

Enhancement of somatic embryogenesis and plant regeneration in Japanese red pine (*Pinus densiflora*)

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Received: 22 January 2014 / Accepted: 16 March 2014 / Published online: 12 April 2014
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Abstract The highest proliferation rate (9.8-fold) of embryogenic suspensor mass (ESM) was obtained from half-strength Litvay ($\frac{1}{2}$ LM, Litvay et al. 1985) medium supplemented with 3.42 mM L-glutamine, while the lowest rate was noted when 0.84 mM L-glutamine (0.6-fold) was added to the medium. The highest growth ratio with brassinolide (BL) was observed for 1.0 μ M (2.3-fold, line 05-21) and 0.05 μ M (2.9-fold, line 06-22). However, in the ESM lines 05-21 and 06-22, high ESM growth rates (2.3-fold, line 05-21 and 2.1-fold, line 06-3) were seen without BL when compared with 1.0 μ M (05-21) or 0.05 μ M (06-22). BL-supplemented medium has been shown to have diverse, genotype-specific effects on the degree of ESM proliferation. For somatic embryo maturation with 0.05 % activated charcoal (AC), the highest number (798 g^{-1} FW) of cotyledonary somatic embryos (line 06-29) were obtained on a maturation medium supplemented with AC. The influence of light-emitting diode (LED) sources on the germination of somatic embryos (four genotypes) in this species was studied and was strongly genotype-dependent. Germination of somatic embryos from ESM line 05-3 was strongly inhibited by fluorescent, and red + blue light, while lines 05-12, 05-29, and 05-37 showed a similar germination frequency for the five LED sources, where red light most stimulated somatic embryo germination.

Keywords ESM proliferation · Germination · L-Glutamine · LED · Somatic embryo

Introduction

The Japanese red pine (*Pinus densiflora*) has a home range that includes Japan, Korea (north-eastern), and the extreme south-east of Russia. In Korea, it is widely cultivated both for timber production and as an ornamental tree, and plays an important part in landscaping. Recently, pine wilt disease, caused by the pinewood nematode *Bursaphelenchus xylophilus*, has attacked and spread to the entire country, and has become a serious problem in some pines (including this species). Therefore, a long-term pine breeding project was initiated to select resistant clones and propagate them in large numbers. In order to accelerate the propagation of these plants, more efficient propagation methods were needed. Among them, somatic embryogenesis (SE) is the most promising technique for mass propagation of clones of the Japanese red pine.

L-Glutamine is a common organic nitrogen source used in plant tissue culture media and provides reduced nitrogen in a form that is energetically less costly to assimilate than nitrate or ammonium (Leustek and Kirby 1988). In conifers, cell suspensions of *Pseudotsuga menziesii* grew rapidly in medium with glutamine as the only source (Lee and Kirby 1986), and the beneficial effect of L-glutamine on SE has been reported elsewhere (Kim and Moon 2007; Nørgaard and Krogstrup 1995). Hristoforoglu et al. (1995) reported embryogenic suspensor mass (ESM) lines of *Abies alba* proliferated faster and matured better on media containing L-glutamine and casein hydrolysate than on media without them. *Picea mariana* somatic embryos matured in media with glutamine as the sole source of nitrogen (Khlifi and Tremblay 1995).

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Brassinolide (BL), the most bioactive form of the brassinosteroids, was first isolated as a steroidal plant hormone from rape pollen (*Brassica napus* L.) (Mitchell et al. 1970), and is now regarded as a plant growth regulator (Stokes 2000). In various bioassays, BL has been known to be 10–1,000-fold more active than (or synergistic with) auxins such as indole-3-acetic acid or NAA (Brosa 1999). The addition of BL to the medium increases callus growth and regeneration in *Spartina patens* (Lu et al. 2003) or stimulates microspore embryogenesis in *Brassica* species (Ferrie et al. 2005). In conifers, Pullman et al. (2003) report that BL increased embryogenic tissue initiation in *Pinus taeda*, *Pseudotsuga menziesii*, and *Picea abies*.

