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Somatic Embryogenesis and Efficient Plant Regeneration in Japanese Cypresses

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1. Introduction

There are six species in the genus *Chamaecyparis* worldwide, of which two, namely the Hinoki cypress (*Chamaecyparis obtusa* Sieb. *et* Zucc.) and Sawara cypress (*Chamaecyparis pisifera* Sieb. *et* Zucc.) are distributed in Japan (Maruyama *et al.,* 2002). The Hinoki cypress is one of the most important commercial timber trees in Japan, representing about 25% of the plantation area in the country. However, the plantation areas are subject to various pests and diseases. In addition, Hinoki cypress pollinosis is reportedly one of the most serious allergic diseases in Japan (Maruyama *et al.,* 2005). The wood quality of Sawara cypress is considered inferior to Hinoki cypress, but grows faster (Fukuhara, 1978), is highly adaptable to humid and poor soils, and is considered more resistant to termite injury (Maeta, 1982) and far more tolerant to cold than the Hinoki cypress (Fukuhara, 1978). Fukuhara (1989) and Yamamoto and Fukuhara (1980) reported the possibility of obtaining natural hybrids between *C. obtusa* and *C. pisifera*.

We are interested in the development of a transgenic Japanese cypress with disease resistance and allergen-free pollen grains. Genetic engineering offers a significant tool to improve forest trees within a relatively short period. However, unfortunately, the major limitation to transformation is the difficulty in regenerating whole plants from target cells, making it vital to develop an efficient and stable plant regeneration system for genetic engineering and somatic hybridization breeding in order to develop disease-resistant hybrids. Somatic embryogenesis is an ideal procedure for effective propagation; not only of plus trees but also target tissue for genetic transformation. Since somatic embryogenesis and the plantlet regeneration of gymnosperm woody species was first reported in Norway spruce (*Picea abies* L. Karst.) (Hakman *et al.,* 1985; Chalupa, 1985; Hakman and von Arnold, 1985), studies in many other conifers have been reported (Tautorus *et al.,* 1991; Attree and Fowke, 1993; Gupta and Grob, 1995; Jain *et al.*, 1995; Hay and Charest, 1999). However, except for the *Larix* or *Picea* species and *Pinus radiata* (Lelu *et al.*, 1994a; Lelu *et al.*, 1994b; Klimaszewska *et al.*, 1997; Kong and Yeung, 1992; Kong and Yeung, 1995; Walter *et al.*, 1998), the regeneration of plants for most species is sometimes difficult or poor and effective utilization remains problematic.

In this chapter we describe a stable and efficient plant regeneration system for the Hinoki and Sawara cypress via somatic embryogenesis. The initiation of embryogenic cultures (EC), their maintenance and proliferation, maturation of somatic embryos, germination

and plant conversion, and *ex vitro* acclimatization and field transfer will be discussed in subsequent sections.

2. Embryogenic culture initiation (ECI)

Immature open-pollinated cones of the Hinoki and Sawara cypress (Fig. 1A and Fig. 2A) were collected in June and July from plus mother trees. The collected cones were subsequently disinfected by 1 min immersion in 99.5% ethanol and dried in the laminar flow cabinet before dissection. The excised seeds were disinfected with 1% (w/v available chlorine) sodium hypochlorite solution for 15 min and then rinsed five times with sterile distilled water. After the seed coats had been removed, the megagametophytes containing immature zygotic embryos were used as explants for ECI initiation.

The explants were cultured in 24-well tissue culture plates (one per well) containing 1/2 MS medium (Murashige and Skoog medium)(Murashige and Skoog, 1962)(MS medium with basal salts reduced to half the standard concentration but replacing all NH_4NO_3 with 1000 mgL^{-1} glutamine) or 1/2 EM medium (Embryo Maturation medium) (Maruyama *et al.*, 2000) (EM medium with basal salts, vitamins, and myo-inositol reduced to half the standard concentration and with KCl concentration reduced to 40 mgL^{-1}), supplemented with 0.5 gL^{-1} casein hydrolysate, 1.0 gL^{-1} glutamine, 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 5 μM 6-benzylaminopurine (BAP), 10 gL^{-1} sucrose, and 3 gL^{-1} gelrite. The pH of the media was adjusted to 5.8 prior to autoclaving for 15 min at 121°C. The cultures were kept in darkness at 25±1°C. The presence or absence of the distinct early stages of embryos characterized by an embryonal head with a suspensor system (Fig. 1C and Fig. 2C) from the explants was observed under an inverted microscope weekly for up to 3 months.

