In Vitro Cell.Dev.Biol.-Plant
DOI 10.1007/s11627-008-9169-3

DEVELOPMENTAL BIOLOGY

Embryogenic tissue initiation and somatic embryogenesis in Fraser fir (*Abies* fraseir [Pursh] Poir.)

Y. W. Kim · R. Newton · J. Frampton · K.-H. Han

Received: 11 April 2007 / Accepted: 5 September 2008/ Editor: R. Newton

Abstract Embryogenic suspensor mass (ESM) was established from immature seeds of Fraser fir. The initiation frequency of ESM was dependent on genotype, collection time, medium, and plant growth regulators (PGR) used. The ESM initiation potential was higher with seeds collected in late June (clone 16-273, 4.7%) or early July (clone 16-45, 2.2%) and decreased as the zygotic embryos matured. Excised proembryo stage of zygotic embryos was most appropriate to initiation of ESM. Most of the ESM arose from the seeds that were at the proembryo stage. From the four different culture media we compared, seven ESM lines were obtained: two lines from Murashige and Skoog (MS) medium with 4.4 μM benzyladenine (BA), one from Schenk and Hildebrandt (SH) medium with 4.5 μM thidiazuron (TDZ), and four from SH with 4.4 μM 6-benzyladenine. However, only one ESM line from clone 16-273 (June 24, SH+TDZ) could be proliferated in subsequent culture. Different concentrations of L-glutamine and casein hydrolysate (CH) in the medium were also compared for their effect on ESM proliferation. The highest proliferation rate (1.16-fold) was obtained from SH medium supplemented with 250 mg/L CH and 3.42 mM L-glutamine. In contrast, the lowest rate was noted when 1,000 mg/L CH plus 3.42 mM L-glutamine (0.17-fold) was added to the medium. As for somatic embryo maturation, the highest number of mature precotyledonary ($100.1/g^{-1}$ FW ESM) or cotyledonary ($64.3/g^{-1}$ FW ESM) somatic embryos was obtained on a medium containing 20 or 80 μM abscisic acid, 10% polyethyleneglycol, 4% maltose, and 0.3% gellan gum. For germination of the somatic embryos, the cotyledonary somatic embryos derived from maturation medium were transferred on half-strength Litvay medium containing 0.3% gellan gum. The somatic plantlets were recovered from the germination medium and transferred to soils.

Keywords Embryogenic tissue · Plant regeneration · Somatic embryo

Y. W. Kim · K.-H. Han (✉)
Department of Forestry, Michigan State University,
126 Natural Resources,
East Lansing, MI 48824-1222, USA
e-mail: hanky@msu.edu

R. Newton
Department of Biology, East Carolina University,
1002 Bate Building, East Fifth Street,
Greenville, NC 27858-4353, USA

J. Frampton
Department of Forestry and Environmental Resources,
N.C. State University,
3024D Biltmore Hall,
Raleigh, NC 27695-8002, USA

Present address:
Y. W. Kim
Division of Biotechnology, Korea Forest Research Institute,
Suwon, Kyonggido 441-350, Republic of Korea

Introduction

Fraser fir (*Abies fraseri* [Pursh] Poir.) is a major Christmas tree species in the USA. Due to their exceptional post-harvest needle retention, many species of fir are used for Christmas tree and decoration greenery production. Firs are propagated mainly by seeds, but vegetative propagation by cuttings or grafting is also possible in some species (Hineslet and Blazich 1985). However, the use of vegetation propagation is limited by the fact that both

cuttings and grafts, particularly from older trees, tend to grow plagiotropically.

Conventional breeding of tree species is a time-consuming process because of their long life cycles and slow sexual maturation. Clonal propagation of high-value forest trees through somatic embryogenesis (SE) has the potential to rapidly capture the benefits of breeding or genetic engineering programs and to improve the uniformity and quality of the nursery stock, particularly in conifer species (Find et al. 1993). Since first reported for spruce (Hakman et al. 1985), SE is favored as a promising tool for mass propagation of coniferous trees (Park 2002).

SE has been demonstrated in some *Abies* species including *A. alba* (Schuller et al. 2000), *A. nordmanniana* (Find et al. 2002), *A. balsamea* (Guevin et al. 1994), *A. numidica* (Vooková and Kormuták 2001; Vooková et al. 2003), and in several hybrids, *Abies alba* × *A. cephalonica* (Salajova et al. 1996; Salaj and Salaj 2003/4) and *A. cilicica* × *A. nordmanniana* (Vooková and Kormuták 2003). Among them, somatic embryo maturation and embryo germination have been also observed using mature zygotic embryos (Guevin and Kirby 1997) or embryogenic suspensor mass (ESM) induction from immature seeds (Rajbhandari and Stomp 1997) in *A. fraseri*. However, attempts to transplant the plants from germinated somatic embryos to soil were not reported until now. Protocols for the different species are relatively similar in that only cytokinin is required for initiation and maintenance of the cultures. Another common feature seems to be the problems with maturation of embryos, germination, and soil establishment.

