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# Somatic embryogenesis from immature and mature zygotic embryos of *Cryptomeria japonica* I: Embryogenic cell induction and its morphological characteristics*

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**Abstract** Embryogenic cells (ECs) of sugi (*Cryptomeria japonica* D. Don) were induced from immature and mature zygotic embryos cultured on different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D)-containing modified Campbell and Durzan medium. The rate of induction of ECs varied depending on the stage of embryos collected. The highest percentage of induction (35%) was obtained with immature zygotic embryos collected July 18 and July 30, 1997, when 1 μM of 2,4-D was added to the induction medium. The ECs easily proliferated when subcultured in a medium of the same composition as the induction medium within 3 weeks. Morphological characteristics of nonembryogenic cells and embryogenic cells of different developmental stages were studied under an inverted fluorescence microscope.

**Key words** *Cryptomeria japonica* · Zygotic embryo · Embryogenic cell · Somatic embryogenesis

## Introduction

Sugi (*Cryptomeria japonica* D. Don) is the most common conifer species in Japan and it is an important source of timber. For this reason, it has long been widely cultivated in approximately 70% of reforested areas since the 1930s.[1] As a consequence of this cultivation, sugi pollen allergies have become a serious ailment in Japanese society. Therefore, if pollen production by sugi could be suppressed or reduced, it would greatly improve the quality of life for many people.

Genetic engineering such as cell fusion and the gene recombinant method is considered to be one of the most useful and important techniques for the alteration of genetic characters of higher plants. To begin such studies, an efficient plant regeneration system, including protoplast culture and/or callus culture, must be established.

Callus cultures of sugi (*C. japonica*) were attempted from excised anthers,[2] hypocotyl,[3,4] stem pieces,[5] and cotyledons.[6] Researchers indicated that sugi calluses have high ability to proliferate and form adventitious roots but little ability to form adventitious buds. Thus a more efficient plant regeneration system, such as somatic embryogenesis, must be developed.

Somatic embryogenesis of coniferous trees was first reported in 1985 from megagametophytes of *Larix decidua* Mill.[7] and from immature zygotic embryos of *Picea abies* (L.) Karst.[8] Since then, additional successes have been reported in many species using the somatic embryogenesis approach.[9] However, there is little information concerning somatic embryogenesis of sugi. In this report, we detail a protocol concerning the induction of embryogenic cells (ECs) from zygotic embryos of *C. japonica* and outline the morphological characteristics.

## Materials and methods

### Plant materials

Immature and mature seeds were collected May 30, June 16, July 18, July 30, September 2, and October 2, 1997 from an approximately 20-year-old tree of *C. japonica*, grown on a campus of Tokyo University of Agriculture and Technology. Seeds were removed from cones and soaked for 10 min in distilled water with several drops of benzalkonium chloride solution (Takeda Chemical Industries, Osaka, 540–8645, Japan) were added. After soaking, they were steril-

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ized with 70% ethyl alcohol for 10 min followed by a 3% $H_2O_2$ solution for another 10 min. After sterilization, they were rinsed five times with sterile distilled water and dried on sterile paper. Zygotic embryos were then dissected from the seeds under a stereoscope and placed on the induction medium.

### Induction and maintenance of embryogenic cells

A modified Campbell and Durzan (mCD) medium was used as the basal medium. The concentration of $NH_4NO_3$ was reduced to 400 mg $l^{-1}$ from 800 mg $l^{-1}$ of the original,[10] and 600 mg $l^{-1}$ of L-glutamine was added. 2,4-Dichlorophenoxyacetic acid (2,4-D) (0, 1, and 10 μM) was added to the mCD medium. The pH of the media was adjusted to 5.6 after the addition of 30 g $l^{-1}$ sucrose. Two g $l^{-1}$ gellan gum was used as the solidifying agent. The complete media were autoclaved for 20 min at 121°C. A 20-ml aliquot of each medium was poured into a 90 × 15 mm petri dish and 5–10 zygotic embryos were placed on each dish.