Embryogenic tissues (ET) extruding from the micropylar ends of explants appeared mostly after 2-4 weeks of culture, while the mean initiation frequency of ET from immature seeds of the Sawara cypress (Fig. 1B) varied from 12.5 to 33.3%. Initiation of ET was also possible in the absence of exogenous plant growth regulators (PGR) as reported for pine species (Smith, 1996; Lelu *et al.* 1999). In the Hinoki cypress, a medium without PGR containing 2 gL^{-1} activated charcoal (AC) was also effective for the induction of EC. The mean initiation frequencies of ET on a medium with and without PGR were 14.5 and 17.2%, respectively, which indicated that when explants are cultured at the appropriate developmental stage, the absence of exogenous PGR did not impede ECI.

The results of experiments for somatic embryogenesis initiation in both cypresses, where relatively small variations were achieved, suggested that the medium was not the most critical factor for ECI when explants were collected from late June to early July. The induction response and the beginning of germination were observed in some explants collected in mid-July. Since the physiological maturation of a seed is determined by its ability to germinate, this result indicates that the zygotic embryos from immature seeds collected in mid-July was the critical limit for ECI on a medium with no PGR. At this time, no germination was observed on PGR-supplemented medium. Among the factors influencing the somatic embryogenesis initiation, the appropriate developmental stage of zygotic embryos seemed the most critical. The optimal developmental stage for many conifer species has been reported in terms of seed collection date or time after fertilization (Becwar *et al.*, 1990; Tautorus *et al.*, 1991; Lelu *et al.*, 1994b; Jain *et al.*, 1995; Zoglauer *et al.*, 1995; Klimaszewska *et al.*, 1997; Lelu *et al.*, 1999; Kim *et al.*, 1999). However, due to the