In the present work, we describe the induction of ESM by culture of immature embryos of Fraser fir on media containing cytokinin. Furthermore, we report the effects of seed collection date on ESM initiation, L-glutamine, or casein hydrolysate (CH) on ESM proliferation, abscisic acid (ABA) on somatic embryo maturation, and the regeneration of plants from the embryos.

Materials and Methods

Plant material. Open-pollinated Fraser fir cones from five clones in the North Carolina Division of Forest Resources' seed orchard near Crossnore, NC, USA were collected weekly. Immature seeds were extracted from the cones and surface-sterilized by immersion in 70% ethanol for 1 min followed by 30-min immersion in 1% (*v*/*v*) sodium hypochlorite. The seeds were then rinsed several times with sterile distilled water. Seed coats of immature seeds were aseptically removed, and whole megagametophytes containing intact zygotic embryos were placed horizontally on ESM initiation media.

Microscopic observation of zygotic embryos of seeds. During the initiation experiments, seven to ten seeds from each collection date were selected, longitudinally dissected with a surgical blade (no. 11, Feather), and the developmental stages of the zygotic embryos monitored. The developmental stages of zygotic embryo were confirmed as previously described for *Pinus pinaster* (Ramarosandratana et al. 2001). The embryo developmental stages were used as indicators for the collection of most responsive explants in initiation of ESM.

Initiation of ESM. Fraser fir ESM was initiated from megagametophytes of immature zygotic embryos on MS (Murashige and Skoog 1962) or SH (Schenk and Hildebrandt 1972) medium supplemented with 4.4 μM benzyladenine (BA) or 4.5 μM thidiazuron (TDZ), 50 mg l^{-1} CH, 58.4 mM sucrose (Sigma S5390, grade I), and 0.3% gellan gum (Phytagel, Sigma). The medium pH was adjusted to 5.7 prior to autoclaving at 121°C for 15 min. TDZ was sterilized by filtration then added to cooled medium (45–50°C) after autoclaving. The megagametophytes were placed on a Petri dish (87×15 mm) containing solidified medium, and the cultures were kept in darkness at 24±1°C for 8 wk.

Proliferation of ESM. For ESM proliferation, the cultures obtained were subcultured onto SH medium supplemented with 4.4 μM BA, 58.4 mM sucrose, 50 mg l^{-1} CH, 58.4 mM, and 0.3% gellan gum. During subculture, ESM was subdivided into small pieces (about 1.0 cm in diameter) and cultured in the dark at 24±1°C. The proliferating ESM was maintained by weekly subculture.

Effect of L-glutamine and CH concentration on ESM proliferation. The effect of L-glutamine (3.42 mM) and/or CH (0, 500 or 1,000 mg l^{-1}) on ESM proliferation was investigated using SH medium supplemented with 4.4 μM BA, 58.4 mM sucrose, and 0.3% gellan gum. Three replications were used in the cultures maintained at 24±1°C in the dark. Before and after 4 wk of culture, weight of ESM derived from each treatment was recorded.

Effect of ABA concentration on somatic embryo maturation. Effect of ABA on embryo maturation was determined by subculturing ESM onto SH medium containing various combinations of ABA ranging from 0 to 120 μM, 10%, polyethyleneglycol (PEG) 4000, 4% maltose, 50 mg l^{-1} CH, and 0.3% gellan gum. Experiments consisted of three replicate plates containing three ESM of approximately 150 mg each. The experiments were repeated three times at 25±1°C in the dark. After 8 wk of culture without subculture onto fresh medium, the number of precotyledonary or fully developed cotyledonary stage somatic embryos was counted under a dissecting microscope. The number of matured somatic embryos per initial fresh weight (g) was determined.

Germination of the somatic embryos. Cotyledonary somatic embryos were selected from ESM cultured on various concentrations of ABA-containing medium and placed horizontally on the surface of SH medium containing 60 mM sucrose and 0.3% gellan gum and lacking CH or ABA. The cultures were kept for 7 d under dim light (1.5 μE m^{-2} s^{-1}), 16:8-h light/dark photoperiod, 24±1°C, and then transferred to higher light (50 μE m^{-2} s^{-1}). For acclimatization, the somatic plants with well-developed epicotyls and roots were transplanted into an artificial soil mixture [perlite/peat moss/vermiculite (1:1:1, *v*/*v*/*v*)] in trays with a transparent lid and were watered daily. Acclimated plants were maintained for 4–5 wk in the tissue culture room (50 μE m^{-2} s^{-1}, 16-h photoperiod, 25±1°C).

