

トウヒ,エゾマツ,アカエゾマツの成熟胚起源のカルスからの 体細胞胚形成と個体の再生

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短 報

Somatic Embryo Formation and Plantlet Regeneration through Embryogenic Callus from Mature Zygotic Embryos of *Picea jezoensis* var. *hondoensis*, *Picea jezoensis*, and *Picea glehnii*

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石井克明：トウヒ、エゾマツ、アカエゾマツの成熟胚起源のカルスからの体細胞胚形成と個体の再生 日林誌 73: 24~28, 1991 トウヒ、エゾマツ、アカエゾマツの成熟胚を $5\mu\text{M}$ の BAP を含有し、 $10\mu\text{M}$ の NAA か 2,4-D を含有する 1/2 LP 培地で培養し不定胚形成能のあるカルスを誘導した。次に $7.6\mu\text{M}$ の ABA を含有する 1/2 LP 培地に移植してサスペンサー部をもつ典型的な針葉樹の体細胞胚を誘導した。体細胞胚を含むカルス塊をホルモン無添加の LP 培地に移植すると、トウヒで 88 体細胞胚含有カルス塊より 1 個体、エゾマツで 10 体細胞胚含有カルス塊より 1 個体の再生植物体を得た。体細胞胚をさらに活性炭含有の 1/2 LP 培地と、 0.005 mg/l NAA 含有の CD 培地に順次移植して個体の再生を向上させた結果、トウヒで 88 個の体細胞胚より 1 個体、エゾマツで 10 個の体細胞胚より 4 個体、アカエゾマツで 16 個の体細胞胚より 2 個体再生した。

I. Introduction

It takes a long time (usually 4~5 years) to care for *Picea* species in the nursery to bring them to a size suitable for planting in the forest. To shorten the time, the application of tissue culture may be one option in the micropropagation of fast-growing clones or for promoting the growth of trees under controlled *in vitro* conditions. Micropropagation using tissue culture techniques is of two types. One is the use of the adventitious-bud formation ability of meristematic tissue, the other is the use of the somatic-embryo formation ability of cultured tissues or cells. Propagation *via* adventitious buds has been much more reliable to date and applied practically with horticultural species. Nevertheless, propagation *via* somatic embryos still has a potential because it is believed that the method is labor-saving, needs less space, and its propagation rate is enormous if it works well.

Recently, many works on the embryogenesis of forest trees, including gymnosperms, have been reported (TULECKE, 1987). *Picea* species are pioneers in this respect. Embryogenesis and further development to plantlets were reported for *P. abies* (L.) KARST. first from immature embryos (HAKMAN *et al.*, 1985), and then from mature embryos (VON ARNOLD, and HAKMAN, 1986). Somatic embryos were induced also from the cotyledons of this species but without successful plant regeneration (TULECKE, 1987). Somatic embryogenesis also was reported from the immature embryos of *P. glauca* (MOENCH) VOSS and *P. mariana* (MILL.) B.S.P. (HAKMAN, and FOWKE, 1987).

In this work reported here, in seeking the probability of micropropagation *via* somatic embryogenesis and plantlet regeneration of other *Picea* species, three Japanese *Picea* species were used with the *in vitro* culture of mature zygotic embryos.

II. Materials and Methods

1. Plant material

Open-pollinated seeds of *P. jezoensis* var. *hondoensis* MAYR collected in Kiso County, Nagano Prefecture, in 1979, *P. jezoensis* CARR. in Sapporo City of Hokkaido in 1987, and *P. glehnii* MAST. in Saru County, Hokkaido, in 1983 were used. They were soaked in cool water at 4°C for 2 days in late 1988, then surface-sterilized for 10 s in 70 % ethyl alcohol and for 13 min in 35 % hydrogen peroxide, and then

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rinsed three times in sterile distilled water.

Embryos were excised aseptically from the surrounding endosperms and placed on a culture medium.