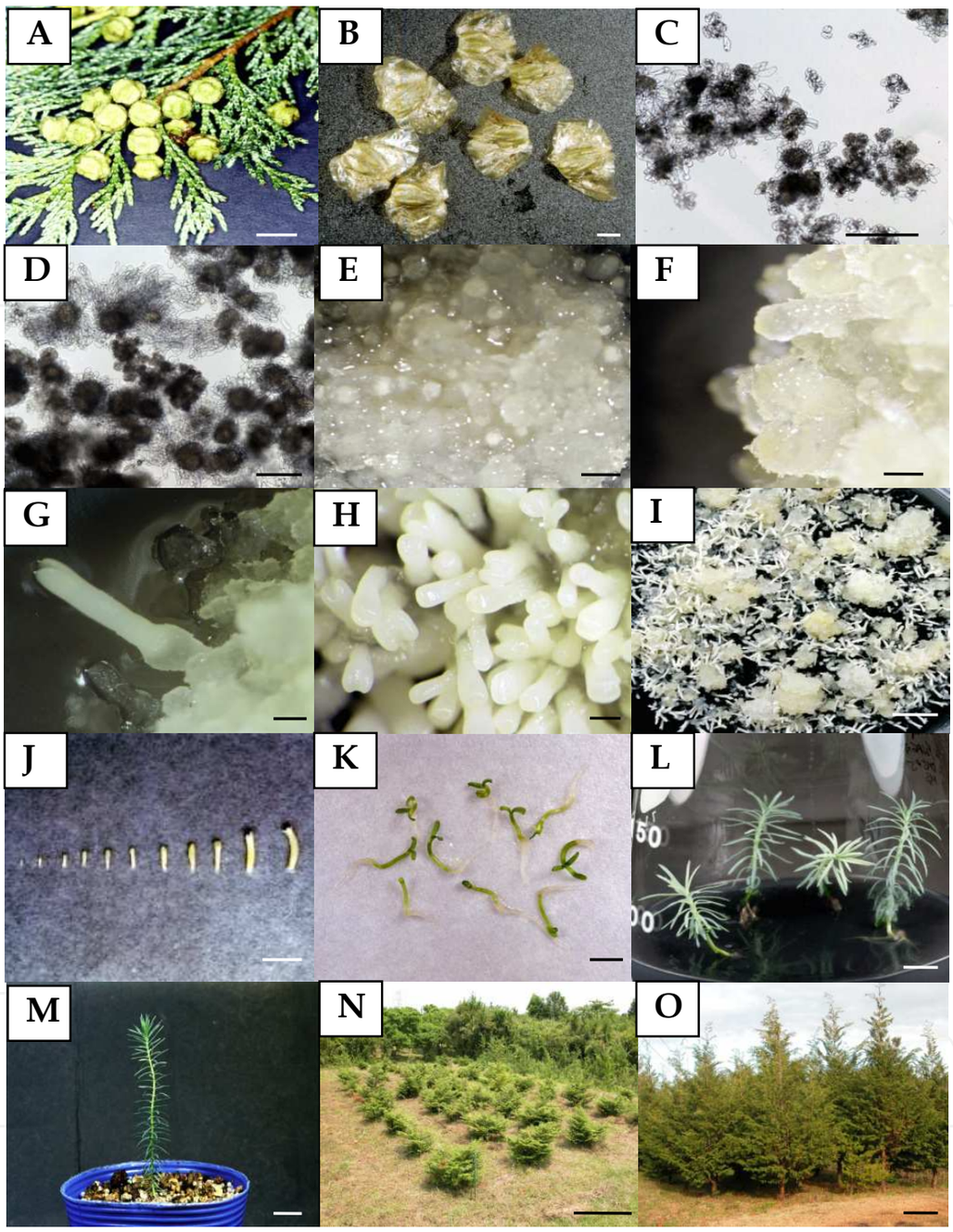

Fig. 1. Somatic embryogenesis in Sawara cypress.
A: Open-pollinated cones. B: Excised immature seeds. C: Embryogenic cells. D-F: Development of embryogenic cells. G-I: Embryo maturation. J: Different maturated embryo sizes in function to PEG concentration in maturation media. K: Germination. L: Plant conversion. M: Acclimatized plant derived from a somatic embryo. N-O: Somatic plants growing out in the field. *Bars* 1mm (B-H), 1cm (A, I-M), 1m (N-O)

difficulty in determining the precise time of fertilization in open-pollinated cones and the fact that the variation in the zygotic embryo development depends on weather and location, the criteria for explant collection for ECI cannot be easily generalized. In addition, variation in the developmental stage of embryos may be observed among trees and even the same tree when individual cones are compared. In the case of the Hinoki and Sawara cypress, most of the immature embryos collected from late June to early July seemed at the late embryogeny stage. Observation of the developmental stage of individual embryos is thus likely to be the most appropriate method to determine the optimal time for embryo selection.

In the present study, relatively high initiation frequencies were achieved for both species and almost all the initiated lines continued to proliferate, even after several years of culture, resulting in stable embryogenic lines. Sometimes however, the initiation of somatic embryogenesis may not result in the establishment of an embryogenic line because the ensuing ET ceases to proliferate, making it important to distinguish between the initial extrusion from an explants and continuous growth, when assessing the success rate (Klimaszewka *et al.*, 2007). Kim *et al.* (1999) reported that from 294 lines initiated in *Larix leptolepis*, only one embryogenic cell line could be proliferated. These results suggest that the capture of stable cell lines should be the optimal criterion by which to compare the ability of somatic embryogenesis initiation among species and families.

3. Maintenance and proliferation of embryogenic cultures

The maintenance and proliferation of EC was possible in several media containing a combination of 2,4-D plus BAP. The principal characteristics of these media were the reduction in the concentration of inorganic components from the standard and the addition of glutamine as an organic nitrogen source. The growth and proliferation of EC on media with a high concentration of inorganic components as a nitrogen source was suboptimal. These media supported growth only for short culturing whereupon the cell condition deteriorated over time. A similar response was also observed for the Japanese cedar EC (Maruyama *et al.*, 2000).

The positive effect of organic nitrogen sources in the medium on the maintenance and proliferation of EC have been reported for many species (Boulay *et al.*, 1988; Finer *et al.*, 1989; Becwar *et al.*, 1990; Gupta and Pullman, 1991; Tremblay and Tremblay, 1991; Smith, 1996; Klimaszewska and Smith, 1997). In our study, filter-sterilized glutamine in a medium combined with a reduction of the nitrate content increased the proliferation rate and the number of mature cotyledonary embryos of the Sawara cypress. In contrast, Zoglauer *et al.* (1995) reported that the continuous subculture of embryogenic suspensions of *Larix decidua* on organic nitrogen-supplemented medium resulted in a dramatic decline in the number of mature embryos obtained. Jalonen and von Arnold (1991) demonstrated the dependence of embryo maturation on the type of embryo morphology during proliferation.

In our culture routines, EC were maintained and proliferated by 2 to 3-week interval subcultures on 90-mm diameter Petri dishes containing 1/2 EM medium or 1/2 LP medium (Aitken-Christie and Thorpe, 1984) supplemented with 30 gL^{-1} sucrose, 3 μM 2,4-D, 1 μM BAP, and 1.5 gL^{-1} glutamine. These media supported the growth of the embryogenic cell lines captured. ET proliferated readily and retained their original translucent and mucilaginous appearance. The fresh weight of ET on the maintenance-proliferation medium

increased about 5- to 12-fold after a 2- to 3-wk culture period. In general, solid media were used for the maintenance routine and liquid media for rapid proliferation of the cultures. The low-density subculture helped maintain suitable conditions for EC (densely embryonal head with a distinct suspensor system) in the suspension culture. Before the maturation step, about 10-20 mg FW of ET from the solid medium were transferred to a 100 mL flask containing 30-40 mL of medium (of a composition equivalent to that used for the maintenance and proliferation but without gelrite) and cultured for about 2 weeks on a rotary shaker at 50-70 rpm, in darkness at 25±1°C.