Statistical analysis. Data presented in the tables were analyzed for significance using analysis of variance and the differences contrasted using a Duncan's multiple range test.

Results and Discussion

Initiation of ESM. Frequency of sound seeds collected form June 10 to July 1 from each of five clones varied (40–100%) depending on collection date and parent clone. The zygotic embryos collected from June 17 to July 1 were dissected to determine the stage of zygotic embryos with ESM initiation efficiency (Table 1). The seeds from all collection dates showed the proembryo stage in terms of zygotic embryo development, except the clone 16-225 that was in globular stage. ESM initiation frequency varied with collection dates, and the highest frequency (4.7%) was obtained with clone 16-273 from cones collected on June 24 followed by clone 16-45 collected on July 16 (2.2%; Table 2). However, the proembryos from clone 16-273 were most responsive to ESM initiation culture conditions. It is well known that the initiation of ESM was critically affected by developmental stage of zygotic embryos at the time of culture. In *Abies alba* hybrids, Salajova et al. (1996) found that ESM induction was best with immature embryos collected in July (38–44.6%). With embryos collected in August, the frequency declined to 4.4–15.8%. NØrgaard et al. (1992) and Hristoforoglu et al. (1992) induced somatic embryogenesis from *A. nordmanniana* and *A. alba* at a frequency of about 40%. Unlike these species, *A. fraseri*, *A. balsamea*, and *A. procera* appear to be more recalcitrant. Guevin et al. (1994) and Guevin and Kirby (1997) reported ESM induction frequencies of only 3.5% from mature embryos of both *A. fraseri* and *A. balsamea*. Rajbhandari and Stomp (1997) reported that the developmental stage of embryo was important for ESM initiation in *A. fraseri*. In their case, ESM was obtained from only immature precotyledonary embryos, not fully formed embryos.

Table 1 Relationship between of developmental stage of zygotic embryo and collection dates of seeds on the initiation of ESM in *A. fraseri*

Collection date	Parent clone	Developmental stage of embryo	ESM initiation (%)
June 10	16-153	–[a]	0
	16-225	–	0
	16-273	–	0
	16-289	–	0
	16-45	–	0
June 17	16-153	–	0
	16-225	Proembryo	0
	16-273	Proembryo	0
	16-289	–	0
	16-45	Proembryo	0
June 24	16-153	Proembryo	0
	16-225	Proembryo	0
	16-273	Proembryo	4.67
	16-289	–	0
	16-45	Proembryo	0
July 1	16-153	Proembryo	0
	16-225	Globular	0
	16-273	Proembryo	0
	16-289	Proembryo	0
	16-45	Proembryo	2.15

[a] Not determined

As an initiation medium, SH medium (0.5%) was only slightly more responsive than MS medium (0.4%) in ESM production (Table 2). SH medium has been reported to be effective for inducing somatic embryos in *Abies* species (Salajova et al. 1996; Rajbhandari and Stomp 1997; Vooková and Kormuták 2003). In the analysis of the effect of PGRs in the initiation medium, BA (0.8%) was more effective than TDZ (0.1%) in inducing ESM for this species (Table 2). Several cytokinins have been used for initiation of ESM in *Abies* spp. With mature zygotic embryos of *A. nordmanniana*, among the four cytokinins (BA, kinetin, 2iP, and TDZ) tested, TDZ was the most effective cytokinin, but it was not significantly different from BA (NØrgaard and Krogstrup 1991). In addition, Salajova et al. (1996) reported that BA was the most efficient ESM in hybrid firs. It is evident that addition of cytokinin to medium is critical for ESM initiation in *Abies* species. On the other hand, in *A. fraseri*, BA and TDZ were found to be equally efficient and better than 2iP (Guevin et al. 1994), as we have observed in Table 2. In general, auxins combined with cytokinins are generally required for ESM initiation in conifer species. However, NØrgaard and Krogstrup (1995) showed that exogenously applied auxins do not play an important role in somatic embryogenesis in *Abies* spp. Cytokinin alone is effective in initiating and maintaining ESM.

