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SHORT COMMUNICATION

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Influence of osmotic pressure on somatic embryo maturation in *Pinus densiflora*

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Abstract The effect of an osmoticum, polyethylene glycol (PEG), on somatic embryo production was examined using embryogenic cells of *Pinus densiflora*. In the basal medium containing 30 μM abscisic acid and 6% maltose, the quality of the embryos formed was poor even though somatic embryos were produced. The addition of PEG with molecular weight of 4000 or 8000 significantly enhanced the development of both the quality and quantity of somatic embryos. Furthermore, higher levels of a constant osmotic pressure with PEG 8000 in a range from about 300 to 450 mmol/kg could remarkably enhance the morphogenesis of somatic embryos and their number of embryos produced. A higher stable osmotic pressure with an appropriate molecular weight of PEG is a key factor for the production of good quality somatic embryos in *P. densiflora*.

Key words Maturation · Osmotic pressure · polyethylene glycol (PEG) · *Pinus densiflora* · Somatic embryogenesis

Introduction

Japanese red pine (*Pinus densiflora*) is an evergreen conifer found in East Asia and is also one of the major species used for forestation in Japan. In recent years, pine wilt disease has attacked this species and the infection is spreading nationally. In order to combat this disease, effort is being made to develop new varieties with resistance to the disease. A project for the breeding of resistant trees began in 1978 (Fujimoto 1990), and a number of resistant varieties have been selected for planting. In order to speed up the propagation of these plants, more efficient propagation protocols have to be developed. Somatic embryogenesis has been successfully used in the micropropagation of conifer species, and this method can be a useful approach in the mass propagation of resistant varieties of red pine.

A protocol for somatic embryogenesis has been developed and used with success in the propagation of a number of *Pinus* species, such as *Pinus elliottii* (Jain et al. 1989), *Pinus palustris* (Nagmani et al. 1993), and *Pinus sylvestris* (Haggman et al. 1999). In Japanese red pine, however, successful reports are limited. Ishii et al. (2001) and Taniguchi (2001) reported successes in embryogenic cell (EC) induction and somatic embryo maturation; however, the conversion frequency was low. Maruyama et al. (2005) proposed an improved procedure for somatic embryogenesis by studying the effects of various additives such as plant growth regulators, different sugars, and polyethylene glycol (PEG). Plant conversion from somatic embryos was achieved at a rate of 30%. Based on these innovative reports, we investigated further the effects of abscisic acid (ABA) levels, maltose, and two different molecular weights and concentrations of PEG on red pine somatic embryo maturation. The stability and fluctuation of osmotic pressure in each medium were also determined to evaluate the importance of an osmotic environment on somatic embryo development.

Materials and methods

Embryogenic culture

Open-pollinated cones were collected in mid-August in 1999 from six independent, 36-year-old mother trees at a seed orchard located in Kobuchizawa, Yamanashi Prefecture. The trees were named Shouchiku 101, Nirasaki 9, Nirasaki 16, Yoshida 16, Yoshida 20, and Yoshida 21. As a

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preliminary experiment, we tested the callus induction rate and found that it varied from 35% to 85% depending on the mother trees used. Because Shouchiku 101 showed the most positive response, we used this clone for initiation of embryogenic cultures. Embryogenic cells were induced from immature seeds that were taken from cones of Shouchiku 101 on 14 July 2001. According to Taniguchi (2001), the Douglas fir cotyledon revised (DCR) medium (Gupta and Durzan 1985) was adopted for the initiation of embryogenic culture with the following modifications. Basal medium elements, i.e., $CaCl_2 \cdot 2H_2O$, 88 mg/l; KH_2PO_4, 340 mg/l; $MgSO_4 \cdot 7H_2O$, 740 mg/l; $MnSO_4 \cdot 4H_2O$, 44.6 mg/l; $ZnSO_4 \cdot 7H_2O$, 17.2 mg/l; $CuSO_4 \cdot 5H_2O$, 0.05 mg/l; nicotinic acid, 1 mg/l; thiamine·HCl, 2.5 mg/l; myo-inositol, 500 mg/l; L-glutamine, 250 mg/l; and casein hydrolysate, 500 mg/l, were added to the original medium and glycine and $NiCl_2$ contained in the original DCR medium were removed. ECs were induced on the modified DCR (mDCR) medium containing 10 µM of 2,4-dichlorophenoxyacetic acid (2,4-D), 3% sucrose, and 1000 mg/l of polyvinylpyrrolidone. Subculture was then carried out on the mDCR medium containing 10 µM of 2,4-D and 1250 mg/l of L-glutamine at 3-week intervals. All the culture media were adjusted to pH 5.7 and solidified with 0.3% gellan gum. The ECs were incubated in a petri dish of 9-cm diameter at 25°C in darkness. Although ca. 5% explants induced ECs, the majority of ECs could barely proliferate or gradually turned normal calluses. Therefore, we chose one EC line that showed the highest growth rate. This EC line also had well-developed early somatic embryos with an embryonal head and well-elongated suspensors (Fig. 1).