Although the initiation of ET was also possible without any additional auxin and cytokinin supplements required, exogenous PGR were found to be essential for the continuous maintenance and proliferation of ET (Fig. 2B). Conversely, the maintenance and proliferation of EC on media with no PGR was reported for *Pinus radiata* (Smith, 1996). He indicated that the use of PGR is not necessary, and that some cell lines maintained on a medium with 2,4-D and BAP lose their plant-forming potential much sooner than others, which have been maintained on a medium without PGR. However, in our experiments, the EC of Japanese cypresses maintained in the absence of PGR showed a tendency to embryo development and a decline in proliferation over time. This result was consistent with the results reported for other Japanese conifers (Maruyama *et al.*, 2000; Maruyama *et al.*, 2005; Maruyama *et al.*, 2007).

4. Maturation of somatic embryos

About 100-200 mg FW of ET suspended in 2-3 mL of medium were plated on 70-mm diameter filter paper disks over 90-mm diameter Petri dishes containing 30-40 mL of maturation medium that contained sugar, abscisic acid (ABA), AC, polyethylene glycol 4,000 (PEG), and EMM amino acids (Smith, 1996) (gL^{-1}: glutamine 7.3, asparagine 2.1, arginine 0.7, citrulline 0.079, ornithine 0.076, lysine 0.055, alanine 0.04, and proline 0.053). The petri dishes were sealed with Novix-II film (Iwaki Glass Co., Ltd., Chiba, Japan) and kept in darkness at 25±1°C for 6-12 weeks.

4.1 Effect of kind and concentration of sugar

Table 1 shows the effect of different kinds of sugar on the maturation of Sawara cypress somatic embryos. At the tested sugar concentrations, optimal results were achieved by using maltose as a carbohydrate source. Although 30 and 50 gL^{-1} did not result in any statistical difference in terms of cotyledonary embryos per Petri dish, the peak embryo maturation frequency resulted from the medium containing 50 gL^{-1} maltose with an average of 372 mature embryos. In contrast, when sucrose was used, 50 gL^{-1} resulted in a decrease of maturation frequency. Maltose has been considered a better carbohydrate and/or osmoticant source than sucrose or glucose for embryo maturation (Uddin *et al.*, 1990; Uddin, 1993). Similarly, Nørgaard and Krogstrup (1995) reported the beneficial effect of maltose for embryo maturation of *Abies* spp. A medium containing maltose as a carbohydrate source and PEG as osmoticum was reported as an effective combination to enhance somatic embryo maturation in the Loblolly pine (Li *et al.*, 1998). These authors inferred that about a 10-fold enhancement was achieved by using maltose to replace sucrose, and that the morphology of cotyledonary embryos was improved. In our results, the morphologies of

cotyledonary embryos induced on the medium with sucrose or maltose were relatively similar. The main difference came in terms of the embryo maturation efficiency.

Kind of sugar	Concentration of sugar	
	30 gL^{-1}	50 gL^{-1}
Sucrose	108 B	158 B
Maltose	316 A	387 A

[1] Cotyledonary embryos per Petri dish. Means followed by same letter are not significantly different at $p < 0.05$. Three dishes for each treatment were used.

Table 1. Effect of kind and concentration of sugar on maturation of Sawara cypress somatic embryos[1]

4.2 Effects of ABA and AC

Table 2 showed the beneficial effect of increased ABA content in media supplemented with AC on the maturation of Sawara cypress somatic embryos. The best result was achieved with 100 μM ABA in the presence of AC, obtaining an average of 348 cotyledonary embryos per petri dish. The higher the ABA concentration, the greater the number of mature embryos. A similar result was reported in *Pinus strobus* (Klimaszewska and Smith, 1997), *Picea glauca-engelmannii* complex (Roberts *et al*., 1990a), and *P. glauca* (Dunstan *et al*., 1991). The addition of AC into the media notably enhanced the maturation efficiency, with around a 4-fold enhancement achieved by using 33.3 to 100 μM in combination with 2 gL^{-1} AC. Pullman and Gupta (1991) reported further improved Loblolly pine embryo development using a combination of ABA and AC, while Gupta *et al.* (1995) reported further improved quality of cotyledonary embryos of Douglas-fir (*Pseudotsuga menziesii*) by a combination of ABA, AC, and PEG. Similarly, Lelu-Walter el al. (2006) indicated that coating the cells with AC reduced ET proliferation and significantly enhanced the maturation of maritime pine somatic embryos. AC is widely used in tissue culture media, where it is believed to function as an adsorbent for toxic metabolic products and residual hormones (von Aderkas *et al*., 2002; Pullman and Gupta, 1991).