After 4–6 weeks, approximately 40–60 mg of ECs was taken from the original explant and subcultured on solid mCD medium of the same components as the induction medium at 3- to 5-week intervals. The cultures were incubated at 25°C, (1) under 4000 lux fluorescent illumination and a 16-h photoperiod or (2) in the dark.

### Development of somatic embryos

To induce further somatic embryo development, ECs derived from immature zygotic embryos collected July 18 were transferred to medium containing (±) abscisic acid (ABA) and/or benzylaminopurine (BAP). The ECs were cultured on mCD medium without L-glutamine, supplemented with each concentration: 0, 0.1, 1, 10, and 50 μM of ABA or 0, 0.1, 1, and 10 μM of BAP (or both) and 30 g $l^{-1}$ sucrose. The pH of the medium was adjusted to 5.6 before solidifying with 2 g $l^{-1}$ gellan gum. Five pieces of 40–60 mg of ECs were cultured on 20 ml of each medium per petri dish and incubated at 25°C under 4000 lux fluorescent illumination and a 16-h photoperiod.

### Microscopy

Morphological characteristics of nonembryogenic cells (NECs) and ECs of sugi were observed using an inverted fluorescence microscope under U- or B-excitation fluorescence light after staining with 2 × $10^{-4}$% solution of 4′-6-diamidino-2-phenylindole (DAPI).[11]

## Results and discussion

### Characteristics of ECs derived from zygotic embryos

An mCD medium prescribed for somatic embryogenesis of *L. leptolepis*[11] was also adaptable for somatic embryogenesis of sugi. Different forms of tissue can be obtained from the same explant. ECs of sugi which are white and friable, as shown in Fig. 1, were derived from immature and mature zygotic embryos on mCD containing only 2,4-D. At the same time, NECs characterized by white-yellow or yellow-green color and compact features were also induced.

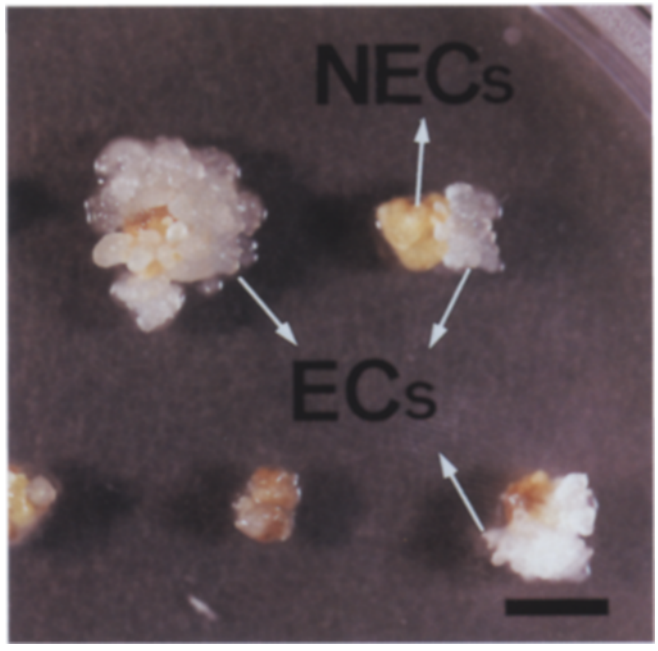


**Fig. 1.** Characteristics of embryogenic (ECs) and nonembryogenic (NECs) cells derived from zygotic embryos of *C. japonica*. *Bar* represents 10 mm

Morphological characteristics of cells derived from explants were recognized by DAPI staining and fluorescence microscopy. Many small clusters of cells characterized by tiny dense cells (embryonal regions) and elongated cells (suspensor regions) were present (Fig. 2A). Strong blue fluorescence could be observed after staining with DAPI, indicating that the embryonal regions are cytoplasmic with large nuclei (Fig. 2B). These clusters were typical structures of early somatic conifer embryos, as shown by Tulecke,[12] Attree and Fowke,[13] and Ogita et al.[11]

In contrast, dense cell clusters or suspensor-like cells could not be detected in the NECs. As shown in Fig. 2CD, the NECs were comprised of cells of similar size containing chloroplasts (chloroplasts are observed as red auto-fluorescence in Fig. 2D).

