combination with 0.5 or 1.0 mg/L meta-topolin; and 2 mg/L 2,4-D in combination with 0.2 mg/L meta-topolin. The immature zygotic embryos were cultured in the dark at 25 $\pm$ 2 ºC.

Cell suspensions and embryo production

Somatic embryogenic tissues produced from explant cultures in the PGR study were placed in a 125 ml Erlenmeyer flask containing 30 mls of liquid WPM supplemented with or without the PGR(s) that produced the somatic embryogenic tissue. The liquid suspension cultures were grown at 25 $\pm$ 2 ºC on a platform shaker in the dark at 100 rpm for 45 days. The

cultures were fed with fresh medium every two weeks. Once one gram of tissue was produced, the embryogenic tissue was collected and size-fractionated using 100 µm pore size metal sieves. The pro-embryogenic masses (PEMs) recovered from the sieves were thoroughly rinsed with 150 ml of embryo maturation medium, WPM devoid of PGRs. The PEMs collected on the sieves were transferred to a new sterile 125ml flask with 30 ml of embryo maturation medium. The cultures were placed back onto the shaker in the dark for five days. Using sterile glass Büchner funnels and vacuum, the PEMs were collected by pipetting 10 ml of the suspension culture evenly onto nylon mesh (30 µm pore size) rafts. The nylon rafts were transferred to semisolid embryo maturation medium and placed in the dark at 25 $\pm$ 2 ºC for one month.

Embryo maturation and pre-germination cold treatm*ent*

Once somatic embryos started to form on the nylon rafts, individual embryos were transferred to fresh embryo maturation medium to allow for further maturation. The embryos were incubated in the dark for one month. In some cases the somatic embryos were exposed to a cold vernalization period whereby the plates were wrapped in foil and placed in a walk-in cooler at 4 ºC for 30, 45, or 90 days.

Germination and Conversion

Following maturation and cold treatment the somatic embryos were placed on germination medium (WPM without glutamine or PGRs, with 0.25 mg/L activated charcoal) in an incubator with a 16:8 hour photoperiod, fluorescent lighting (30 µmol m−2 s −1), and temperature set at 25 $\pm$ 2 ºC until the embryos produced roots from the radicle and primary shoots from the apical meristem.

Transfer to soil and acclimation

Each somatic seedling was carefully transferred to a 10.2 cm (4-in.) square pot containing 1 part Fafard 3B: 1 part vermiculite soil mix, misted with a dilute solution of Miracle Gro (1/2 teaspoon/L tap water), and covered by an inverted GA7 vessel (Magenta Corp.) to create a humid micro-environment. The pots were placed in a dome-covered tray on moist perlite and incubated in a growth chamber with a 16:8 hour photoperiod, fluorescent lighting (30 µmol m−2 s −1), and temperature of 25 ± 2 ºC. The somatic seedlings were gradually exposed to lower humidity by removing the GA7s after 2-3 weeks and gradually opening the vents on the lid of the dome tray. Once the dome was removed, plantlets were transferred to the greenhouse and eventually potted up into 1-gal pots containing 3 parts mini nugget bark chips: 1 part peat: 2 parts perlite, and top dressed with Osmocote slow release fertilizer. The final step was transfer of the plants to the shade house and monitoring of survival.

Cryopreservation

The cryopreservation method used with the Stewartia embryogenic cultures was described by Vendrame et al. (2001). Embryogenic tissue of each Stewartia species was grown in culture flasks on a shaker at 100 rpm in the dark containing liquid WPM maintenance medium with PGRs for at least one week. The day before freezing, the tissue was transferred to flasks containing the same medium plus 0.4M sorbitol. The culture flasks were incubated on the shaker overnight and the next day, they were placed in the cold room at 4 ºC. After 30 minutes in the cold, the flasks were placed on ice until the cells were ready to transfer to cryovials. Enough WPM maintenance medium containing 0.4 M sorbitol with DMSO was added to each culture flask so that the final concentration of DMSO in the medium was 10%. The cells were mixed thoroughly in the DMSO/sorbitol solution and 1.8 ml of cells were pipetted into the 2 ml

cryovials and placed in a -1 ºC "Mr. Frosty" freezing container (Nalgene, Rochester, NY). The freezing container was placed in an ultralow freezer set at -80 ºC overnight. The next day the cryovials were put in a cryobox and placed in the liquid nitrogen freezer at -196 ºC. Following removal from liquid nitrogen, the cryovials were transferred from the cryobox to a pre-frozen "Mr. Frosty" container at -80 ºC for 1.5h. Cryovials were then placed in a floating tray in a 40 ºC water bath for 2 minutes to facilitate rapid thawing. Once the cells were thawed, the embryogenic cell clusters were collected on sterile nylon mesh raft overlaying several layers of filter paper to soak up DMSO solution. The nylon rafts were then transferred to fresh WPM maintenance medium supplemented with PGRs. The transfer was repeated the next day for continued removal of remaining DMSO. Then, the cultures were placed in the dark at 25 $\pm$ 2 ºC and observed over the following weeks for re-growth of the embryogenic cultures. Once the cells were successfully recovered, they were tested for ability to make somatic embryos following the cryopreservation treatment.

