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Note

Plant regeneration from embryogenic tissue of *Pinus luchuensis* Mayr, an endemic species in Ryukyu Island, Japan

Yoshihisa Hosoi*, Tsuyoshi E. Maruyama

Department of Molecular and Cell Biology, Forestry and Forest Products Research Institute, Tsukuba, Ibaraki 305-8687, Japan
*E-mail: yh2884@ffpri.affrc.go.jp Tel: +81-29-829-8266 Fax: +81-29-873-1542

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Abstract Somatic embryogenesis in Ryukyumatsu (*Pinus luchuensis* Mayr.), an endemic species in Ryukyu Island, Japan, was initiated from megagametophytes containing zygotic embryos on a medium supplemented with 10 μM 2,4-dichlorophenoxyacetic acid and 5 μM 6-benzylaminopurine. Embryogenic cultures were maintained and proliferated by subcultures at 2- to 3-week-intervals on the same fresh medium. The maturation of somatic embryos occurred on media containing maltose, activated charcoal, abscisic acid and polyethylene glycol. High frequencies of germination were obtained after the post-maturation treatment of somatic embryos under conditions of high relative humidity, and around 90% of them were converted into plants. Growth of the somatic embryo-derived regenerated plants has been monitored in the field.

Key words: Micropropagation, *Pinus luchuensis*, Ryukyu pine, somatic embryogenesis, somatic embryo-derived plants

Ryukyumatsu (*Pinus luchuensis* Mayr.), a two-needle pine species endemic to the Ryukyu Islands, is the Japanese representative of a group of three closely related taxa, including *P. taiwanensis* of Taiwan and *P. hwangshanensis* of mainland China. Ryukyumatsu is also one of the most economically important trees in Okinawa Prefecture and is valued for the construction of homes and furniture, the production of decorative veneer, and for landscaping. However, in recent years, Ryukyumatsu populations have further declined as a result of pine wilt disease, caused by the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle, which is primarily transmitted by the Japanese pine sawyer, *Monochamus alternatus* Hope (Akiba and Nakamura 2005). The pinewood nematode and pine sawyer are among the most serious pests in Japan, while pine wilt disease has been a key critical factor in the mass mortality, not only of the Ryukyumatsu populations but also in other important Japanese pine forests consisting of *P. densiflora*, *P. thunberghii* or *P. armandii* var. *amamiana*.

The application of biotechnology techniques is one of the alternatives for propagation, *ex situ* conservation *in vitro*, and for the development of transgenic Ryukyumatsu for resistance to pine wilt disease. The development of an efficient and stable plant regeneration system is essential for genetic engineering of any given species. Somatic embryogenesis through tissue and cell culture is the most attractive plant regeneration system for the purposes of genetic transformation. However, for many species, the low efficiency on plant conversion from somatic embryos has been one of the factors hindering widespread utilization. In this report, we describe the initiation of embryogenic cultures from seed explants, production of somatic embryos, and efficient regeneration of plants.

Open-pollinated cones were collected in August 2007 from a mother tree at the Iriomote Tropical Forest Tree Breeding Technical Garden (Taketomi, Okinawa, Japan) (Figure 1A). Collected cones (Figure 1B) were disinfected by 5 min immersion in 99.5% ethanol, and then dried in a laminar-flow cabinet before dissection. Excised seeds were disinfected with 2.5% (v/v available chlorine) sodium hypochlorite solution for 30 min, and then rinsed 5 times with sterile distilled water. After the seed coats had been removed, the megagametophytes (containing zygotic embryos at the cotyledonary developmental stage) were used as explants for the initiation of embryogenic cultures.

The explants were cultured in 4-compartment plates (100×15 mm) (Kord-Valmark Labware, Ontario, Canada) containing somatic embryogenesis induction medium. The induction medium was EM medium (Maruyama et al. 2000), modified as follows: basal salts, vitamins, and myo-inositol were reduced to half the standard concentrations; the concentrations of KCl was reduced to 40 mg l^{-1}; and 500 mg l^{-1} casein hydrolysate

Abbreviations: ABA, abscisic acid; AC, activated charcoal; BA, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid ; FW, fresh weight; PEG, polyethylene glycol.