2. Initiation of embryogenic callus and further development

Embryogenic-callus cultures were initiated from zygotic embryos cultured on modified half-strength Lepoivre (LP) medium (AITKEN-CHRISTIE, and THORPE, 1984) containing 30 mM of sucrose, 15 mM of ammonium nitrate, $5 \mu\text{M}$ of N^6 -benzylaminopurin (BAP), and $10 \mu\text{M}$ of α -naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D). For development of somatic embryos, the calli were transferred to modified half-strength LP medium containing 90 mM of sucrose and $7.6 \mu\text{M}$ of abscisic acid (ABA). For maturation of somatic embryos, modified full-strength LP medium, containing 60 mM of sucrose, was used.

For further development to plantlets from somatic embryos, half-strength LP medium, containing 5 g/l of activated charcoal, and CAMPBELL, and DURZAN's medium (CD) (CAMPBELL, and DURZAN, 1975), containing 0.005 mg/l of NAA, were used. All media were solidified with 0.7% agar (Wako Pure Chem. Ind. Ltd.).

Rooted plantlets obtained were transferred to perlite soil containing 0.1% hyponex in a flask.

Twenty embryos and embryogenic calli were cultured in 18 mm diameter test-tubes, and germinated somatic embryos were cultured in the same test tubes or in 200~300 ml culture flasks. Initial cultures were incubated at 20°C in the dark. Established embryogenic calli were maintained at 20°C with a 16 h photoperiod of 3,000 lx for somatic embryogenesis and plantlet regeneration. Subculturing was conducted bimonthly on fresh medium. Rooted plantlets obtained were cultured at 25°C with a 16 h photoperiod of 5,000 lx.

III. Results and Discussion

1. Embryogenic callus formation

Within the months of culture, zygotic embryos of *P. jezoensis* var. *hondoensis*, *P. jezoensis*, and *P. glehnii* had initiated callus formation on the surfaces. *P. glauca* was reported in another paper to have a embryogenic callus type which was white to translucent and mucilaginous; whereas, nonembryogenic callus was green, and firm to friable (WANN *et al.*, 1987). In this experiment, both types of calli were observed. As shown in Table 1, NAA, a different auxin from that used in the experiments with *P. glauca* and *P. abies* was also effective in inducing embryogenic callus formation from zygotic embryos, and the effect was slightly better than 2,4-D in the case of *P. jezoensis* var. *hondoensis* and *P. glehnii*.

2. Somatic embryos formation

The morphology of somatic embryos induced in tissue cultures from *P. jezoensis* var. *hondoensis*, *P. jezoensis*, and *P. glehnii* resembled that of somatic embryos previously described for *P. glauca*, *P. mariana*, and *P. abies* (HAKMAN, and FOWKE, 1987; BECWAR *et al.*, 1987): namely, a somatic region consisting of small embryonal heads with long suspensor cells extending from them as shown in Fig. 1. The lengths of the somatic embryos were about 0.6~1 mm. ABA is supposed to be effective for somatic embryo formation as described for *P. glauca* (DUNSTAN *et al.*, 1988).

Table 1. The effect of different auxins on embryogenic callus formation in the *Picea* species after two months ($N=20$) * (%)

Species	No. of mature embryos forming embryogenic callus	
	NAA, $10 \mu\text{M}$	2,4-D, $10 \mu\text{M}$
<i>P. jezoensis</i>		
var. <i>hondoensis</i>	8(40)	6(30)
<i>P. jezoensis</i>	6(30)	7(35)
<i>P. glehnii</i>	16(80)	12(65)

* Cultured on modified half-strength LP medium containing $5 \mu\text{M}$ of BAP.

Table 2 indicates the number of somatic embryos in the initial medium. The average numbers of NAA-induced somatic embryos from *P. jezoensis* var. *hondoensis* were 7.6 per zygotic embryo, and greater than those of 2,4-D-induced ones. But in the other two species, *P. jezoensis* and *P. glehnii*, the use of NAA for inducing somatic embryos was less effective than 2,4-D, and the average numbers of somatic embryos were small. In the case of *P. glehnii*, although NAA was effective for mucilaginous callus formation, fur-