Table 2 Effect of collection dates, parent clones and culture medium on ESM initiation in *A. fraseri*

Collection date	Clone	Medium	No. of cultured explants	No. of initiated ESM (%)
June 24	16-153	MT[a]	18	0
		MB[b]	22	0
		ST[c]	24	0
		SB[d]	17	0
	16-225	MT	27	0
		MB	30	0
		ST	23	0
		SB	24	0
	16-273	MT	28	0
		MB	30	2 (6.7) b[e]
		ST	30	1 (3.3) c
		SB	19	2 (10.5) a
	16-289	MT	24	0
		MB	24	0
		ST	30	0
		SB	42	0
	16-45	MT	18	0
		MB	12	0
		ST	24	0
		SB	18	0
July 1	16-153	MT	23	0
		MB	12	0
		ST	12	0
		SB	17	0
	16-225	MT	21	0
		MB	12	0
		ST	12	0
		SB	18	0
	16-273	MT	30	0
		MB	30	0
		ST	36	0
		SB	30	0
	16-289	MT	18	0
		MB	30	0
		ST	24	0
		SB	24	0
	16-45	MT	24	0
		MB	15	0
		ST	24	0
		SB	30	2 (6.7) b

[a] MS+1.0 mg/L TDZ
[b] MS+1.0 mg/L BA
[c] SH+1.0 mg/L TDZ
[d] SH+10 mg/L BA
[e] Different *letters* within *columns* indicate significant differences at $P=0.05$

Proliferation of ESM. A total of seven ESM lines were initiated from megagametophytes with immature embryos during the experiment. However, only one ESM derived from clone 16-273, when cultured on SH medium with 4.5 μM BA collected from June 24, survived and proliferated in subsequent culture. During the process of initiation, a mucilaginous ESM protruded from the micropylar end of the megagametophyte within 6 wk in culture (Fig. 1*a*). This translucent and mucilaginous ESM was composed of a few somatic embryos at the early stage of development (Fig. 1*b–e*). The ESM lines were proliferated onto solid SH medium containing 4.4 μM BA, 58.4 mM sucrose, and 0.3% gellan gum (Fig. 1*f*). The transferred ESM proliferated rapidly on the medium and was subcultured weekly onto fresh medium. The subcultured ESM formed numerous somatic embryos with distinct embryonal heads and suspensors (Fig. 1*e*). We have tried to establish stable embryogenic suspension cultures; however, it has proven to be difficult for several reasons. The suspensor of *A. fraseri* is very long, which causes the cultures to form large clumps and so not scatter from each other in the liquid medium (Fig. 1*e*). Furthermore, the suspensors are fragile and sensitive to the osmotic stress and/or the shear stress experienced in a shaker flask. The results have often been a gradual loss of the suspensor cells resulting in a slowly growing, completely non-embryogenic suspension culture consisting of small clumps of isodiametric cells (NØrgaard and Krogstrup 1995).

Effect of CH and L-glutamine on ESM proliferation. The effect of CH and L-glutamine concentration on ESM proliferation is shown in Table 3. The highest proliferation rate of ESM (1.16-fold) was obtained with the combination of 3.42 mM L-glutamine and 500 mg l^{-1} CH (Table 3). In addition, ESM cultured on medium with 50 mg l^{-1} CH alone had a similar proliferation rate (1.14-fold) to that of the combination of 3.42 mM L-glutamine and 500 mg l^{-1} CH (Table 3). However, a lower proliferation rate was obtained on the medium with 3.42 mM L-glutamine and 1,000 mg l^{-1} CH (0.17-fold). The beneficial effect of L-glutamine on somatic embryogenesis has been reported elsewhere. Hristoforoglu et al. (1995) reported that ESM lines of *A. alba* proliferated faster and matured better on medium containing L-glutamine and CH than on medium without them. Besides the effect of L-glutamine and CH on proliferation of ESM, there are some reports about the positive effect of L-glutamine or CH inclusion in the SE maturation process. According to NØrgaard and Krogstrup (1995), L-glutamine up to 1,000 mg l^{-1} stimulates the formation of stage 2 embryos, whereas only 10,000 mg l^{-1} improved the number of stage 3 embryos compared to the control. On the other hand, CH had a negative effect on the number of stage 2 embryos, and no significant improvement on the number of stage 3 embryo achieved in *A. nordmanniana*. Therefore, the outcome responses with L-glutamine were regarded to be highly beneficial on ESM proliferation or SE in *Abies* species. *Picea mariana* somatic embryos matured in medium with L-glutamine as the sole source of nitrogen (Khlifi and Tremblay 1995). It has been