Activated charcoal (AC) is known for its adsorption of residual plant growth regulators (von Aderkas et al. 2002). As ESM had been cultured on a medium with 2,4-D and BA prior to transfer onto maturation medium, the beneficial effect of coating the cells with AC particles might be attributed to this particular property. Previous studies have shown the potential influence of AC on the elemental composition of liquid medium for conifer ESM initiation (Van Winkle et al. 2003; Van Winkle and Pullman 2003, 2005) or somatic embryo development (Pullman et al. 2005; Lelu et al. 2006).

Light conditions play an important role in plant cell and tissue cultures, and cool-white fluorescent lamps are the primary light source for plant tissue cultures, cited by 90 % of the research literature. The use of light-emitting diodes (LEDs) has attracted considerable interest in recent years because of the vast potential for commercial applications. Light quality has been shown to influence callus growth, shoot regeneration, and rooting (Economou and Read 1987). Red light stimulates shoot elongation of geraniums (Appelgren 1991) and rooting of *Prunus* (Rossi et al. 1993), while blue light promotes rooting and acclimatization of birch (Saeba et al. 1995). However, Latkowska et al. (2000) revealed that the effect of light quality on the growth of embryogenic tissue in Norway spruce was strongly genotype-dependent.

There have been a few reports on SE in Japanese red pine (Maruyama et al. 2005; Shoji et al. 2006); however, those experiments were focused on developing brief SE protocols for this species. Additionally, the studies were unable to provide critical data that may influence the successful mass production of somatic embryos and enhance germination frequency from somatic embryos. Therefore, the main objective of the present study was to describe optimal factors that would support SE in *P. densiflora*. This study reports the effects of L-glutamine or BL concentration on ESM proliferation, AC treatment on somatic embryo production, and finally the effects of LED on somatic embryo germination in this species.

Materials and methods

Plant material

Open-pollinated, immature seeds of 10-year-old *P. densiflora* were collected from twelve trees grown in the experimental garden of the Korea Forest Research Institute (Suwon, Gyeonggi province, Korea) between June 21 and July 5. Seeds extracted from cones were disinfected by immersion in 70 % (w/v) ethanol for 2 min and aqueous 2 % NaClO (w/v) for 10 min, followed by rinsing 5 times with sterile distilled water.

Initiation of ESM and culture medium formulation

Entire megagametophytes (without seed coat) were cultured on a P6 (Teasdale et al. 1986) medium which contained full-strength macrosalts, microsals, vitamins, 10.3 mM L-glutamine (Sigma), 87.6 mM sucrose, μM 2,4-D, and 4.4 μM BA; and solidified with 0.2 % (w/v) gellan gum (PhytigelTM, Sigma). The pH of the medium was adjusted to 5.7 prior to autoclaving at 121 °C for 15 min. L-Glutamine was sterilized by filtration, then added to the partially cooled medium (45–50 °C) after autoclaving. For the initiation of ESM, seed coat and nucellus tissue were removed aseptically without damage, and intact megagametophytes containing zygotic embryos were placed directly on the ESM initiation medium. The cultures were kept in darkness at 24 ± 1 °C for 8 weeks without subculture.

Effect of L-glutamine or brassinolide concentration on ESM proliferation

The effect of L-glutamine (0, 1.71, 3.42, 6.84, and 13.68 mM) on ESM proliferation was investigated using $\frac{1}{2}$ LM (Litvay et al. 1985, Duchefa) medium supplemented with 9.0 μM 2,4-D, 4.4 μM BA, and 58.4 mM sucrose. and solidified with 0.4 % gellan gum. In addition, the effect of BL on ESM proliferation was studied. BL was obtained from Sigma (E1641) and dissolved in dimethyl sulfoxide (DMSO, Duchefa) to make 0.05, 0.1, 0.5, and 1.0 μM stock solutions which were stored at -20 °C until needed.

ESM proliferation was performed using $\frac{1}{2}$ LM medium supplemented with 9.0 μM 2,4-D, 4.4 μM BA, and 58.4 mM sucrose, and solidified with 0.4 % gellan gum in the experiments testing the effects of L-glutamine or BL. The ESM was placed on each piece of filter paper (90 mg per each) and then placed onto the proliferation medium containing different concentrations of L-glutamine or BL. The cultures were maintained at 24 ± 1 °C in dark for 4 weeks without subculturing. The weights of each ESM for the treatments were recorded and the growth rate

calculated on the basis of fresh weight (FW) according to: growth rate (fold) = [(FW at the end of treatment – FW at the start of treatment)/FW at the start of treatment]. For each test, there were three replicates with at least three Petri dishes for each treatment.