Investigations of stimulatory factors for maturation

The mDCR medium was used for a basal medium for maturation of *Pinus densiflora* with modifications as follows.

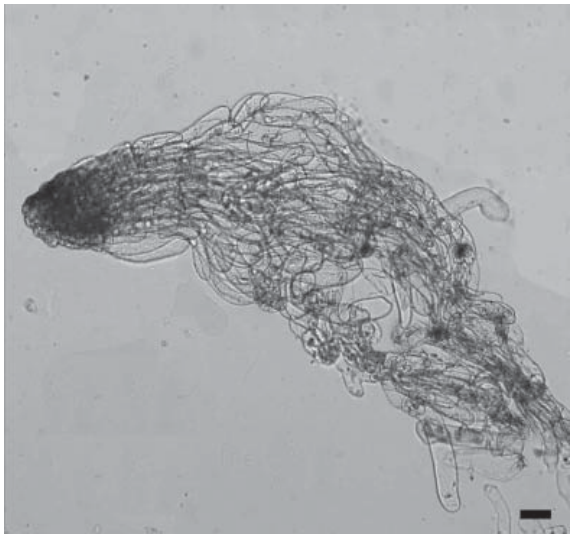

Fig. 1. Early somatic embryo of *Pinus densiflora* derived from immature zygotic embryo. *Bar* 100 µm

Concentration of ABA

To determine the optimal concentration of ABA, filter-sterilized ABA was added to the maturation mDCR medium in the following concentrations: 0, 1, 5, 10, 30, 60, 100, 120, 150, and 200 µM. In these experiments, 6% maltose was used instead of sucrose.

Types and concentration of sugar

Sucrose and maltose were added to the maturation mDCR medium with an adjustment of two different concentrations, 3% and 6%, to determine their effectiveness on somatic embryo maturation. Based on the ABA results, 30 µM ABA was added to the maturation media.

Molecular weight and concentration of PEG

For investigation of the effective molecular weight and concentration of PEG, 5% and 10% of PEG 4000 (Wako, Osaka; average molecular weight 3000) and PEG 8000 (Sigma, St Louis, MO, USA; average molecular weight 8000) were added to the maturation mDCR medium. The concentrations of ABA and sugar were fixed at 30 µM and 6% maltose, respectively. In this experiment, ECs were first cultured on a regular maturation medium containing 30 µM ABA and 6% maltose for 4 weeks to prevent the ECs from necrotic damage. The cultured cell masses were then transferred to PEG-containing media and cultured for another 4 weeks.

Five to ten independent ECs (50 mg in fresh weight) were cultured in each experiment. After 8 weeks of culture, morphological changes of ECs were observed under a stereomicroscope to confirm the development of somatic embryos. The number of mature somatic embryos produced was counted on each cell mass.

Measurement of osmotic pressure

Osmotic pressure in the maturation media was recorded to determine its importance in embryo maturation. To do this, the preparation of ECs and culture conditions were the same as mentioned above, however, the maturation process was carried out in a liquid medium condition. Osmotic pressure in each liquid medium was periodically measured by the Vapor Pressure Osmometer 5520 (Wescor, Logan, UT, USA) at 0 and 8 h, and at 1, 3, 5, 7, 14, 21, and 28 days of culture. The measurements were repeated six times per sample.

Statistical analysis

The results were examined statistically with consideration of variation. The Fisher's test was performed using Stat View (SAS Institute, Cary, NC, USA).