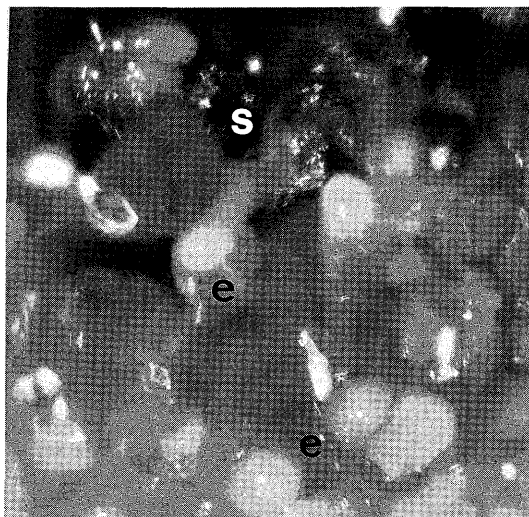


Fig. 1. Somatic embryogenesis in *P. jezoensis* var. *hondoensis* two months after transfer from 1/2 LP medium containing BAP and NAA to 1/2 LP medium containing ABA

Legend: s, Suspensor cells; e, Embryonal heads.

Table 2. Number of somatic embryos one month after transfer from callus-forming medium containing different auxins to half-strength LP medium containing 90 mM of sucrose and 7.6 μ M of ABA

Species	Auxins in the initial callus-forming medium	No. of mature embryos cultured	No. of embryos surviving	No. of embryos inducing somatic embryos per embryo	Average no. of somatic embryos per embryo	Ranges of no. of somatic embryos
<i>P. jezoensis</i>						
var. <i>hondoensis</i>	2,4-D	20	8	5	5.4	2~12
	NAA	20	11	8	7.6	1~30
<i>P. jezoensis</i>	2,4-D	20	9	2	4.5	4~5
	NAA	20	6	1	1	1
<i>P. glehnii</i>	2,4-D	20	18	6	2.2	1~4
	NAA	20	16	2	1.5	1~2

ther development to somatic embryos was poor.

3. Budding, rooting, and plantlet regeneration

Budding and rooting of somatic embryos were observed rarely two weeks after transfer from ABA-containing medium to full-strength LP medium without a plant-growth regulator under light conditions.

Although embryo development was slow, further budding and rooting were observed later. Some of them developed further into plantlets. Figures 2~5 show the specimens developed by the three *Picea* species. At this stage, one regenerated small plantlet was obtained from 88 embryogenic calli containing somatic embryos of *P. jezoensis* var. *hondoensis*, and one was obtained from 10 embryogenic calli containing

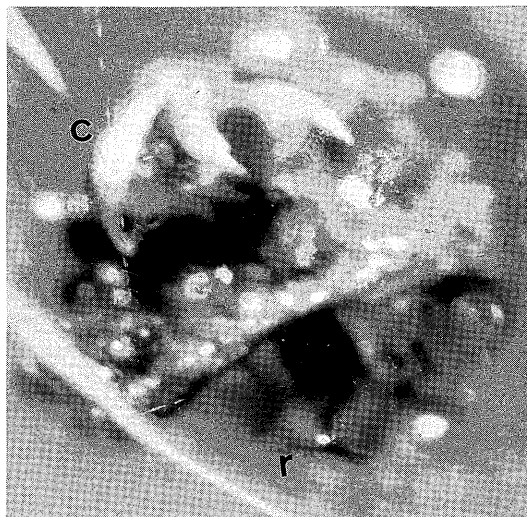


Fig. 2. Cotyledon and small root development from a somatic embryo of *P. jezoensis* var. *hondoensis* after six months culture

Legend: c, Cotyledon; r, Root.

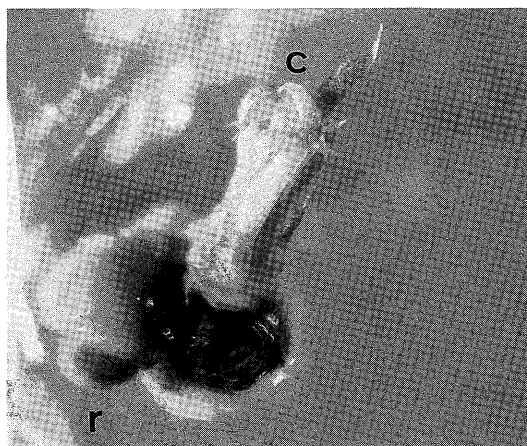


Fig. 3. Cotyledon and small root development from a somatic embryo of *P. jezoensis* after six months culture

Legend: c, Cotyledon; r, Root.