Statistical analysis

All statistical analyses were performed using R statistical package, R-3.5.1.tar.gz (2018-07-02). The frequencies of somatic embryogenesis induction by the different PGR treatments were compared using a 2-sample test for equality of proportions with continuity correction. The test variables for the germination and conversion response were tested for significance using the ANOVA function following transformation of data using the arcsine function. Differences among dependent variables were determined using Tukey's HSD multiple comparison test at a significance of α=0.05.

RESULTS

Induction of somatic embryogenesis

The North American species of Stewartia were more responsive to picloram than other PGRs in producing somatic embryos, although only at low concentrations. Table 1 shows that picloram concentrations above 2 mg/L proved to be ineffective for inducing somatic embryos. In fact, upon exposure to high concentrations of picloram, the tissue turned brown and died within a very short time. Interestingly, somatic embryogenesis was induced from the North American native cultivar *S. malacodendron* 'Delmarva' when exposed to no PGRs. Thirty-three percent of the immature embryos of this cultivar produced somatic embryos upon exposure to 0.1 mg/L picloram. Immature embryos of another North American native cultivar, *S. ovata* 'Red Rose', were most responsive at the lower concentrations of picloram, 0.05 and 0.1 mg/L, at frequencies of 10% and 20%, respectively (Table 1). Immature embryos from the Asian species, *S. rostrata*, produced somatic embryos upon exposure to 0.1 and 2.0 mg/L picloram at frequencies of 65% and 25%, respectively (Table 1).

Two additional PGRs, 2, 4-dichlorophenoxyacetic acid (2, 4-D) and indolebutyric acid (K salt-IBA or KIBA) were tested for induction of somatic embryos with several Asian species, including *S. monadelpha*, *S. pseudocamellia*, *S. rostrata*, and *S. sinensis*, along with the two North American native species, an Alabama cultivar of *S. malacodendron* and the Polly Hill Arboretum cultivar *S. ovata* 'Royal Purple'. In one experiment, two concentrations of 2, 4-D (2.0 and 4.0 mg/L), one concentration of KIBA, 0.1 mg/L, the control with no PGRs, and two concentrations of picloram (0.1 and 1.0 mg/L), were tested for induction of embryogenesis. Interestingly, The Polly Hill Arboretum cultivar *S. ovata* 'Royal Purple' also produced somatic embryos without PGRs and upon exposure to 0.1 mg/L picloram and to 4.0 mg/L 2,4-D at

frequencies of 33.3 percent , 14.3 percent, and 16.7 percent, respectively (Table 2). Induction of embryogenesis was only observed on medium containing 2.0 mg/L 2, 4-D for the Asian species *S. sinensis* and the Blount County Alabama cultivar of *S. malacodendron,* at frequencies of 30 percent and 20 percent, respectively (Table 2).

Three Stewartia species produced somatic embryos upon exposure to KIBA, *S. rostrata* (Table 3), *S. ovata* 'Otto, NC' and *S. koreana* (data not shown). Table 3 shows a PGR experiment with *S. rostrata* where KIBA alone or in combination with the cytokinins meta-topolin and benzylaminopurine (BAP) induced embryogenesis from immature embryo explants. In this experiment, 2, 4-D was also tested at two different levels, 2.0 and 4.0 mg/L, as well as a no-PGR treatment. Chi-Square analysis with continuity correction showed no significant differences at $\alpha = 0.05$ for treatment comparisons for induction frequency of somatic embryogenesis. Eighty percent of the explants exposed to 2.0 mg/L 2, 4-D, 0.1mg KIBA, and 0.1 KIBA with 0.5 mg/L of BAP produced somatic embryos. No somatic embryos were produced on 4.0 mg/L 2, 4-D, or when the medium was devoid of PGRs. Embryogenesis was only induced from 20 percent of the immature embryos when KIBA at 0.1 mg/L was in used in combination with the cytokinin meta-topolin at 0.5 or 1.0 mg/L. However, KIBA at 1.0 mg/L induced embryogenesis at a frequency of 60 percent, while KIBA at 0.1 mg/L in combination with 1.0 mg/L BAP induced embryogenesis from 40 percent of the explants. There is a a summary showing the various Stewartia species that produced somatic embryos and/or embryogenic callus from these PGR induction experiments (Fig. 2). See the summary of the various steps from the production of somatic embryos to germination and conversion to somatic seedlings in soil (Fig. 3).