### Seasonal variation on induction of ECs from zygotic embryos

As shown in Table 1, ECs could be obtained in both the light and dark conditions after 4 weeks of culture. Furthermore, it was recognized that the induction rate varied depending on the collection date of the seeds, especially in the light. ECs were induced at frequencies varying from 5% to 35% from zygotic embryos collected between June 16 and October 2. The highest response was observed from imma-

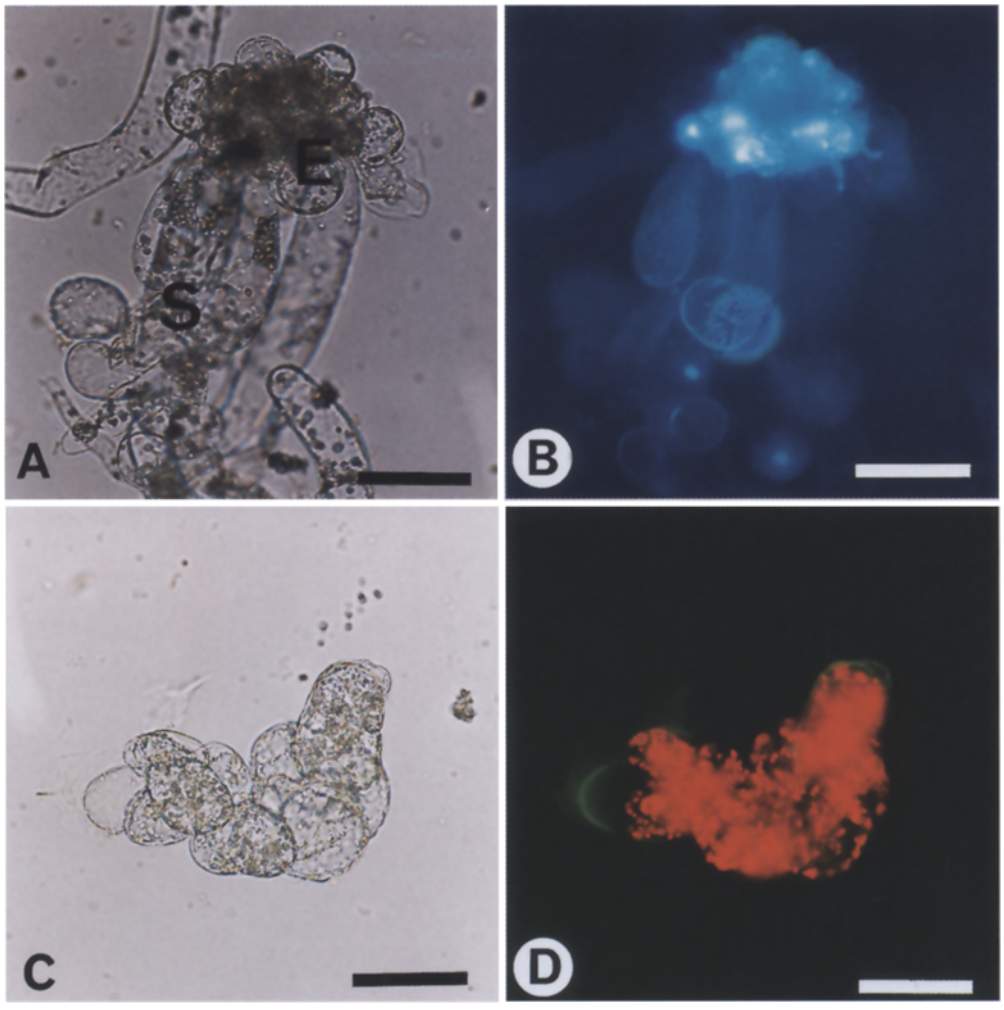


**Fig. 2.** Morphological characteristics of ECs and NECs derived from zygotic embryos of *C. japonica*. *E*, embryonal region; *S*, suspensor region. **A** ECs are characterized by having many small clusters of cells. **B** DAPI staining results in blue fluorescence from the cell clusters indicating that the cells are cytoplasmic with large nuclei. **C** NECs are characterized by cells of similar size and the absence of cytoplasmic cell clusters. **D** Chloroplasts are present within the NECs as red auto-fluorescence. *Bars* represent 100 μm