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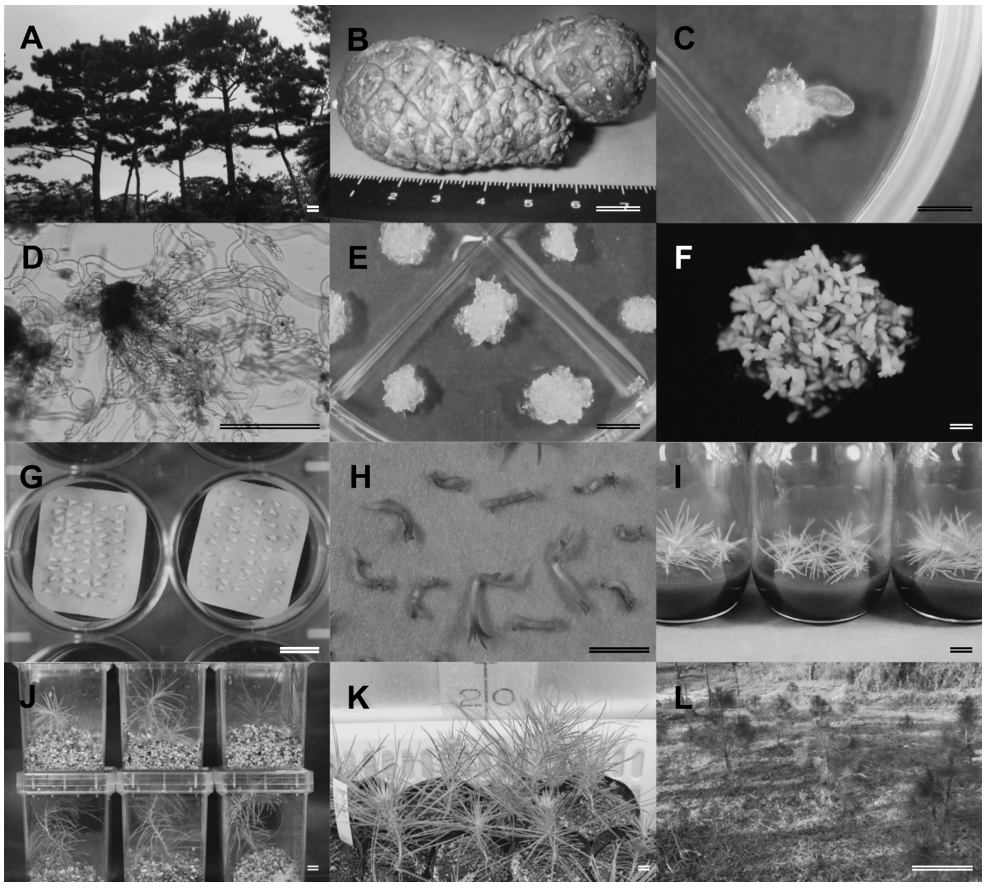

Figure 1. Somatic embryogenesis and plant regeneration in *Pinus luchuensis*. (A) Mother trees. (B) Collected cones. (C) Induced embryogenic tissue. (D) Embryogenic cell. (E) Proliferation of embryogenic tissue. (F) Mature somatic embryos. (G) Post-maturation treatment of somatic embryos. (H) Germination of somatic embryos. (I–J) Plantlets growing in vitro. (K) Acclimatized plants. (L) Somatic embryo-derived plants growing in the field. Bars: 1 m (A, L), 1 cm (B–C, E–K), 1 mm (D)

as well as 1 g l^{-1} L-glutamine, 10 g l^{-1} sucrose, 10 μM 2,4-D, and 5 μM BA were added. The medium was solidified with 3 g l^{-1} gellan gum (Gelrite®; Wako Pure Chemical, Osaka, Japan). The pH of the medium was adjusted to 5.8 prior to autoclaving, and the cultures were kept in darkness at 25°C. The presence or absence of somatic embryos at distinct early stages characterized by an embryo part (smaller and denser cells) with a suspensor (long vacuolated cells) was determined weekly under an inverted microscope up to 12 weeks. Induced embryogenic tissues were transferred onto the same fresh medium supplemented with 30 g l^{-1} sucrose, 3 μM 2,4-D, and 1 μM BA, but lacking casein hydrolysate. Once embryogenic cultures increased in mass, they were maintained and proliferated by subculturing 10–20 pieces of tissue per plate at 2- to 3-week-intervals and incubating under conditions equivalent to those for callus initiation.