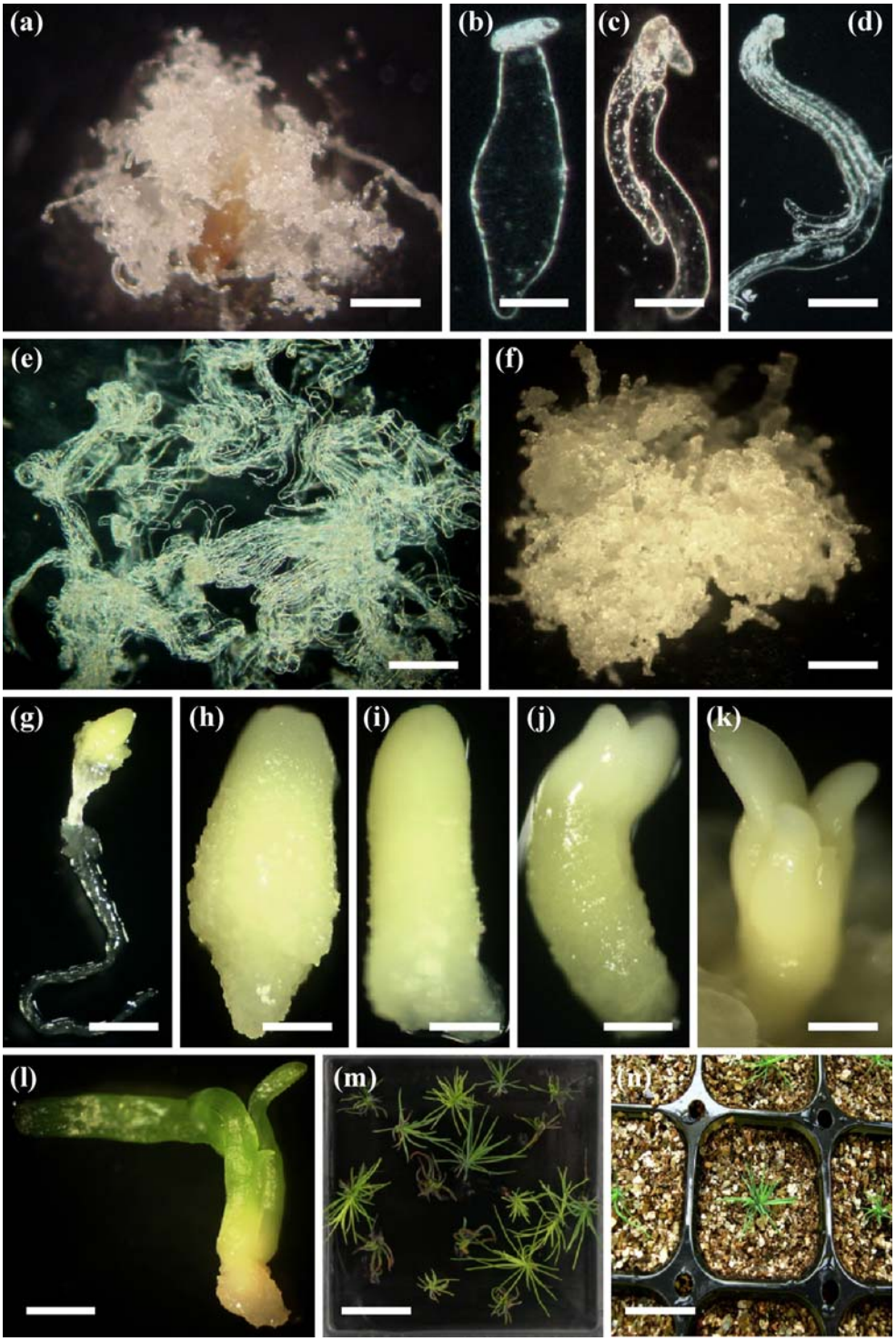

Figure 1. ESM initiation, somatic embryo maturation, and plant regeneration in *A. fraseri*. (*a*) Mucilaginous ESM extruded from micropyle end of megagametophyte after 6-wk culture (*bar*, 2 mm). (*b*) An early stage somatic embryo with embryonic head and long suspensor (*bar*, 0.8 mm). (*c*) More differentiated early stage somatic embryo having two long suspensors divided from original one (*bar*, 0.9 mm). (*d*) A whole single proembryo with several long suspensors (*bar*, 1.3 mm). (*e*) ESM dissociated in liquid showed some proembryos entangled with each other (*bar*, 1.5 mm). (*f*) Well-maintained ESM grown on SH medium containing 4.5 μM BA (*bar*, 2.5 cm). (*g*) A globular stage of somatic embryos maturing on SH medium with 80 μM ABA (*bar*, 0.8 mm). (*h*) A more matured somatic embryo showing dense head and radicle end (*bar*, 0.6 mm). (*i*) A somatic embryo with long elongated hypocotyls (*bar*, 2.5 cm). (*j*) A precotyledon stage somatic embryos differentiated (*bar*, 2.1 cm). (*k*) A typical cotyledon-staged somatic embryo with distinct cotyledons (*bar*, 1.6 cm). (*l*) A somatic germinant produced on germination medium without ABA (*bar*, 1.6 cm). (*m*) More developed germinants after 5 wk of culture (*bar*, 2.7 cm). (*n*) Regeneration of somatic seedlings transplanted into soil (*bar*, 13.2 cm).