Effect of AC on somatic embryo maturation

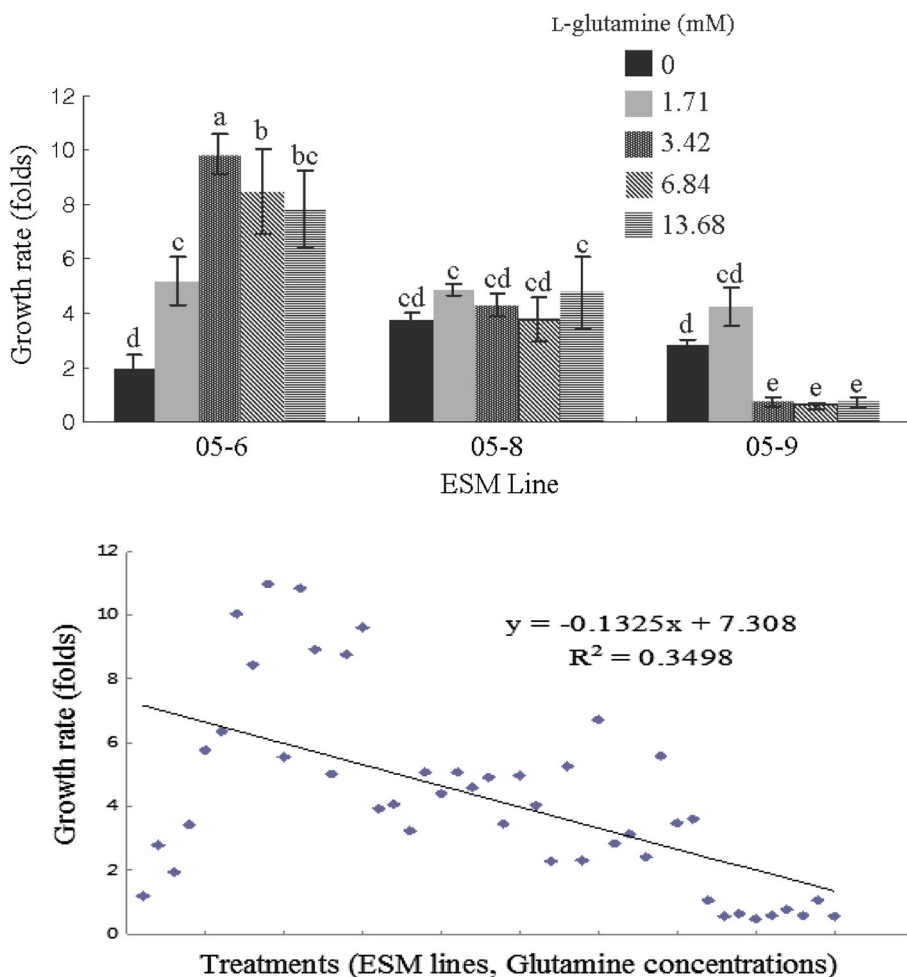
The goal of the somatic embryo maturation experiment was to assess the effects of the presence or absence of AC at 0.05 % (Sigma) in the maturation medium. For maturation of somatic embryos, 250 μM (±)-ABA (Sigma), 1.2 % gellan gum, and 0.05 % AC were added to ½LM medium supplemented with 0.2 M maltose (Duchefa) and 6.8 mM L-glutamine. ABA solution was filter-sterilized (0.22 μm, Millipore) and added to the cooling medium after autoclaving. The plating technique for maturation of somatic embryos was previously described by Kim et al. (2007). Briefly, the ESM were weighed and dispersed in liquid ½LM medium without growth regulators. After the ESM suspensions were homogenized, 3 ml of the liquid medium containing 90 mg fresh weight (30 mg/ml) of dispersed

tissue was poured over filter paper (Whatman #2, 5.5 cm) and placed in a Büchner funnel. After draining the medium with a low pressure pulse vacuum, the filter paper with the ESM was placed on the maturation medium and cultured in darkness for 12 weeks (without subculturing onto fresh medium). After 12 weeks in culture, the numbers of somatic embryos induced were counted under a stereomicroscope. For each test, there were three replicates, each consisting of five Petri dishes for each treatment.