Embryogenic tissues, about 2 weeks after subculture, were used for maturation experiments. Five pieces of embryogenic tissue (each about 200 mg in FW) were transferred to 90×20 mm monoplates containing 30–40 ml of semi-solid maturation medium. The maturation medium contained salts and vitamins from the original EM medium (Maruyama et al. 2000), 30 g l^{-1} maltose, 100 μM ABA, 0–200 g l^{-1} PEG 6000 (Av. Mol. Wt.: 7,300–9,300; Wako Pure Chemical, Osaka, Japan), 2 g l^{-1} AC (Wako Pure Chemical, Osaka, Japan), 3 g l^{-1} gellan gum, and amino acids (g l^{-1}: glutamine 0.75, asparagine 0.5, arginine 0.25, citrulline 0.04, ornithine 0.04, lysine 0.03, alanine 0.02 and proline 0.02). The pH of the medium was adjusted to 5.8 prior to autoclaving. Plates were sealed with Parafilm and kept in darkness at 25°C. Mature somatic embryos represent cotyledonary embryos with an elongated embryonic region and well-developed cotyledons, resembling zygotic embryos at the late cotyledonary stage.

Mature somatic embryos collected from the maturation medium after 8 weeks of culture were subjected to slow desiccation at high relative humidity prior to germination. Somatic embryos were placed on a rectangular filter paper (about 40×30 mm in size) equipped in 2 (central) wells of a 6-well multiplate (Iwaki, AGC Techno Glass Co., Ltd., Chiba, Japan) in which the remaining 4 (side) wells were filled with 5–6 ml of sterile water, sealed tightly with Parafilm,

and kept in darkness at 25°C for 3 weeks. Under these conditions, the generated relative humidity inside the plate was approximately 98%, which was registered with a thermo-hygrometer recorder (RS-10, ESPEC MIC Corp. Aichi, Japan). After post-maturation treatment, somatic embryos were transferred to 6 g l^{-1} gellan gum-solidified germination medium, which composition was equivalent to that for the maintenance and proliferation, except for the elimination of plant growth regulators and supplementation with 30 g l^{-1} glucose, 2 g l^{-1} AC, 0.4 g l^{-1} glutamine, 0.25 g l^{-1} arginine, and 0.1 g l^{-1} proline. Cultures were kept at 25°C under a photon flux density of about 65 μmol m^{-2} s^{-1} provided by cool, white fluorescent lamps (100 V, 40 W; Toshiba, Tokyo, Japan) for 16 h. The numbers of somatic embryos that germinated (root emergence) and grew into plantlets (development of epicotyls from germinated embryos) were recorded after 6 and 12 weeks, respectively.

The regenerated plantlets were transferred into 300-ml flasks containing 100 ml of fresh germination medium with no amino acids and containing 30 g l^{-1} sucrose or glucose, 5 g l^{-1} AC and 10 g l^{-1} agar (Wako Pure Chemical Industries, Osaka, Japan) or into Magenta® vessels (Sigma, St. Louis, USA) containing Florialite® (Nisshinbo Industries, Tokyo, Japan) irrigated with a plant food solution modified from Nagao (1983) (containing in mg l^{-1}: NH_4NO_3 143, $NaH_2PO_4 \cdot 2H_2O$ 55.1, KCl 47.1, $CaCl_2 \cdot 2H_2O$ 52.5, $MgSO_4 \cdot 7H_2O$ 61, Fe-III EDTA 25, Cu EDTA 0.1, Mn EDTA 0.1, Zinc EDTA 0.1, H_3BO_3 1.5, KI 0.01, $CoCl_2 \cdot 6H_2O$ 0.005, and MoO_3 0.005), and kept under conditions equivalent to those described above for about 20 weeks prior to *ex vitro* acclimatization. The developed plants were transplanted into plastic pots filled with Kanuma soil and acclimatized inside a growth chamber at 25°C and 80% relative humidity. During the first 2 weeks, the somatic embryo-derived plants were kept in plastic boxes with transparent covers. Subsequently, the covers were gradually opened during a further 2-week-period and removed completely about 1 month after transplanting. The plants were irrigated with tap water for the first 2 weeks and then with a 0.1% (v/v) Hyponex® 6-10-5 plant-food solution (Hyponex Japan Co., Ltd., Osaka, Japan).

The standard mean errors were calculated from five to thirty replications as described in each Table or Figure. Statistically significant differences between means were determined using Tukey's multiple comparison test at the 95% significance level.