suggested that the positive effect of organic nitrogen, in comparison to that of inorganic nitrogen, is due to organic nitrogen being metabolized at a lower energy cost than inorganic nitrogen (NØrgaard and Krogstrup 1991). However, von Arnold (1987) found a strong negative effect of L-glutamine, L-arginine, and L-asparagine on induction of somatic embryogenesis in *P. abies*. Kirby et al. (1987) reported that the addition of CH inhibited growth in cell suspension of *Picea menziesii*.

Effect of ABA on somatic embryo maturation. The effect of both ABA on somatic embryo maturation is shown in Table 4. Maturation of somatic embryos was carried out with one ESM line (derived from clone 16-273) on SH

Table 3 Effect of various L-glutamine or CH concentrations on proliferation of ESM in *A. fraseri*

Treatment	Proliferation rate of ESM (fold increase)
50 mg/L CH[a]	1.14±0.07 ab[b]
500 mg/L CH+3.42 mM L-glutamine	1.16±0.18 a
1,000 mg/L CH+3.42 mM L-glutamine	0.17±0.01 c

[a] Casein hydrolysate
[b] Different *letters* within *columns* indicate significant differences at P=0.05

medium supplemented with 10% PEG 4000, 4% maltose, 3.42 mM L-glutamine, 500 mg l^{-1} CH, and various concentrations of ABA (Table 4). The greatest mean number of precotyledonary or cotyledonary somatic embryos was obtained on the medium containing 20 (100.1/g^{-1} FW ESM) or 80 μM (64.3/g^{-1} FW ESM) ABA, respectively (Table 4). On the other hand, the poor production of embryos was observed on the medium containing 10 μM ABA (17.5/g^{-1} FW ESM) for cotyledonary embryo production. However, the mean number of precotyledonary embryos produced showed nearly the same value (48.8–57.9/g^{-1} FW ESM) for precotyledonary embryo production on all concentrations of ABA except 20 μM ABA (100.1/g^{-1} FW ESM). Maturation of somatic embryos of Fraser fir was not observed on medium lacking ABA for both precotyledonary and cotyledonary embryos. Therefore, addition of ABA was a necessary step for embryo maturation in conifer species including Fraser fir. There were no clear optima with respect to ABA concentration in this experiment. Several scientists have established optimal ABA concentrations for the maturation of somatic embryos in *A. nordmanniana* (NØrgaard 1997) at 40 μM ABA and 80 μM ABA in *A. cilicica* × *A. nordmanniana* (Vooková and Kormuták 2003). According to Guevin and Kirby (1997), after 8 wk of culture on medium containing 5–80 μM ABA, cotyledonary stage somatic embryos developed from ESM, and maximum number of cotyledon-stage embryos was observed at 80 μM ABA in *A. fraseri*. Considering our result, the 80 μM ABA was most effective in producing cotyledonary stage embryos in *A. fraseri*. Therefore, the optimal ABA concentration was highly variable depending on species or ESM lines even in the same species. Besides the effect of ABA on the maturation process, osmotica such as PEG (Vooková and Kormuták 2001), lactose (Schuller et al. 2000), etc., are well known as critical factors for stimulating maturation of somatic embryos in *Abies* species. However, interestingly, Gebhardt (1992) reported maturation on medium containing both BA and auxin, but no ABA. Application of ABA inhibited cleavage polyembryony (Durzan and Gupta 1987) and synchronized SE maturation (Krogstrup et al. 1988). Higher ABA concentrations led to faster SE maturation in *Picea rubens* (Harry and Thorpe 1991) and in *Picea glauca engelmannii* (Roberts 1991). ABA concentrations of 30–40 μM promoted the development of cotyledonary embryos. In our experiment, higher ABA concentrations above 20 or 80 μM favored the formation of SE up to the for precotyledonary or cotyledonary embryo production, respectively.