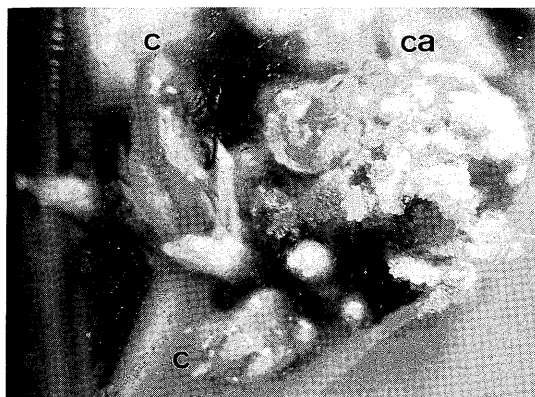


Fig. 4. Cotyledons development from somatic embryos of *P. glehnii* after six months culture

Legend: c, Cotyledon; ca, Callus.

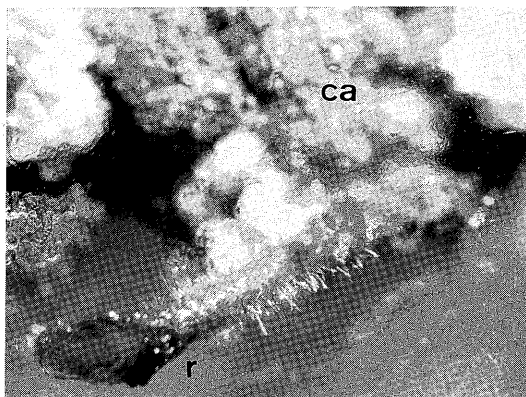


Fig. 5. Root development from a somatic embryo of *P. glehnii* after six months culture

Legend: r, Root; ca, Callus.



Fig. 6. Shoots development from somatic embryos of *P. glehnii* after two months culture on 1/2 LP medium containing 5 g/l of activated charcoal

Table 3. Retention of plantlets, originated from embryos of *Picea* species, five months after transfer from full-strength LP medium to half-strength LP medium containing 5 g/l of activated charcoal for two months and then into CD medium containing 0.005mg/l of NAA for three months.

Species	Auxins in the initial callus forming medium	No. of somatic embryos cultured	No. of shoots obtained	No. of plantlets obtained
<i>P. jezoensis</i>				
var. <i>hond-</i>	2,4-D	27	4	0
<i>oensis</i>	NAA	61	16	1
<i>P. jezoensis</i>	2,4-D	9	10	3
	NAA	1	4	1
<i>P. glehnii</i>	2,4-D	13	18	1
	NAA	3	5	1

somatic embryos of *P. jezoensis*.

Somatic embryo development on the full-strength LP medium without a plant-growth regulator was relatively poor, and in some cases buds or roots ceased to grow. To grow plantlets beyond this stage, developing somatic embryos were transferred to the half-strength LP medium containing 5 g/l of activated charcoal for two months and then into CD medium containing 0.005 mg/l of NAA for three months. On activated charcoal medium, buds from somatic embryos were promoted to grow as shown in Fig. 6. The results after the culturing was completed are shown in Table 3.

The effect of this treatment on bud growth was clear, and the increase in the number of shoots compared with the number of initial somatic embryos in *P. jezoensis* and *P. glehnii* suggests the existence of invisible somatic embryos inside the callus that were not counted in the subculture stage. However, the root development still was very poor, and regenerated rooted plantlets were rare. Regenerated plantlets from



Fig. 7. Plantlet regeneration from a somatic embryo of *P. jezoensis* var. *hondoensis*



Fig. 8. Plantlet originating from a somatic embryo of *P. glehnii* subcultured in perlite containing 0.1 % hyponex

somatic embryos were one out of 88 somatic embryos in *P. jezoensis* var. *hondoensis* (Fig. 7), 4 out of 10 in *P. jezoensis*, and 2 out of 16 in *P. glehnii*.

Rooted plantlets subcultured in perlite soil survived as shown in Fig. 8.

So far, although the numbers of regenerated plantlets were small, it might be said that somatic embryo induction and further development into plantlets by three Japanese *Picea* species (*P. jezoensis* var. *hondoensis*, *P. jezoensis*, and *P. glehnii*) are possible much like *P. abies*.

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