Results and discussion

Effects of ABA on somatic embryo maturation

As shown in Fig. 2, ABA has a vital role to play in somatic embryo formation from ECs. In the absence of ABA, ECs started to brown rapidly. On the other hand, somatic embryos were observed on the surface of ECs cultured on the ABA-containing media in the concentration range of 1–60 µM. The optimal ABA concentration for maturation was 30 µM and the number of somatic embryos was ca. 20 per 50 mg fresh weight (FW) of ECs in *Pinus densiflora*. The number of somatic embryos decreased to 15 per 50 mg FW of ECs when 60 µM ABA was added to the maturation medium. Further addition of ABA, in concentrations of 100 µM and 200 µM, did not induce any developed somatic embryos. The stimulatory effect of ABA on the maturation of somatic embryo was reported in various species, including conifers. Klimaszewska and Smith (1997) pointed out that the numbers of somatic embryos are closely related to ABA concentration. According to von Arnold et al. (2002), although the effective concentration of ABA depends on the species, it is generally recognized that concentrations in a range of about 10–50 µM are the most effective. The effect of ABA found in *P. densiflora* could support this theory.

Effects of sugars on somatic embryo maturation

It is known that sugar does not only serve as a carbon source for the growth of plant cells, but also as an osmoticum. Therefore, the effects of sugar type and concentration on somatic embryo maturation were examined (Fig. 3A). Maltose was more effective in stimulating the production of somatic embryos than sucrose, regardless of concentration. The higher level of maltose concentration (6%) increased the number of somatic embryos to 22 per 50 mg FW ECs; however, the embryos produced were morphologically defective. The somatic embryos developed from the maltose-containing medium had swollen cotyledons and poorly elongated hypocotyls (Fig. 4A). The effect of maltose on the maturation of somatic embryos was reported in *Pinus* sp., e.g., *Pinus nigra* (Salajova et al. 1999) and *Pinus taeda* (Li et al. 1998). These reports also indicated abnormal somatic embryo formation when maltose was used

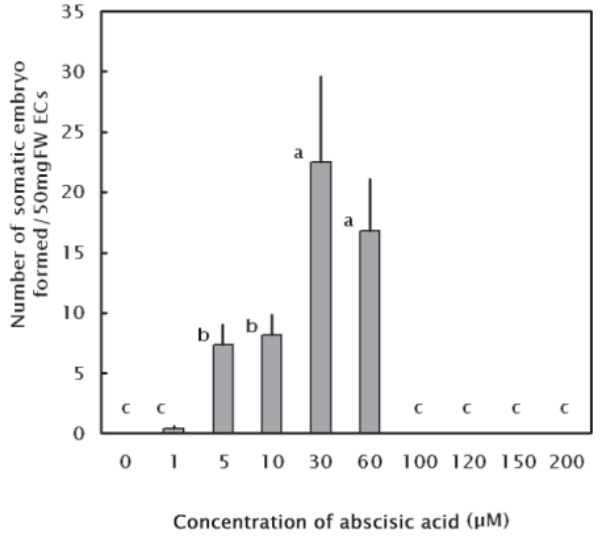

Fig. 2. The effect of abscisic acid on the maturation of *P. densiflora* somatic embryos after 8 weeks in culture. Standard errors were calculated from ten independent measurements. Data marked with the same lowercase letter are not significantly different ($P < 0.05$). *FW ECs*, fresh weight embryogenic cells