ABA-free media or those supplemented with a low concentration (10 μM) failed to stimulate appropriate embryo maturation, producing only a few mature cotyledonary embryos (Table 2). Embryogenic cells on media without ABA did not develop beyond the embryo stage 1, as described elsewhere (von Arnold and Hakman, 1988). Most of the proembryos arrested development, with the proliferation of EC evident. Lelu *et al*. (1999) reported that mature embryos of *Pinus sylvestris* and *P. pinaster* were produced in far higher numbers and that the development of cotyledonary somatic embryos versus abnormal, shooty ones was enhanced with the addition of 60 μM ABA in comparison with media without ABA. Somatic embryos of the hybrid larch (*Larix x leptoeuropaea*) developed normally on a medium supplemented with 60 μM ABA, but abnormally on a medium with no ABA (Gutmann *et al*., 1996). Most of the studies on somatic embryogenesis in conifers have reported ABA as a key hormone in embryo development and that the number and quality of embryo produced was vastly reduced in its absence (Durzan and Gupta, 1987; von Arnold and Hakman, 1988; Hakman and von Arnold, 1988; Attree and Fowke, 1993; Dunstan *et al*., 1998).

ABA (μM)	AC (0 gL^{-1})	AC (2 gL^{-1})
0	1 D	3 D
10	7 D	16 D
33.3	48 CD	178 B
100	84 C	348 A

[1] Cotyledonary embryos per Petri dish. Means followed by same letter are not significantly different at $p < 0.05$. Three dishes for each treatment were used.

Table 2. Effect of ABA and AC on maturation of Sawara cypress somatic embryos [1]

Several authors have suggested that the role of ABA in somatic embryogenesis is to inhibit cleavage polyembryony with the consequent development of individual somatic embryos (Durzan and Gupta, 1987; Boulay *et al*., 1988; Krogstrup *et al*., 1988; Gupta *et al*., 1991), to stimulate the accumulation of nutrients, lipids, proteins, and carbohydrates (Hakman and von Arnold, 1988), and suppress precocious germination (Roberts *et al*., 1990a). In addition, Gupta *et al*. (1993) reported improved desiccation tolerance to less than 10% water content with 80 to 90% germination rates in Norway spruce embryos produced with a combination of ABA and AC. The use of ABA for somatic embryo maturation in gymnosperms is extensively reported in the compilation of Jain *et al*. (1995).

4.3 Effect of PEG

As shown in Table 3, the addition of PEG stimulated the mature embryo production of Sawara cypress (Fig.1D-I), with a higher concentration of PEG in the medium resulting in a higher maturation frequency. The best result was obtained at a concentration of 150 gL^{-1} with an average number of 1,043 cotyledonary embryos collected per Petri dish, in comparison with 382, 215, and 13 embryos per dish at concentrations of 75, 50, and 0 gL^{-1}, respectively. In the absence of PEG, most of the proembryos did not develop into cotyledonary embryos. Embryogenic cell proliferation was evident and most of them developed into structures consisting of small embryonal heads from which elongated suspensors extended (stage 1 somatic embryos).

Although in recent years, several studies have reported promotion of the maturation of somatic embryos by the addition of ABA into media solidified with a high concentration of gellan gum (gelrite) in the absence of PEG (Klimaszewska and Smith, 1997; Lelu *et al*., 1999), the use of PEG in combination with ABA has become routine for stimulating somatic embryo maturation in many gymnosperms. In our study, a high concentration of gellan gum in the absence of PEG was not effective in promoting the somatic embryo maturation of Hinoki and Sawara cypress as described above (data not shown). In contrast, some authors have reported that PEG promotes maturation but inhibits the further development of *Picea glauca* (Kong and Yeung, 1995) and *P. abies* somatic embryos (Bozhkov and von Arnold, 1998). The results of our experiments indicated that the positive effect of PEG on maturation did not inhibit the further development of somatic embryos in Japanese cypresses. Almost all mature cotyledonary embryos germinated (Fig. 1K) and developed normal plants (Fig. 1L). The beneficial effect of PEG on embryo maturation may be related to a water stress induction similar to that generated by desiccation (Attree and Fowke, 1993), to an increase in the accumulation of storage reserves, such as storage proteins, lipids, and

polypeptides (Roberts *et al*., 1990a; Attree *et al*., 1992; Misra *et al*., 1993), and to a tolerance to water loss (Attree *et al*., 1991).

Morphological differences among somatic embryos of Sawara cypress obtained on media supplemented with different concentrations of PEG was restricted to size (Fig. 1J). The higher the PEG concentration, the smaller the resulting embryos (Table 3). However, the embryo size was not found to be influential in germination and subsequently plant conversion. Cotyledonary embryos germinated and converted in plants at high frequencies independent of their size (Table 3). More compact PEG-treated embryos were also reported for *Larix laricina* (Klimaszewska *et al*., 1997) and *Picea abies* (Find, 1997). Iraki *et al.* (1989) reported that small cell size was a typical symptom of PEG-induced osmotic stress. Low external osmotic potential may have led to alterations in the cell wall composition, decreasing the ratio of cellulose to hemicellulose. This results in decreased cell wall tensile strength and the reduced ability of cells to expand (Iraki *et al*., 1989). Therefore, the presence of PEG in the maturation medium may have influenced the subsequent growth of somatic embryos. Bozhkov and von Arnold (1998) determined that the morphology of mature somatic embryos of *Picea abies* had changed after PEG-treatment (smaller, irregularly shaped embryos, smaller cell size, larger root caps with intercellular spaces in pericolumn, degraded quiescent center), which could further restrict the embryo growth. However, in our study the subsequent development of PEG-treated embryos was no different to untreated ones. Germination frequencies and plant conversion rates of Sawara cypress were similar in somatic embryos derived from different PEG-treated media (Table 3).