Effect of LED on germination from somatic embryo

To examine the effects of LED light sources on germination, somatic embryos were cultured using an LED system (GF-320, Good Feeling, Sungnam, South Korea). The temperature was 24 ± 2 °C, and the photoperiod was adjusted to 16/8 h photoperiod. Somatic embryos were germinated under fluorescent light (FL, control group) (50 μEm⁻² s⁻¹, LUMILUX, 40W, OSRAM) and four types of LED treatments: 100 % red LED (peak wavelength 660 nm), 100 % blue LED (peak wavelength 450 nm), 50 % red + 50 % blue, and 50 % red + 50 %

Fig. 1 Effect of L-glutamine concentrations on ESM weights from 3 genotypes of *P. densiflora* (upper graph). Error bars standard error the mean. Different letters within columns indicate significant differences at *P* = 0.05. Relationship between the treatments (ESM lines, glutamine concentrations) and the growth rate of ESM (down graph). The treatments were plotted against the growth rate of ESM

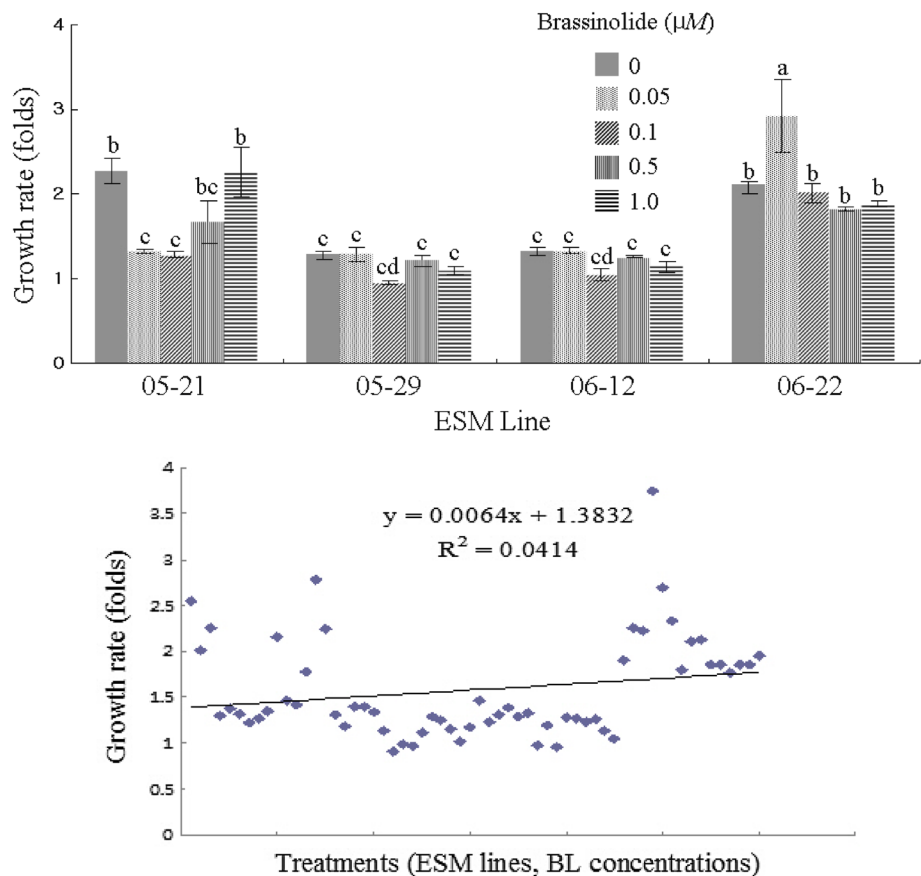


far red (peak wavelength 730 nm). For each test, there were three replicates, each consisting of 30 somatic embryos derived from four ESM lines for each treatment.