**Table 1.** Effects of collection date, 2,4-D, and culture conditions on induction of ECs in culture for 4 weeks in *Cryptomeria japonica*

| Collection date | 2,4-D (μM) | Light condition | | | Dark condition | | |
|---|---|---|---|---|---|---|---|
| | | Zygotic embryos cultured (no.) | Zygotic embryos forming ECs (no.) | (%) | Zygotic embryos cultured (no.) | Zygotic embryos forming ECs (no.) | (%) |
| May 30 | 1 | 10 | 0 | 0 | 10 | 0 | 0 |
| | 10 | 10 | 0 | 0 | 10 | 0 | 0 |
| June 16 | 1 | 20 | 1 | 5 | 20 | 0 | 0 |
| | 10 | 20 | 2 | 10 | 20 | 0 | 0 |
| July 18 | 1 | 20 | 7 | 35 | 20 | 4 | 20 |
| | 10 | 20 | 0 | 0 | 20 | 0 | 0 |
| July 30 | 1 | 40 | 14 | 35 | 20 | 4 | 20 |
| | 10 | 20 | 0 | 0 | 20 | 0 | 0 |
| September 2 | 1 | 100 | 14 | 14 | NT | – | – |
| October 2 | 1 | 10 | 1 | 10 | 10 | 1 | 10 |
| | 10 | 10 | 3 | 30 | 10 | 2 | 20 |

2,4-D, 2,4-dichlorophenoxyacetic acid; ECs, embryogenic cells; NT, not tested; –, no data.

ture zygotic embryos collected July 18 and July 30 on mCD containing 1 μM 2,4-D in the light. Furthermore, Table 1 shows that for mature zygotic embryos collected October 2, culture on medium containing 10 μM of 2,4-D is more effective for inducing ECs than on 1 μM 2,4-D. At high concentrations of 2,4-D, the induction rate rose from 10% to 30% in the light and from 10% to 20% in the dark.

Many reports suggested that the frequency of EC induction is highly dependent on the collection date of the explants. In *Pinus* species such as *P. caribaea*[14] and *P.*

*strobus*,[15] the precotyledonary stage of zygotic embryos was optimal for the induction. Similarly, in *Larix* species such as *L. decidua*, *L. leptolepis*, *L.* × *eurolepis* and *L.* × *leptoeuropaea*,[16–19] the precotyledonary stage of zygotic embryos was also optimal. In contrast, for *Picea* species such as *P. glauca, P. mariana*, and *P. engelmannii*,[20–22] the optimum response occurred from the cotyledonary developmental stage of zygotic embryos.

In *C. japonica* the induction rate of ECs was influenced by the collection date of seeds. The highest rate (35%) was obtained with immature zygotic embryos collected July 18 and July 30, 1997. These zygotic embryos, characterized by being semitransparent or white and 2–4 mm in length, were in the cotyledonary developmental stage.

## Maintenance of ECs

After 4–6 weeks of initial culture, the explants with ECs grew to more than 5 mm in diameter. ECs from the original explants were then dissected and subcultured. The light condition was equivalent to the dark condition in terms of the frequency of EC proliferation. Furthermore, the tendency of proliferation changed according to collection date of seeds. All ECs derived from zygotic embryos collected June 16 and October 2 were difficult to maintain when subcultured on the same medium composition as that of induction. They lost their embryogenic potency after 12–20 weeks of culture. The other ECs derived from zygotic embryos collected between July 18 and September 2 grew actively, although they tended to turn brown if the subculture period was longer than 4 weeks. More frequent transfer to fresh medium (i.e., within 3 weeks) was better for maintenance of the ECs.

## Development of somatic embryos

When ECs were transferred from the maintenance medium to ABA- or BAP-containing medium or ABA/BAP medium, four medium conditions in particular (0 or 0.1 μM ABA and 1 μM BAP, 1 μM ABA and 10 μM BAP, or 10 μM ABA and 0.1 μM BAP) helped somatic embryos begin to develop. Densely packed embryonal regions with distinct suspensor could be found to differentiate from the surface of the ECs (Fig. 3A).