PEG (gL^{-1})	Somatic embryos	Size range (mm)	Germination (%)	Conversion (%)
0	13 C	3-10	97 A	92 A
50	215 BC	2- 8	98 A	93 A
75	382 B	2- 6	97 A	93
150	1,043 A	1- 3	97 A	92 A

[1] Cotyledonary embryos per Petri dish. Means followed by same letter are not significantly different at $p < 0.05$. Three dishes for each PEG concentration were used.

Table 3. Effect of PEG concentration in maturation media on production, size, germination and plant conversion of Sawara cypress somatic embryos [1]

The development of a proembryo mass of Hinoki cypress was encouraged by the transfer of EC onto a PGR free-medium. Cells developed gradually to form an individual and compact mass showing the early stages of somatic embryos going to a mature stage (Fig. 2D-E). Embryo maturation was induced by the transfer of cultures onto a medium containing maltose, PEG, AC, ABA and a higher concentration of amino acids. The embryos continue to develop and after 3-4 weeks of culture the initial formation of the cotyledonary embryo was observed (Fig. 2F). For most cell lines, the development of somatic embryos to the cotyledonary stage was observed after about 6-8 weeks of culture (Fig. 2G).

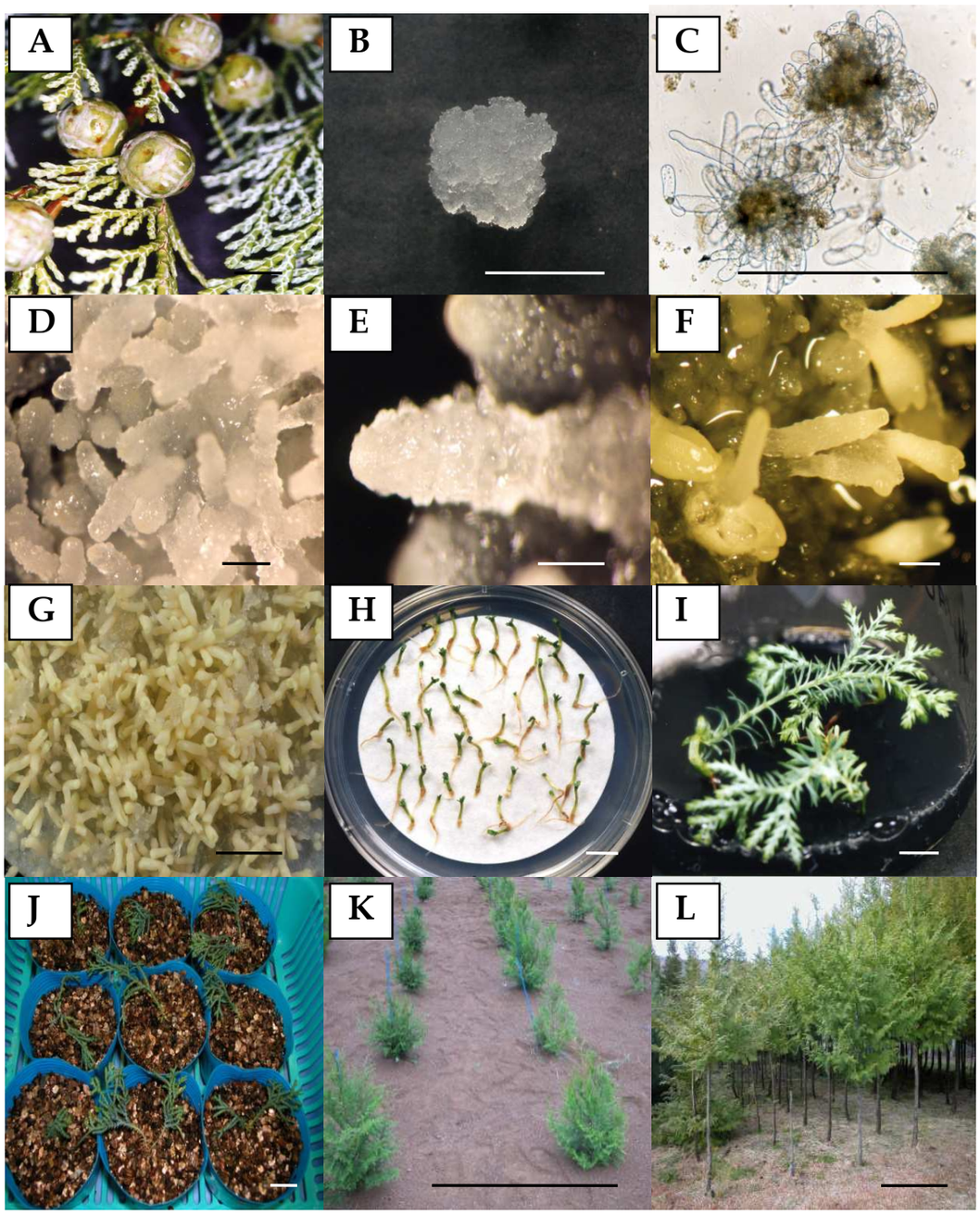

Fig. 2. Somatic embryogenesis in Hinoki cypress. A: Collected open-pollinated cones. B: Proliferation of induced embryogenic tissue on medium containing auxin and cytokinin. C: Embryogenic cells. D-F: Different developmental maturation stages of somatic embryos. G: Production of somatic embryos. H: Germination of somatic embryos. I: Plantlets growing *in vitro*. J: Acclimatized plants derived from somatic embryos. K-L: Somatic plants growing out in the field. *Bars* 1mm (C-F), 1cm (A-B, G-J), 1m (K-L)

Cell line	Total number of somatic embryos	Number of somatic embryos per Petri dish (Mean ± SE [1])	Germination frequency (%) (germinants/total tested)	Conversion frequency (%) (plants/total tested)
HNO7-1	1403	467.7 ± 21.3	94 (659/700)	91 (637/700)
HNO7-2	32	10.7 ± 1.2	50 (5/10)	40 (4/10)