Germination and Conversion

A comparison of cold versus no cold treatment was tested for several somatic embryo genotypes of *S. rostrata*. There is a comparison of the no cold (25 °C) versus cold (4 °C) treatment with the six *S. rostrata* genotypes, SR-1, SR-2, SR-4, SR-10, SR-31, and SR-32, with regard to the effect on percentages of germination and conversion of somatic embryos (Table 4). Analysis of variance results showed that the treatment effect for percent germination was significant ($p= 1.5e^{-11}$) and there was a significant difference among genotypes following Tukey's HSD multi-comparison test ($p= 0.0309$) which was only due to a difference between two genotypes, SR1 and SR10. Analysis of variance results also showed that the cold (4 °C) treatment gave significantly higher percent somatic embryo conversion than the no cold (25 °C) treatment ($p< 0.001$).

Cryopreservation

Somatic embryogenic cultures of 17 genotypes across six species of Stewartia were put into cryopreservation: *S. rostrata*, *S. koreana*, *S. monadelpha*, *S. pseudocamellia*, *S. pseudocamellia* var. *koreana* (UMaine-cultivar), and *S. ovata* 'Red Rose'. Cultures of four out of the six tested species were recovered from cryopreservation and five genotypes were recovered at 100%. Recovery was measured by growth of cells, maintenance by these cultures, and the ability to produce somatic embryos that were able to mature, germinate, and convert into plants.

DISCUSSION

Somatic embryogenesis was successfully induced from immature zygotic embryo explants of seven Stewartia species—five Asian and two North American. Additionally, three unique North American cultivars, *S. ovata* 'Red Rose', *S. malacodendron* 'Delmarva', and *S. ovata* 'Royal Purple' from the Polly Hill Arboretum were successfully propagated by somatic

embryogenesis using no or a variety of PGRs. Picloram at low concentrations, such as 0.05 and 0.1 mg/L, was more successful at producing somatic embryos with the North American Stewartia species. Most of the Asian Stewartia species responded best to 2, 4-D at concentrations of 2.0 or 4.0 mg/L in producing somatic embryos. This result is in contrast to results reported for the closely related genus *Camellia* in the Theaceae family. Somatic embryos of *Camellia japonica* were induced from immature zygotic embryos, but using a different basal medium Murashige and Skoog (MS) (1962) and different PGR combination, BAP plus IBA (Vieitez and Barciela, 1990). Somatic embryo conversion and somatic seedling acclimatization to the greenhouse were achieved with all seven tested Stewartia species. Results with several of the species showed that a cold treatment enhanced germination and conversion of somatic embryos into somatic seedlings. Somatic embryogenic tissue from five Stewartia species was successfully recovered from cryopreservation - suggesting feasibility of long-term storage of valuable germplasm. This includes unique cultivars of the Polly Hill Arboretum selected North American Stewartia species: *S. malacodendron* 'Delmarva', *S. ovata* 'Red Rose', *and S. ovata* 'Royal Purple'. This is the first report of somatic embryogenesis in the genus Stewartia.

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Figure 1a. *Stewartia malacodendron* ‘Delmarva’ courtesy of The Polly Hill Arboretum, Tisbury, MA

Figure 1b. *Stewartia ovata* ‘Red Rose’ courtesy of The Polly Hill Arboretum, Tisbury, MA