Table 4 Effect of various concentrations of ABA on maturation of somatic embryos in *A. fraseri*

ABA (μM)	Precotyledonary embryos (no./g^{-1} FW ESM)	Cotyledonary embryos (no./g^{-1} FW ESM)
0	0	0
5	48.8±26.7 d [a]	24.2±10.7 ef
10	57.9±5.9 b	17.5±5.3 f
20	100.1±2.7 a	46.2±13.6 c
40	55.8±4.4 b	33.4±2.0 de
60	52.3±5.6 bc	31.3±2.8 de
80	55.4±2.8 b	64.3±9.2 a
100	48.9±7.9 d	56.5±28.2 b
120	52.0±6.4 bc	39.6±4.7 d

[a] Different *letters* within *columns* indicate significant differences at P=0.05

Many somatic embryos had abnormally short cotyledons and hypocotyls. Cotyledonary-stage embryos with elongated hypocotyls and cotyledons were developed only at an ABA concentration greater than 40 μM (Fig. 1*l*, *m*). A low percentage (less than 5%) of somatic germinants survived following transplanting to soil (Fig. 1*n*); however, most of them did not show further growth.

In conclusion, development of clonal propagation techniques such as SE has the potential to dramatically enhance the genetic quality of Fraser fir Christmas tree plantations. Not only does this system offer the capability to produce unlimited numbers of high-valued plants but it also enables the use of genetic transformation to improve traits such as disease and insect resistance. The present work demonstrates that induction of SE in Fraser fir and regeneration of plants is feasible. Further work will be focused on enhancing the efficiency of ESM initiation, embryo maturation, and plant regeneration from proliferating ESM of Fraser fir.

Acknowledgments This work was supported by the Michigan Agricultural Experimentation Station GREEEN Project grant to K.-H.H. (grant no. GR03-015). The authors are grateful to the North Carolina Division of Forest Resources for providing the immature seeds used for this investigation. We also gratefully acknowledge the efforts of Avery County Extension Agent, Jerry Moody, in making the weekly cone collections.

References

Durzan D. J.; Gupta P. K. Somatic embryogenesis in Duglas-fir cell suspension cultures. *Plant Sci.* 52: 229–235; 1987. doi:10.1016/0168-9452(87)90056-2.

Find J.; Grace L.; Krogstrup P. Effect of anti-auxins on maturation of embryogenic tissue cultures of Nordmanns fir (*Abies nordmanniana*). *Physiol. Plant.* 116: 231–237; 2002. doi:10.1034/j.1399-3054.2002.1160213.x.

Find J. I.; Floto F.; Krogstrup P.; Dahl Mollern J.; NØrgaard J. V.; Kristensen M. M. H. Cryopreservation of an embryogenic suspension culture of *Picea sitchensis* and subsequent plant regeneration. *Scand. J. For. Res.* 8: 156–162; 1993.

Hakman I.; Fowke L. C.; von Arnold S.; Eriksson T. The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce). *Plant Sci.* 38: 53–59; 1985. doi:10.1016/0168-9452(85)90079-2.

Harry I. S.; Thorpe T. A. Somatic embryogenesis and plant regeneration from mature zygotic embryos of red spruce. *Bot. Gaz.* 152: 446–452; 1991. doi:10.1086/337905.

Hineslet L. E.; Blazich F. A. Vegetative propagation of *Abies fraseri* by stem cuttings. *HortSci.* 15: 96–97; 1985.

Hristoforoglu, K.; Grahsl, A.; Schmidt, J. Somatic embryogenesis from mature embryos of *Abies alba.* Poster at Conifer Biotechnology Working Group Meeting, Raleigh, North Carolina, April 23–28; 1992.

Hristoforoglu K.; Schmidt J.; Bolhar-Nordenkampf H. Development and germination of *Abies alba* somatic embryos. *Plant Cell Tissue Organ Cult.* 40: 277–284; 1995. doi:10.1007/BF00048134.

Gebhardt, K. Somatic embryogenesis in silver fir (*Abies alba*). Poster at Conifer Biotechnology Working Group 6th Meeting, North Carolina, April 23–28; 1992.

Guevin T. G.; Kirby E. G. Induction of embryogenesis in cultured mature zygotic embryos of *Abies fraseri* (Pursh) Poir. *Plant Cell Tissue Organ Cult.* 49: 219–222; 1997. doi:10.1023/A:1005747026269.

Guevin T. G.; Micah V.; Kirby E. G. Somatic embryogenesis in cultured mature zygotic embryos of *Abies balsamea*. *Plant Cell Tissue Organ Cult.* 37: 205–208; 1994. doi:10.1007/BF00043618.

Kirby E. G.; Leustek T.; Lee M. S. Nitrogen nutrition. In: Bonga J. M.; Durzan D. J. (eds) General principles and biotechnology. Cell and tissue culture in forestry, vol. 1. Nijhoff, Dordrecht, pp 67–88; 1987.