HNO7-3	450	150.0 ± 15.0	82 (41/50)	78 (39/50)
HNO7-4	312	104.0 ± 18.6	83 (33/40)	80 (32/40)
HH2-1	47	15.7 ± 4.3	80 (8/10)	80 (8/10)
HH2-2	30	10.0 ± 1.7	70 (7/10)	70 (7/10)
HN1-1	54	18.0 ± 5.7	50 (5/10)	40 (4/10)
HN1-2	57	19.0 ± 3.8	76 (38/50)	72 (36/50)
HHA2-1	1536	512.0 ± 34.8	93 (219/236)	91 (215/236)
HHA2-2	188	62.7 ± 22.6	86 (43/50)	80 (40/50)
HHA2-5	14	4.7 ± 1.2	70 (7/10)	60 (6/10)
HHA2-6	1724	574.7 ± 78.3	94 (317/336)	92 (308/336)
HF4-1	565	188.3 ± 34.6	95 (123/130)	94 (122/130)
HF4-11	12	4.0 ± 2.1	NT[2]	NT
HF4-15	170	56.7 ± 9.0	98 (47/48)	96 (46/48)
HF4-19	181	60.3 ± 11.8	90 (18/20)	90 (18/20)
HF4-21	7	2.3 ± 1.9	NT	NT
HK7-17	4	1.3 ± 0.9	NT	NT
HK7-25	209	69.7 ± 11.0	93 (28/30)	93 (28/30)
HK7-29	8	2.7 ± 1.5	NT	NT
HK7-30	1511	503.7 ± 86.0	100 (130/130)	100 (130/130)
HK7-33	1052	350.7 ± 47.7	99 (286/290)	97 (280/290)
HK7-39	33	11.0 ± 1.0	70 (7/10)	60 (6/10)
HK7-45	19	6.2 ± 1.9	50 (5/10)	50 (5/10)
HK7-46	3	1.0 ± 0.6	NT	NT
HK7-57	280	93.3 ± 19.3	98 (41/42)	95 (40/42)
HK7-58	10	3.3 ± 0.3	NT	NT
HK7-60	18	6.0 ± 2.6	NT	NT
HK7-72	10	3.3 ± 1.9	NT	NT
HK7-75	1428	476.0 ± 56.0	100 (50/50)	100 (50/50)
HK7-83	7	2.3 ± 1.5	NT	NT
HK7-88	30	10.0 ± 5.1	NT	NT
HK7-105	66	22.0 ± 6.2	NT	NT
HK7-107	66	22.0 ± 1.5	60 (6/10)	60 (6/10)
Total	11536	113.1±18.1	93 (2123/2282)	91 (2067/2282)

[1]SE: standard errors of means of 3 replicates for each cell line
[2]NT: non-tested

Table 4. Somatic embryo production, germination and plant conversion for 34 cell lines of Hinoki cypress

Mature cotyledonary embryos were produced in 34 of 50 embryogenic cells lines tested (68%), and the mean number of somatic embryos per Petri dish produced varied from 1 to 575 (Table 4). This result indicates that the potential to develop cotyledonary somatic embryos varied among the cell lines. Similar results were reported for the Japanese cedar (Igasaki *et al*., 2003), maritime pine (Ramarosandratana *et al*., 2001; Miguel *et al*., 2004; Lelu-Walter *et al*., 2006), and Japanese pines (Maruyama *et al*., 2005; Maruyama *et al*., 2007).