In Norway spruce [*P. abies* (L.) Karst], different embryogenic cell lines (polar, solar, and undeveloped types) were recognized based on their morphology and growth habit.[23] It has been suggested that well-developed mature somatic embryos can be obtained from the polar and solar types, characterized by somatic embryos with densely packed embryonal regions, when transferred to the ABA-containing medium. In the ECs of sugi, similar polar or solar types were present after transferring the ECs from the maintenance medium to ABA-, BAP-, or ABA/BAP-containing medium. Furthermore, after 30 weeks in the maintenance medium, somatic embryos with green cotyledonary structures (Fig. 3B) were found on the surface of the ECs derived from immature zygotic embryos collected July 30. This indicates that the potential for further development into mature somatic embryos may be similar to those of Norway spruce.

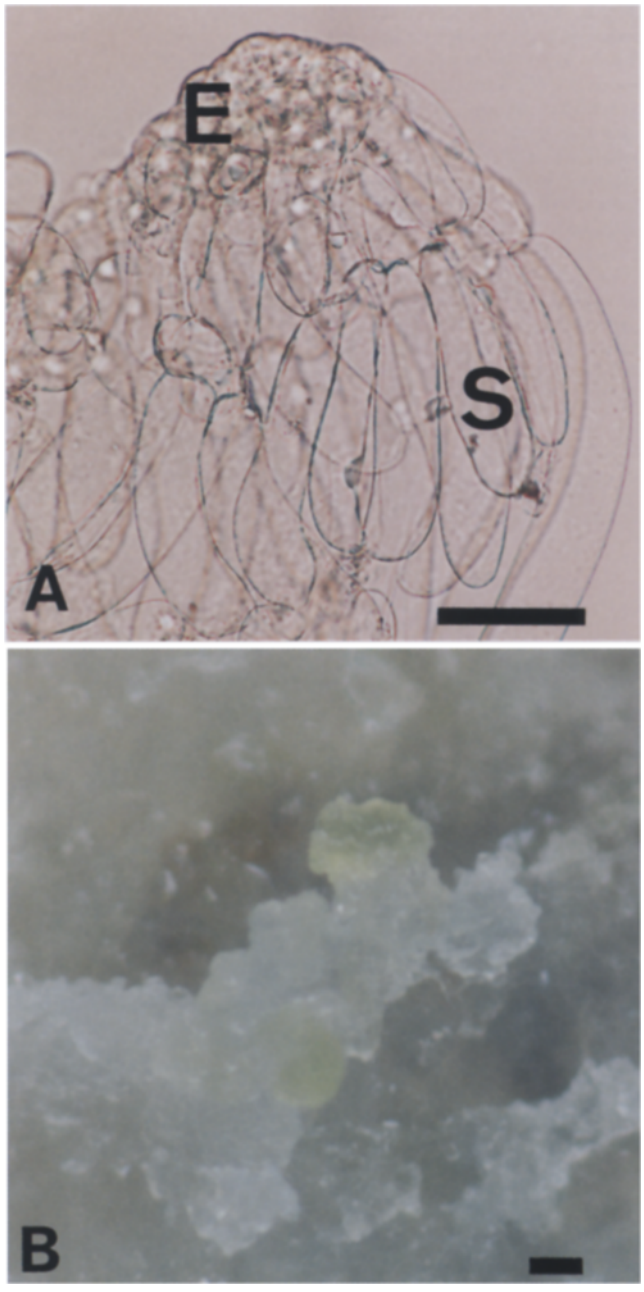


**Fig. 3.** Somatic embryogenesis from zygotic embryos of *C. japonica*. *E*, embryonal region; *S*, suspensor region. **A** After transferring to the maturation medium, somatic embryos with a densely packed embryonal region and a distinct suspensor begin to appear. *Bar* represents 100 μm. **B** In more mature culture, a somatic embryo with a green cotyledonary structure appears. *Bar* represents 1 mm

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