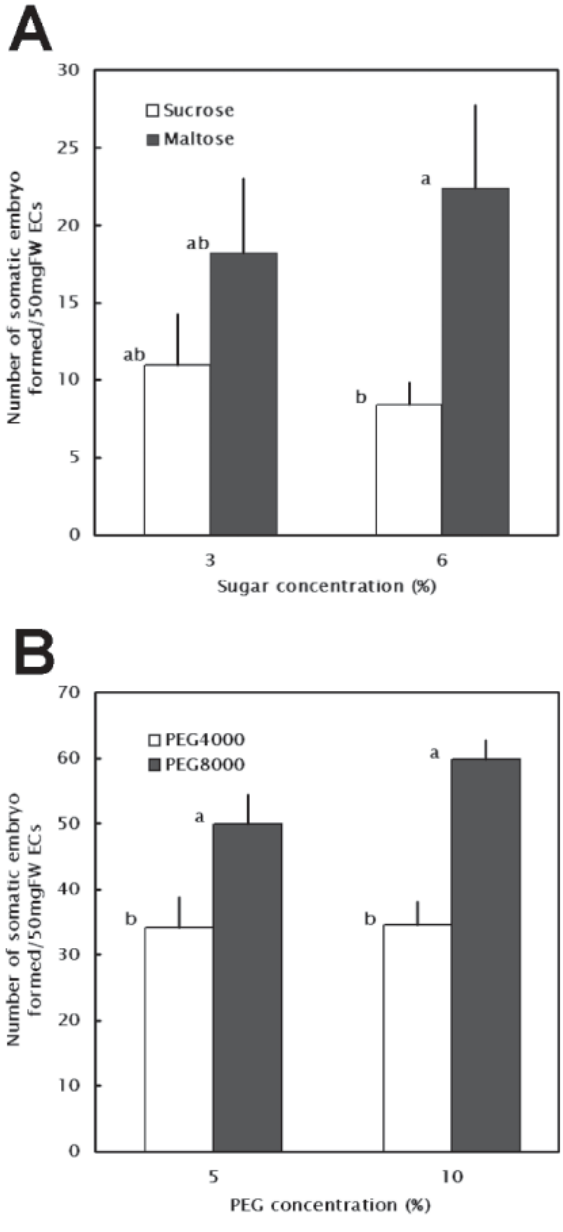

Fig. 3. The effects of sucrose and maltose (**A**), and polyethylene glycol (*PEG*) (**B**) on the maturation of *P. densiflora* somatic embryos after 8 weeks in culture. Standard errors were calculated from five independent measurements. Data marked with the same letter are not significantly different ($P < 0.05$)

alone. It is clear that, although maltose enhances the total number of embryos produced, further improvements are needed to improve embryo quality. In order to overcome this problem, we first investigated the osmotic pressure in each sugar-containing medium. As shown in Fig. 5A, the initial osmotic pressures were in the range of 150–250 mmol/kg. For sucrose, the pressures gradually increased up to ca. 250 and 450 mmol/kg for concentrations of 3% and 6%, respectively. On the other hand, in maltose-containing media, stable values were found during the maturation period. Because fluctuations and/or stabilities of osmotic pressure in a medium were correlated with development of somatic embryos, we also examined the influence of osmotic pressure on embryo development using PEG.

Effects of PEG on somatic embryo maturation

The number of somatic embryos increased conspicuously on medium containing PEG in comparison with medium without the substance (Fig. 3). In addition, normal cotyledons and hypocotyls were observed in somatic embryos formed on all media containing PEG as shown in Fig. 4B. The effects were significantly different with the varying molecular weights of PEG. The higher molecular weight of PEG 8000 was more effective on somatic embryo formation than PEG 4000, especially in a 10% concentration. In the medium containing 10% PEG 8000, the number of somatic embryos was 60 per 50 mg FW ECs (Fig. 3B), whereas in conditions without PEG, embryo production

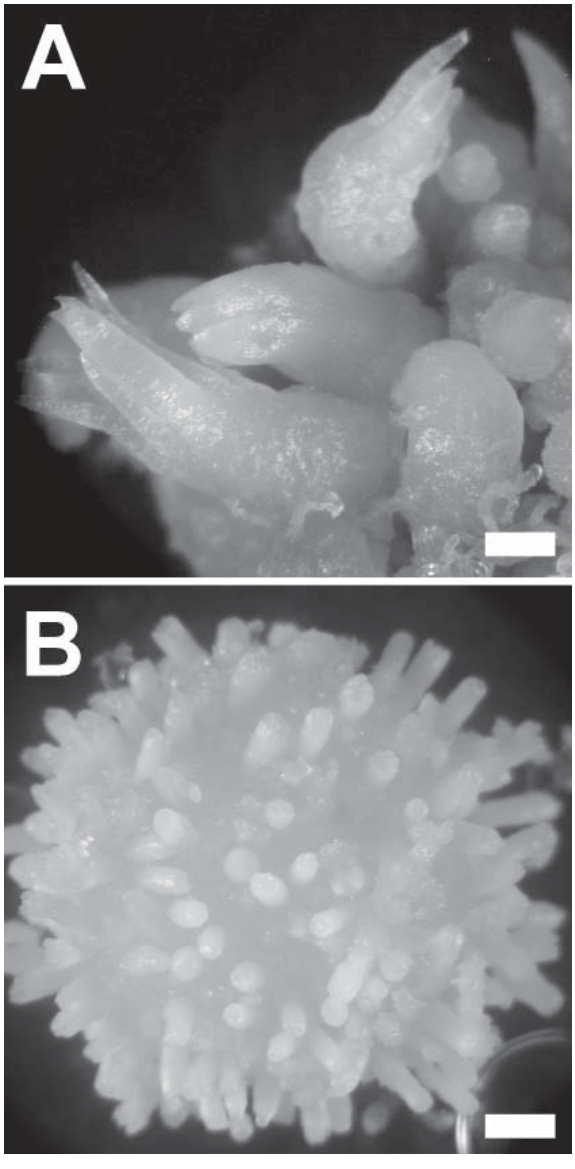

Fig. 4A,B. Morphological characteristics of mature *P. densiflora* somatic embryos. **A** Swollen somatic embryos developed on a maturation medium containing 3% sucrose and 30 µM abscisic acid (ABA); *Bar* 1 mm. **B** Well-developed somatic embryos formed on a maturation medium containing 6% maltose, 30 µM ABA, and 10% PEG 8000; *Bar* 2 mm