5. Germination and plant conversion

Mature cotyledonary somatic embryos were collected from the maturation medium and transferred to the germination medium (1/2 LP or a 1/2 EM PGR free-medium with 2 gL^{-1} AC and 10 gL^{-1} agar). Cultures were kept at 25±1°C under a photon flux density of about 65 $\mu mol\ m^{-2}s^{-1}$ with cooling and fluorescent lamps for 16 h daily.

The start of germination (Fig. 2H) was observed as early as 3-5 days after transfer to the germination medium, and after 2-4 weeks of culture, most of the somatic embryos germinated and were converted into plantlets. The mature cotyledonary somatic embryos from 23 embryogenic cell lines of the Hinoki cypress were tested, with mean germination and plantlet conversion frequencies of 93 and 91%, respectively (Table 4). This result was similar to that achieved for the Sawara cypress (Table 3). No morphological difference among the germinants and plantlets was observed among the genotypes.

Regenerated emblings of Hinoki (Fig. 2I) and Sawara cypress (Fig. 1L) were transferred to 300 ml flasks containing 100 mL of fresh medium (same composition used for the germination and conversion but with 30 gL^{-1} sucrose and 5 gL^{-1} AC) and kept under the same conditions described above for 8-12 weeks before *ex vitro* acclimatization.

6. *Ex vitro* acclimatization and field transfer

The developed emblings of the Hinoki (Fig.2J) and Sawara cypress (Fig.1M) were transplanted into plastic pots filled with vermiculite and acclimatized in plastic boxes inside a growth cabinet. During the first 2 weeks, emblings were kept under high relative humidity by covering the plastic boxes with transparent plastic covers and irrigating with tap water. Subsequently, the cover was gradually opened and the pots were fertilized with a nutrient solution modified from Nagao (1983) containing in mgL^{-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. The covers were completely removed about 4 weeks after transplanting. Survival rates ranging from 90 to 100% were achieved after acclimatization. Subsequently, the acclimatized plants were transferred to a greenhouse and grown under controlled conditions for 6-8 months before transplanting to the field. No indication of any morphological abnormality was reported, and the growth of established plants is currently being monitored in the field (Fig. 1N-O and Fig. 2K-L).

7. Concluding remarks

An effective plant regeneration system has been achieved for Japanese cypresses via the specified procedure. In addition to high somatic embryo maturation efficiency, the

subsequent high germination and plant conversion frequencies attained demonstrated the high quality of the somatic embryos produced. These somatic embryos have a zygotic embryo-like morphology, are generally longer than they are wide, with radial symmetry, and have the ability to produce normal plants like the zygotic one. The maturation frequency and the quality of embryo produced are the key criteria for the optimization of an efficient plant regeneration system via somatic embryogenesis. The cotyledonary somatic embryos of the Hinoki and Sawara cypress readily germinated after transfer to a PGR-free medium without any kind of post-maturation treatment, as was previously reported as necessary to promote the germination of somatic embryos of some other species (Roberts *et al.*, 1990b; Roberts *et al.*, 1991; Kong and Yeung, 1992; Kong and Yeung, 1995; Jones and van Staden, 2001). Thus, most of the germinants developed epycotyl and grew into normal plants. The present system should permit, in the near future, the large-scale clonal propagation of selected trees and the genetic engineering of Japanese cypresses.

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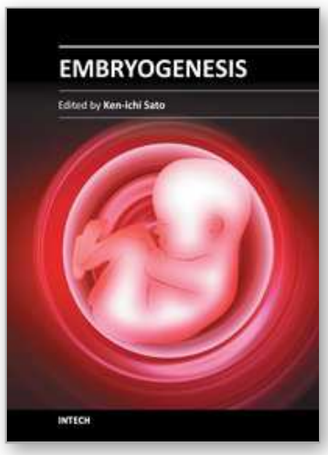

Embryogenesis
Edited by Dr. Ken-Ichi Sato

ISBN 978-953-51-0466-7
Hard cover, 652 pages
Publisher InTech
Published online 20, April, 2012
Published in print edition April, 2012

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How to reference
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Tsuyoshi E. Maruyama and Yoshihisa Hosoi (2012). Somatic Embryogenesis and Efficient Plant Regeneration in Japanese Cypresses, Embryogenesis, Dr. Ken-Ichi Sato (Ed.), ISBN: 978-953-51-0466-7, InTech, Available from: http://www.intechopen.com/books/embryogenesis/somatic-embryogenesis-and-efficient-plant-regeneration-in-japanese-cypresses

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