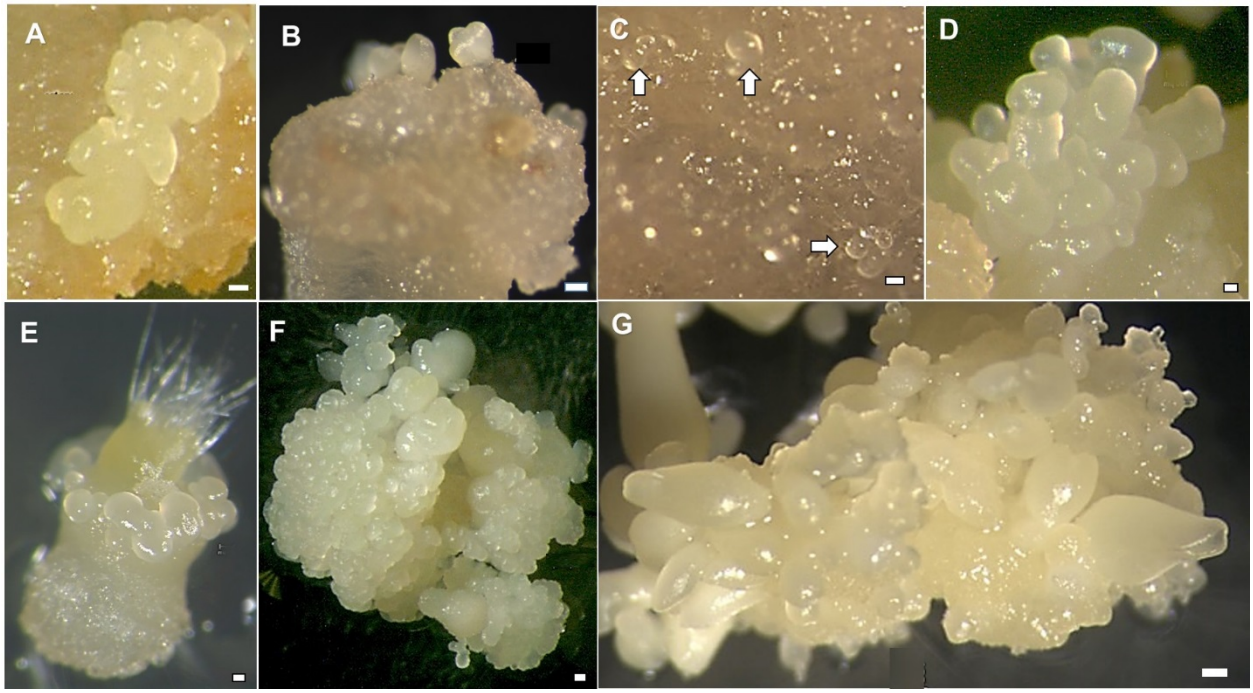

Figure 2. Somatic embryos and embryogenic callus of Stewartia species (A) *S. monadelpha* on WPM medium with 4.0 mg/L 2,4-D, (B) *S. malacodendron* 'Delmarva' with 0.1 mg/L picloram, (C) *S. rostrata* with 0.1mg/L picloram , (D) *S. pseudocamellia* var. *koreana* 'UMaine' with 2.0 mg/L 2,4-D, (E) *S. sinensis* with 2.0 mg/L 2,4-D, (F) *S. koreana* with 2.0 mg/L 2,4-D, and (G) *S. ovata* 'Red Rose' with 0.1 mg/L picloram.