Plantlet regeneration and acclimatization

Cotyledonary somatic embryos were selected from embryogenic masses cultured on ABA-containing medium for 12 weeks and placed on the surface of $\frac{1}{2}$ LM medium containing 60 mM sucrose and 0.4 % gellan gum. The cultures were kept for 7 d under dim lighting ($1.5 \mu\text{Em}^{-2} \text{s}^{-1}$) with a 16/8 h photoperiod, and at a temperature of $24 \pm 1 \text{ }^\circ\text{C}$; they were then transferred to higher light ($50 \mu\text{Em}^{-2} \text{s}^{-1}$). After 8 weeks of germination treatment, the plantlets with well developed epicotyls (at least 20 mm) and roots were transplanted into an artificial soil mixture (perlite/peat moss/vermiculite 1:1:1) in trays with a transparent lid and watered once a day. After an acclimation period of 4–6 weeks, the lid was gradually opened to reduce humidity in the tray and removed when new shoot growth started. Acclimated plants were maintained for 4–5 weeks in the tissue culture room ($50 \mu\text{Em}^{-2} \text{s}^{-1}$, 16/8 h photoperiod, $25 \pm 1 \text{ }^\circ\text{C}$) and then transferred to the greenhouse.

Fig. 2 Effect of BL concentrations on ESM weight from four genotypes of *P. densiflora* (upper graph). Error bars standard error of the mean. Different letters within columns indicate significant differences at $P = 0.05$. Relationship between the treatments (ESM lines, BL concentrations) and the growth rate of ESM (down graph). The treatments were plotted against the growth rate of ESM



Statistical analysis

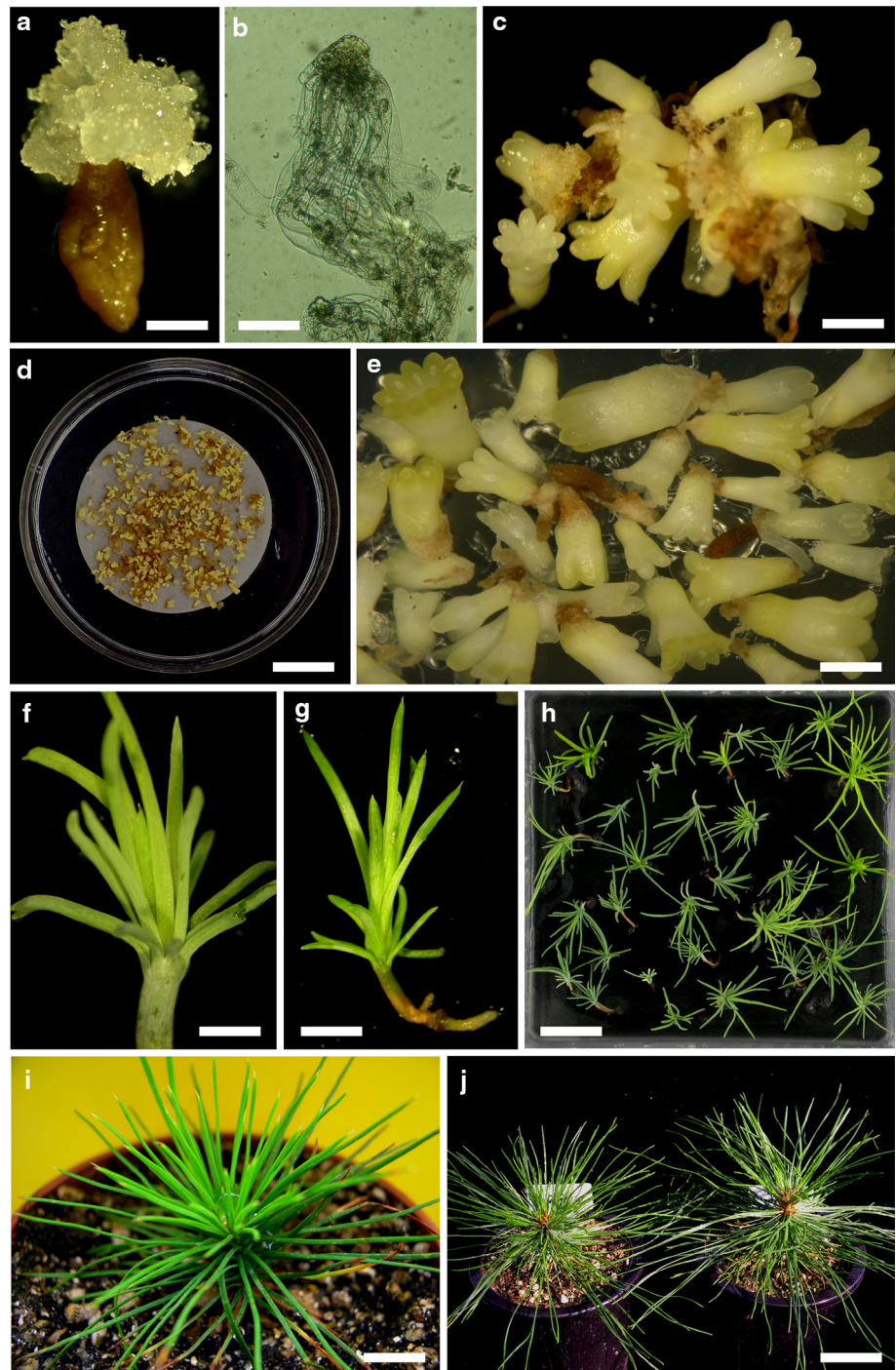
Data recorded during in vitro culture were analysed by ANOVA, and significant differences between means were tested by Duncan's multiple range test at $P = 0.05$.