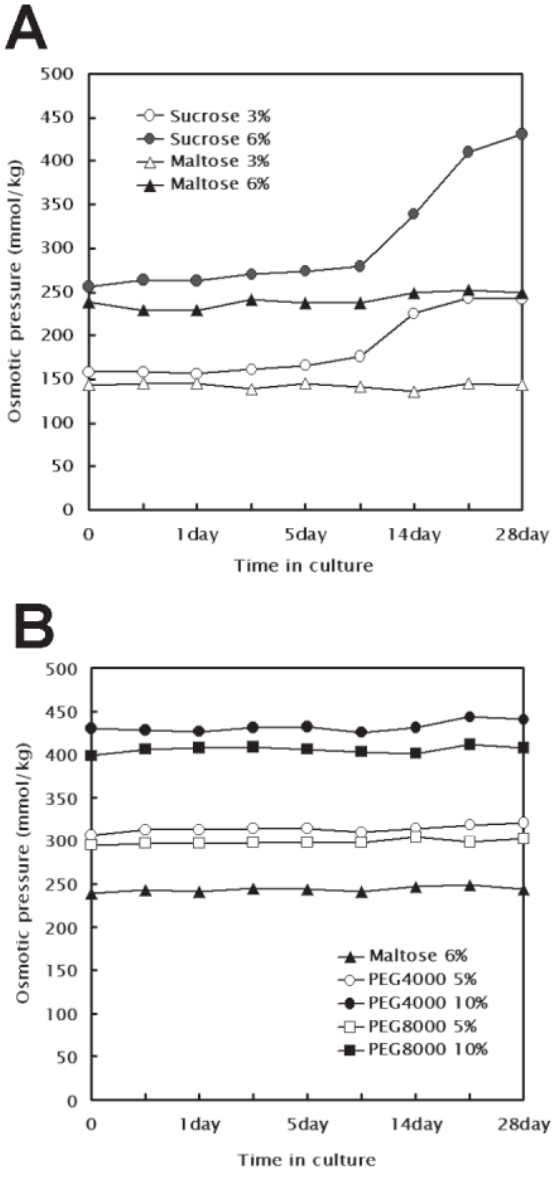

Fig. 5A,B. Differing osmotic pressure on various maturation media during somatic embryo maturation. **A** The effect of sucrose and maltose. **B** The effect of PEG in medium containing 6% maltose. Osmotic pressure in each liquid medium was measured at 0 and 8 h, and at 1, 3, 5, 7, 14, 21, and 28 days. The plotted data indicate the mean values of six independent measurements

was less than 22 embryos produced per 50 mg FW ECs (Fig. 3A).

In PEG-containing media, the higher values of osmotic pressure were observed during the culture period. The values were over 400 mmol/kg for 10% additions of both molecular weights and 300 mmol/kg for 5% additions in comparison with ca. 250 mmol/kg in the control medium without PEG (Fig. 5B). Klimaszewska et al. (1997) investigated the effects of sucrose, PEG 3000–3500, and ABA concentration in a medium on the maturation of *Larix laricina* somatic embryos. Although the stability of osmotic pressure during a culture period was not reported, they suggested that higher osmotic pressures of the medium in a range from 315.0 to 543.6 mmol/kg were effective for stimulating the maturation process. In our experiment, higher constant osmotic pressure levels with maltose and PEG 8000, in a range from about 300 to 450 mmol/kg, could remarkably enhance morphogenesis of somatic embryos and increase the number of embryos produced. Regarding the significant difference between PEG 4000 and PEG 8000 in the number of somatic embryos formed, we currently favor the following theory. Attree and Fowke (1993) expressed that PEG with molecular weight over 4000 could not pass through the cell wall and hence could generate an appropriate osmotic pressure without inducing plasmolysis of the cells. Accordingly, a stable osmotic pressure with an appropriate molecular weight of PEG must be an important factor allowing for the proper development of somatic embryos in *P. densiflora*.

When the somatic embryos were transferred to a hormone-free mDCR medium, the cotyledons developed normally and the hypocotyls elongated (data not shown). However, rooting was suppressed in all seedlings tested. Further improvement for germination is therefore needed.

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