Khlifi S.; Tremblay F. M. Maturation of black spruce somatic embryos. I. Effect of L-glutamine on the number and germinability of somatic embryos. *Plant Cell Tissue Organ Cult.* 41: 23–32; 1995. doi:10.1007/BF00124083.

Krogstrup P.; Erikson E. N.; Moller J. D.; Roulund H. Somatic embryogenesis in Sitka spruce (*Picea sitchensis* (Bong.) Carr.). *Plant Cell Rep.* 7: 594–597; 1988. doi:10.1007/BF00272766.

Murashige T.; Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473–497; 1962. doi:10.1111/j.1399-3054.1962.tb08052.x.

NØrgaard J. V. Somatic embryo maturation and plant regeneration in *Abies nordmanniana* LK. *Plant Sci.* 124: 211–221; 1997. doi:10.1016/S0168-9452(97)04614-1.

NØrgaard, J. V.; Baldursson, S.; Krogstrup, P. Somatic embryogenesis in *Abies nordmanniana*. Induction and maturation of somatic embryos. Poster at Conifer Biotechnology Working Group 6th Meeting, North Carolina, April 23–28; 1992.

NØrgaard J. V.; Krogstrup P. Cytokinin induced somatic embryogenesis from immature embryos of *Abies nordmanniana* LK. *Plant Cell Rep.* 9: 509–513; 1991.

NØrgaard, J. V.; Krogstrup, P. Somatic embryogenesis in *Abies* spp. In: Jain S.; Gupta P.; Newton R. (eds) Somatic embryogenesis in woody plants, vol. 3. Kluwer, Dordrecht, Netherlands, pp 341–355; 1995.

Park Y. S. Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. *Ann. For. Sci.* 59: 651–656; 2002. doi:10.1051/forest:2002051.

Rajbhandari N.; Stomp A. Embryogenic callus induction in Fraser fir. *HortSci.* 32: 737–738; 1997.

Ramarosandratana A.; Harvengt L.; Bouvet A.; Calvaryrac R.; Paques M. Effects of carbohydrate source, polyethylene glycol, and gellan gum concentration of embryonal-suspensor mass (ESM) proliferation and maturation of maritime pine somatic embryos. *In Vitro Cell. Dev. Biol., Plant* 37: 29–34; 2001. doi:10.1007/s11627-001-0006-1.

Roberts D. R. Abscisic acid and mannitol promote early development, maturation and storage protein accumulation in somatic embryos of interior spruce. *Physiol. Plant.* 83: 247–254; 1991. doi:10.1111/j.1399-3054.1991.tb02149.x.

Salaj, T.; Salaj, J. Somatic embryo formation on mature *Abies alba* × *Abies cephalonica* zygotic embryo explants. *Biol. Plant.* 47: 7–11; 2003/4.

Salajova T.; Jasik J.; Kormuták A.; Salaj J.; Hakman I. Embryogenic culture initiation and somatic embryo development in hybrid firs (*Abies alba* × *Abies cephalonica*, and *Abies alba* × *Abies numidica*). *Plant Cell Rep.* 15: 527–530; 1996.

Schenk R. V.; Hildebrandt A. C. Medium and techniques for induction and growth of monocotyledonous plant cell cultures. *Can. J. Bot.* 50: 199–204; 1972. doi:10.1139/b72-026.

Schuller A.; Kirchner-NEβ R.; Reuther G. Interaction of plant growth regulators and organic C and N components in the formation and maturation of *Abies alba* somatic embryos. *Plant Cell Tissue Organ Cult.* 60: 23–31; 2000. doi:10.1023/A:1006429428170.

von Arnold S. Improved efficiency of somatic embryogenesis in mature embryos of *Picea abies* (L.) Karst. *J. Plant Physiol.* 128: 233–244; 1987.

Vooková B.; Kormuták A. Effect of sucrose concentration, charcoal, and indole-3-butyric acid on germination of *Abies numidica* somatic embryos. *Biol. Plant.* 44: 181–184; 2001. doi:10.1023/A:1010278704613.

Vooková B.; Kormuták A. Plantlet generation in *Abies cilicica* Carr. and *Abies cilicica* × *Abies nordmanniana* hybrid via somatic embryogenesis. *Turk. J. Bot.* 27: 71–76; 2003.

Vooková B.; Matusova R.; Kormuták A. Secondary somatic embryogenesis in *Abies numidica*. *Biol. Plant.* 46: 513–517; 2003. doi:10.1023/A:1024899124774.