Results and discussion

Effect of L-glutamine on ESM proliferation

The effects of L-glutamine concentrations on ESM proliferation are shown in Fig. 1 where the highest ESM proliferation rate was obtained with 3.42 mM L-glutamine (9.8-fold, line 05-6) (Fig. 1). However, a lower proliferation rate was obtained on the medium with 3.42 mM (0.7-fold), 6.84 mM (0.6-fold), and 13.48 mM (0.7-fold) L-glutamine in the 05-9 ESM line. In the treatments at the higher level (3.42, 6.84, or 13.68 mM), the ESM weight was observed to decrease (05-9), with the exception of lines 05-6 and 05-8 (Fig. 1). In the 05-8 line, no significant ESM weight increase or decrease was found for the five different concentrations. These data suggest the weight was greatly affected by the nitrogen source, which was also

Fig. 3 SE and plant regeneration in *P. densiflora*. **a** White-mucilaginous ESM extruded from the micropyle end of the megagametophyte after 8 weeks in culture (bar 2 mm). **b** Single proembryo with several long suspensors (bar 1.3 mm). **c** Somatic embryos maturing on $\frac{1}{2}$ LM medium with 1.0 % gellan gum, 0.2 M maltose, and 250 μ M ABA (bar 0.6 mm). **d** Somatic embryos on filter paper placed on maturation medium after 8 weeks in culture (bar 2.5 cm). **e** Collection of cotyledon-stage somatic embryos before germination treatment (bar 1.2 mm). **f** Newly produced epicotyl shoots from the germinant (bar 2 mm). **g** Germinant from the cotyledonary somatic embryo after 4 weeks in culture (bar 2.7 mm). **h** More developed germinants after 5 weeks in culture (bar 1.0 cm). **i** Acclimated somatic plant recovered from SE growing in the greenhouse (bar 1.0 cm). **j** Greenhouse-grown 8-month-old somatic plants during a spring flush of new growth (bar 3.5 cm)



dependent on the genotype of ESM (Fig. 1). ESM from *Abies alba* (Hristoforoglu et al. 1995) or *Cryptomeria japonica* (Ogita et al. 2001) proliferated faster on medium containing glutamine than on medium lacking it.