The extrusion of embryogenic tissue from the micropylar end of explants occurred after about 3–4 weeks of culture. An average of 1% (2/192) of megagametophytes cultured extruded embryogenic tissues, which were then removed and transferred to a maintenance-proliferation medium. However, after several subcultures, only one could be proliferated and converted into stable embryogenic line (Figure 1C). Subsequently, embryonic masses containing cells characterized by dense cytoplasm cells (embryonic heads) and elongated cells (suspensor systems), as shown in Figure 1D, proliferated readily by subculturing at 2- to 3-week-intervals on a medium supplemented with 3 μM 2,4-D and 1 μM BA, retaining their original translucent and mucilaginous appearance (Figure 1E). The FW of tissue on the maintenance-proliferation medium increased 5- to 10-fold after a 2- to 3-week-culture period (data not shown).

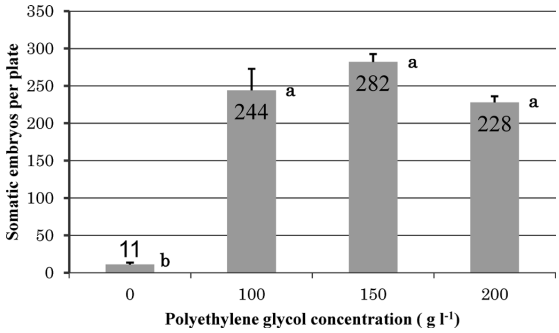

Figure 2. Effect of polyethylene glycol concentration on maturation of *Pinus luchuensis* somatic embryos. Values represent the mean of five plates for each concentration. Bars indicate the standard errors. Means followed by same letter are not significantly different at $p<0.05$.

Somatic embryo development and maturation were promoted on maturation medium supplemented with 30 g l^{-1} maltose, 100 μM ABA, 2 g l^{-1} AC and 100–200 g l^{-1} PEG. About 2 week after transfer onto maturation medium, embryogenic cells developed gradually to form an individual and compact mass (pro-embryo). Cotyledonary somatic embryos were first observed about 4 weeks after the transfer of the embryonic mass, and were clearly distinct after 6 weeks of culture (Figure 1F). The PEG in the medium stimulated the embryo maturation, and the number of mature embryos increased with increased PEG concentration up to 150 g l^{-1}, but decreased at 200 g l^{-1} (Figure 2). Although the PEG concentration from 100 to 200 g l^{-1} did not result in any statistical difference in terms of the somatic embryo yield per plate, the highest embryo maturation frequency was obtained on a medium supplemented with 150 g l^{-1}, with an average of 282 cotyledonary embryos collected per plate, in comparison with 244 and 228 embryos per plate at concentrations of 100 and 200 g l^{-1}, respectively (Figure 2). In the absence of PEG the embryogenic cell proliferation was prominent and only a few early somatic embryos developed into the cotyledonary stage (average of 11 embryos per plate).

Table 1 shows the germination and conversion frequencies after post-maturation treatment (Figure 1G) of somatic embryos that matured on media with different concentrations of PEG. Radicle emergence

was observed to commence 1-2 weeks after transfer to the germination medium (Figure 1H), and after 6 weeks of culture, the germination frequencies varied from 85 to 90%. Subsequently, epicotyls developed from germinants, and after 12 weeks of culture, the plantlet conversion frequencies ranged from 82 to 88%. Although the germination and conversion frequencies decreased slightly with increasing PEG concentration in maturation media, no morphological differences in germinants and plantlets were observed among PEG-derived treatments. Regenerated plants (Figure 1I) were cultured *in vitro* for about 20 weeks before *ex vitrum* acclimatization. The survival rate and the growth of plantlets after 15 weeks of culture are shown in Table 2. Survival rates of 100% in all treatments were achieved, and the optimal result in term of growth was obtained using Magenta® vessels containing Florialite® substrate irrigated with Nagao's plant food solution (Figure 1J). For growth on semi-solid media, at the concentration of sugar tested, the growth of plantlets improved when supplementing the germination medium with sucrose in lieu of glucose. Subsequently, developed plantlets were acclimatized successfully under the conditions described above (Figure 1K) (data not shown). The growth of somatic embryo-derived plants is currently being monitored in the field (Figure 1L).