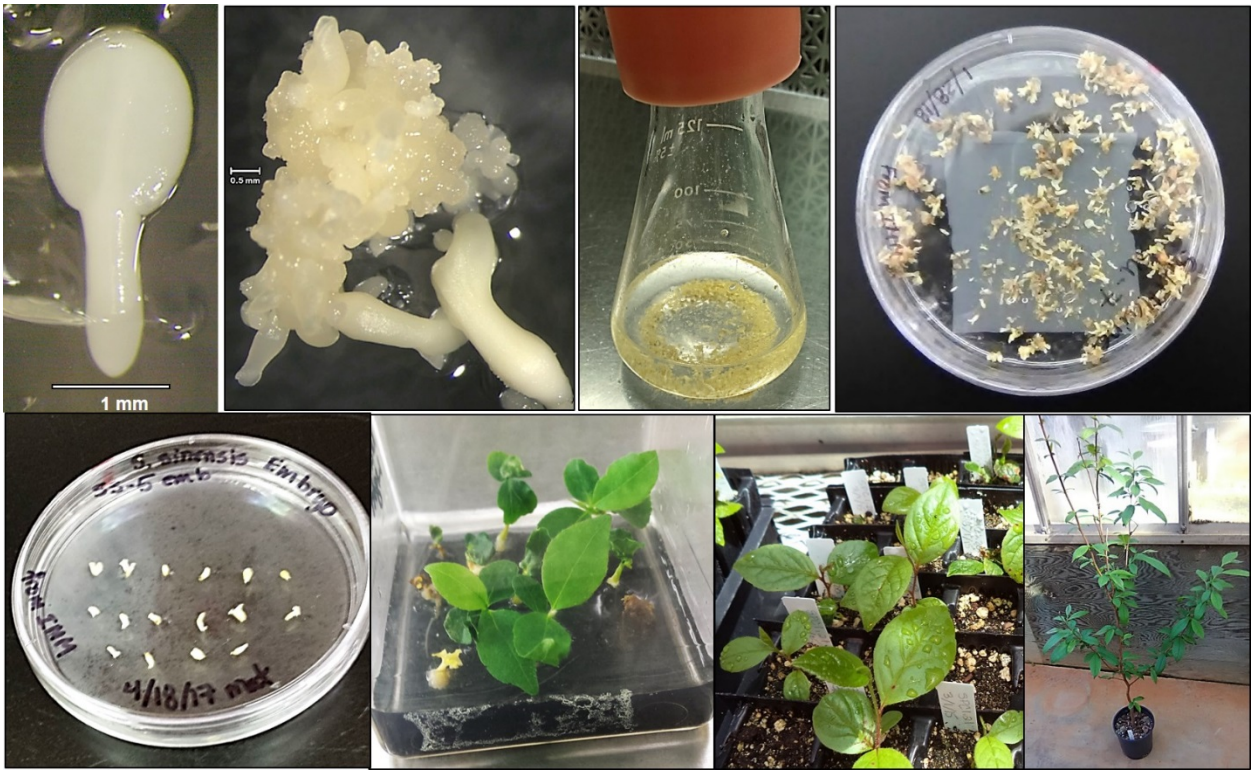

Figure 3. Somatic embryogenesis of Stewartia species from explant to plants. (A) explant source is the immature zygotic embryo, (B) embryogenic callus proliferation, (C) liquid cell suspension, (D) embryo development, (E) embryo maturation, (F) conversion and germination, (G) *ex-vitro* acclimation, (F) 2 year-old tree derived from somatic embryogenesis.

Table 1: Percent induction of somatic embryogenesis in three Stewartia species, *S. malacodendron* 'Delmarva', *S. ovata* 'Red Rose', and *S. rostrata*, following exposure to a gradient of picloram concentrations from 0 to 10mg/L in the nutrient medium.

Species	**Picloram (mg/L)**							
	0	**0.05**	**0.1**	**1.0**	**2.0**	**4.0**	**8.0**	**10.0**
S. malacodendron 'Delmarva'	100%	0	33%	0	0	0	0	0
S. ovata 'Red Rose'	0	10%	20%	0	0	0	0	0
S. rostrata	0	0	65%	0	25%	0	0	0

Table 2. Percent induction of somatic embryogenesis in five Stewartia species, *S. malacodendron*, *S. monadelpha*, *S. ovata* 'Royal Purple', *S. pseudocamellia* var. *koreana* (UMaine), and *S. sinensis* following exposure to three different PGRs at various concentrations versus no PGRs in the nutrient medium.

Species	**Plant Growth Regulators (mg/L)**					
	No PGRs	0.1 Picloram	1.0 Picloram	2.0 2,4-D	4.0 2, 4-D	0.1 KIBA
S. malacodendron	0	0	0	20%	0	0
S. monadelpha	0	20%	20%	50%	80%	0
S. ovata 'Royal Purple'	33.3%	14.3%	0	0	16.7%	0
S. pseudocamellia var. *koreana*	0	0	0	72%	75%	0
S. sinensis	0	0	0	30%	0	0

Table 3. Percent induction of somatic embryogenesis of *S. rostrata* following exposure to varying concentrations and combinations of auxins with and without cytokinins. The negative control is exposure to no PGRs.

Plant Growth Regulators (mg/L)	**SE induction (%)**
No PGRs	0
2.0 2,4-D	80
4.0 2,4-D	0
0.1 KIBA	80
0.1 KIBA + 0.5 meta-Topolin (mT)	20
0.1 KIBA + 1.0 meta-Topolin (mT)	20
0.1 KIBA + 0.5 BAP	80
0.1 KIBA + 1.0 BAP	40
1.0 KIBA	60

Table 4. Comparison of no cold (25 °C) versus cold (4 °C) treatment with six *S. rostrata* genotypes, SR-1, SR-2, SR-4, SR-10, SR-31, SR-32, and the effect on percentage of germination and conversion of somatic embryos.

Genotypes	**Germination (%)**		**Conversion (%)**	
	25 °C	**4 °C**	**25 °C**	**4 °C**
SR-1	31.7 *c*	65.0 *a*	19.2 *c*	39.2 *a*
SR-2	20.0 *d*	46.7 *ab*	4.2 *d*	21.7 *b*
SR-4	24.2 *d*	55.8 *ab*	3.3 *d*	18.3 *b*
SR-10	21.7 *d*	41.7 *b*	4.2 *d*	10.8 *b*
SR-31	23.3 *d*	51.7 *ab*	3.3 *d*	14.2 *b*
SR-32	25.0 *d*	54.2 *ab*	5.0 *d*	17.5 *b*

Tukey's HSD multi-comparison test indicated by letters *a*, *ab*, *c*, and *d* and those letters that differ are significant at α=0.05.