Effect of BL on ESM proliferation

Growth rates for ESM at various concentrations (0, 0.05, 0.1, 0.5 and 1.0 μ M) of BL are shown in Fig. 2. Based on

these results, the highest growth rate was observed for 0.05 μ M (2.9-fold, line 06-22). However, in the 05-21 line, the treatment without BL also had a high ESM growth rate (2.3-fold) (Fig. 2). Since the highest BL concentration (1.0 μ M) tested showed the greatest ESM growth in the 05-21 line, this suggested that BL concentrations >1.0 μ M should be tested (Fig. 2). Two ESM lines (05-29 and 06-12) responded poorly compared with the other two lines, regardless of BL concentrations

(Fig. 2). Therefore, these data showed that the growth of ESM lines was dependent on the original ESM genotypes, rather than the concentrations of BL. Though little research has been done with BL on conifer species, Pullman et al. (2003) reported that BL profoundly influences the weight increment of loblolly pine ESM by 66 %.

ESM initiation and proliferation

The mucilaginous cell mass protruded from the micropylar end of the megagametophyte within 6–8 weeks in culture (Fig. 3a). The translucent and mucilaginous ESM was composed of a few proembryos in an early stage of development (Fig. 3b). The ESM lines were proliferated on ½LM medium containing 2,4-D and BA. The transferred ESM proliferated rapidly on the medium and were sub-cultured weekly onto fresh medium.

Fig. 4 Effect of AC supplementation on ESM somatic embryo maturation for 15 genotypes of *P. densiflora*. Error bars standard error of the mean. Different letters within columns indicate significant differences at $P = 0.05$

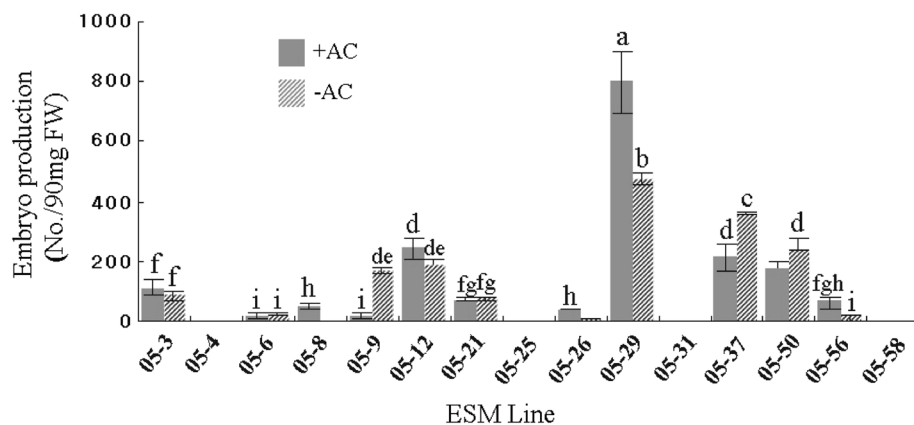
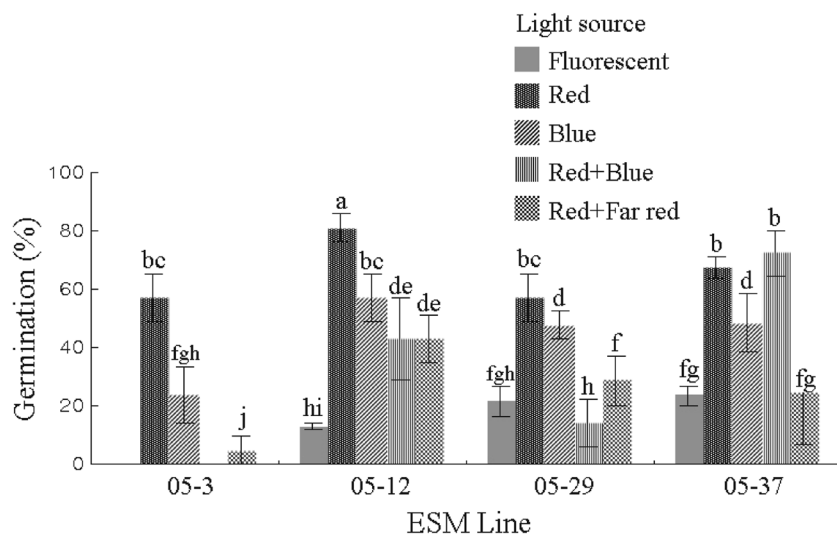