Of the 192 megagametophytes containing zygotic embryos cultured, only one resulted in a stable embryogenic cell line (0.5%). This low initiation rate was similar to the results reported for other pine species such as *Pinus banksiana* (0.37%) (Park et al. 1999), *Pinus patula* (0.04%) (Jones and van Staden 1999), and *Pinus rigida*×*P. taeda* (0.44%) (Kim and Moon 2007). The low initiation frequency of somatic embryogenesis is one of the key problems to resolve for practical applications. Although the condition of the mother tree and the quality of seeds have been reported to affect the frequency of somatic embryogenesis in several species, it is important in establishing productive embryogenic cultures, since seed genotypes and culture procedures may impact on the initiation frequencies of somatic embryogenesis (Garin et al. 1998; Klimaszewska et al. 2001; Lelu et al. 1999; Miguel et al. 2004; Park et al. 2006).

Somatic embryo maturation was obtained by culturing embryogenic tissue on media supplemented with maltose, PEG, ABA, and AC. The supplement of PEG to the media was essential to obtain high maturation frequencies of somatic embryos as reported for other Japanese pines (Maruyama et al. 2005a, 2005b, 2007a). The stimulating effect of PEG on somatic embryo maturation in conifers has been extensively reported (Gupta and Pullman 1991; Jain et al. 1995; Li et al. 1997, 1998; Pullman et al. 2003; Maruyama et al. 2002, 2005c, 2007b), and may be related to water stress induction similar to that generated by desiccation and to an increase in the accumulation of storage reserves (Attree et al. 1992; Misra et al. 1993; Roberts et al. 1990a). In the present study, the best result obtained on somatic embryo production (282 embryos per plate) represented an improvement of more than 25-fold compared to that achieved on a PEG-free medium (11 embryos per plate) (Figure 2).

Slow desiccation at high relative humidity was found to be effective in promoting the germination of somatic embryos. Desiccated embryos germinated rapidly and after 12 weeks of culture, more than 80% were converted into plants. In contrast, when somatic embryos without post-maturation treatment were placed directly on the germination medium, the root emergence of embryos occurred at a very low frequency (data not shown). Post-maturation treatment after the PEG-mediated maturation of somatic embryos has also been reported to successfully improve germination frequencies in *Picea engelmanni-glauca* (Roberts et al. 1990b), *Picea sitchensis* (Roberts et al. 1991), *Picea glauca* (Attree et al. 1995; Kong and Yeung 1992, 1995), *Larix*×*leptoeuropaea* (Dronne et al. 1997; Lelu et al. 1995), and *Pinus patula* (Jones and van Staden 2001). The regenerated somatic embryo-derived plants were successfully acclimatized and are growing in the field without any abnormal

Table 1. Germination and conversion frequencies in somatic embryos of *Pinus luchuensis* after maturation on media with different concentrations of polyethylene glycol (PEG).

Somatic embryos from maturation media containing PEG ($g l^{-1}$)	Frequency of germination (%±SE)	Frequency of conversion (%±SE)
0	NT	NT
100	90±1.2	88±2.1
150	90±4.1	87±6.0
200	85±2.4	82±1.8

Germination and conversion frequencies represent the mean±SE (standard error), $n=5$ replicates of 30 to 50 somatic embryos per plate. NT: no tested due to insufficient number of somatic embryos. Frequency of germination: percentage of somatic embryos that showed root emergence. Frequency of conversion: percentage of somatic embryos that were converted into plantlets.

Table 2. Effect of kind of media/substrate on survival and growth of *Pinus luchuensis* somatic embryo-derived plants.

Media/Substrate	Survival (%)	Growth (cm±SE)
Medium with sucrose	100	3.5±0.2
Medium with glucose	100	2.9±0.1
Florialite	100	4.4±0.2

Survival and growth of somatic embryo-derived plants were evaluated after 15 weeks of culture on germination media containing $30 g l^{-1}$ sucrose or glucose, and into Magenta® vessels containing Florialite® irrigated with Nagao's plant food solution. Values were calculated from 30 somatic plants for each treatment. SE: standard error.

appearance in morphologic features.

To our knowledge, this is the first report on somatic embryogenesis in Ryukyumatsu. Therefore, the regeneration system described in this paper represents a promising tool for efficient propagation and for future genetic engineering approaches to develop transgenic Ryukyumatsu resistance to the pinewood nematode.

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