Fig. 5 Effect of light quality on ESM somatic embryo germination for four genotypes of *P. densiflora*. Error bars standard error of the mean. Different letters within columns indicate significant differences at $P = 0.05$



Effect of AC on somatic embryo maturation

As the ESM had been cultured for a long time on a medium containing 2,4-D and BA prior to transfer onto maturation medium, the beneficial effect of producing somatic embryos on the medium with AC was studied. Addition of AC to the medium is known to adsorb residual plant growth regulators (von Aderkas et al. 2002). Data showed somatic embryo yields ranged from 0 (05-4, 05-25, 05-31 and 05-58 with/without AC) to 798 (05-29 with AC) from plated ESM cells (Fig. 4). In general, maturation medium containing AC (05-9, 05-21, 05-37, and 05-50) did not produce as many somatic embryos as media without AC (Fig. 4), leading to the conclusion that the somatic embryo production was greatly dependent on the genotype of ESM in *P. densiflora* (Fig. 4). Pullman et al. (2005) reported that the addition of AC to the maturation medium resulted in increases for Norway spruce, maritime pine (Lelu et al. 2006), and loblolly pine (Pullman and Gupta 1991).

After the 6–8 weeks maturation, microscopic observations revealed somatic embryos with cotyledons on the AC-containing medium (Fig. 3c). After 2–3 additional weeks, a large number of fully developed somatic embryos were produced on the maturation medium with filter-paper (Fig. 3d, e).

Effect of LEDs on germination of somatic embryo

The various light sources strongly influenced the germination frequency of somatic embryos (Fig. 5). The highest frequency of germination was obtained with red light for lines 05-12 (80.9 %), 05-37 (67.5 %), and 05-3 or 05-29 (57.1 %), suggesting germination from somatic embryos was positively affected by red light. In contrast, fluorescent light resulted in lower frequencies (0, 12.9, 21.5, and 23.4 % for 05-3, 05-12, 05-29, and 05-37, respectively) regardless of the ESM lines tested in the experiments. For red + blue light, no germinants were obtained from the 05-3 line; however, 05-37 had a high frequency (72.2 %) for the same treatment (Fig. 5). All lines except 05-3 responded similarly to the different light sources (Fig. 5), suggesting significant interactions between ESM lines and light sources. The germination of *P. densiflora* somatic embryos was positively affected by application of red light (Fig. 5). Our results closely paralleled those reported by Merkle et al. (2005), which found that red wavelengths provided by LEDs resulted in higher plantlet conversion rates for southern pine somatic embryos than light from cool white fluorescent bulbs. In addition, Kvaalen and Appelgren (1999) reported that red wavelengths improved germination frequencies for Norway spruce (*Picea abies*) somatic embryos and enhanced hypocotyl and taproot length, which were strongly inhibited by blue light and less strongly by fluorescent and cool white light. Conversely, Tisserat et al. (2000) reported that germination of loblolly pine seeds was promoted by blue–green wavelengths, rather than by red light.

Germination of somatic embryos and plant regeneration

Two weeks after being transferred to the germination medium ($\frac{1}{2}$ LM containing 58.4 mM sucrose, solidified with 0.4 % gellan gum) without ABA, mature somatic embryos (produced from the ESM) started to induce epicotyl shoots (Fig. 3f). One week later, the cotyledons turned a deep green colour with elongated hypocotyls and roots. Upon transfer to fresh germination medium, plantlets with well-developed cotyledons, elongated hypocotyls, and roots developed (Fig. 3h), and by keeping them on the germination medium under light conditions, some new apical shoots were induced from the terminal bud (Fig. 3h). The somatic plants that developed were transplanted into a

soil mixture (Fig. 3i), where they grew well and could be transferred to larger pots and grown under greenhouse conditions (Fig. 3j).

In conclusion, as shown above, somatic plants were regenerated from somatic embryos in *P. densiflora*. However, there are some problems: low initiation rate of ESM, decrease or loss of ESM maturation ability after long subculture periods, and low maturation rate. To solve these problems and use the SE system in genetic transformation, improvement of the protocol is needed. Screening families in search of high potential ESM lines for embryogenesis is also important.

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