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Somatic Embryogenesis in Sawara Cypress (*Chamaecyparis pisifera* Sieb. et Zucc.) for Stable and Efficient Plant Regeneration, Propagation and Protoplast Culture

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Somatic embryogenesis in *Chamaecyparis pisifera* was initiated from immature seeds collected from the end of June to early July. We obtained initiation frequencies ranging from 12.5 to 33.3% using whole seed explants in liquid media. Embryogenic cultures were maintained and proliferated for more than a year in solid and liquid media. High maturation frequencies of "high quality" embryos were obtained on maturation media containing abscisic acid (ABA), activated charcoal (AC), and polyethylene glycol (PEG) as osmotic agent. More than one thousand cotyledonary embryos on average per 100 mg initial fresh weight of embryogenic cells were attained on medium containing 100 μM ABA, 2 gL^{-1} AC, and 150 gL^{-1} PEG. About 97% germination frequencies and 92% plant conversion rates were achieved without any pretreatment. Growing of plants regenerated from somatic embryos has been monitored in the field. Furthermore, a procedure for culture of protoplasts isolated from embryonal masses was also described.

Key words: *Chamaecyparis pisifera*, protoplast, Sawara cypress, somatic embryogenesis

Somatic embryogenesis is an ideal procedure for effective propagation of not only plus trees but also target tissue for genetic transformation. Up to now, there are many examples of somatic embryogenic regeneration with conifers (Hay and Charest, 1999). In almost all woody gymnosperm species, zygotic embryos are the frequently used explants for initiation of somatic embryogenesis (Raemakers *et al.*, 1999). However, regeneration of plants is usually difficult or at very low frequency except in the case of *Larix* species (Lelu *et al.*, 1994a, b, c).

The Sawara cypress belonging to the Taxodiales is one of the six species in the genus *Chamaecyparis* all over the world. Two of them, namely Hinoki cypress and Sawara cypress, are distributed in Japan (Ishii, 1991). Micropropagation of Hinoki cypress (*Chamaecyparis obtusa* Sieb. et Zucc.) using shoot culture was reported (Ishii and Sato, 1989). Somatic embryo induction and plantlet regeneration at low rate was reported with Hinoki cypress only among *Chamaecyparis* species (Ishii and Maruyama, 1998). We here succeeded in the production of many plantlets by somatic embryogenesis from immature seeds of Sawara cypress (*Chamaecyparis picifera* Sieb et Zucc.).

The wood quality of Sawara cypress is considered inferior to Hinoki cypress; however, it grows faster in its young stage (Fukuhara, 1978). It also has high adaptability to humid and unproductive soil and resistance against termite injury (Maeta, 1982). It is considered much cold hardier and easy to propagate from cuttings (Fukuhara, 1978). Yamamoto and Fukuhara (1980) report the possibility of obtaining natural hybrids between *C. obtusa* and *C. pisifera*.

Plant regeneration through somatic embryogenesis of Sawara cypress is important for genetic engineering and somatic hybridization breeding to create hybrids resistant to disease. A stable and efficient plant regeneration system is important for genetic engineering. Somatic embryogenesis is the most attractive plant regeneration system for genetic transformation.

Since somatic embryogenesis and plantlet regeneration of woody gymnosperm 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). However, information on somatic embryogenesis in Sawara cypress cannot be found.

In this report we describe a stable and efficient plant regeneration system for propagation and conservation of Sawara cypress by somatic embryogenesis from immature seeds.

Materials and Methods

1 Initiation of embryogenic cultures

Immature, open-pollinated cones were collected in 1997 and 1998 (late June to early July), from 25–30 year-old trees (mixed cones from three mother trees) at the Forestry and Forest Products Research Institute, Tsukuba, Japan (Fig. 1A). Experiments to induce embryogenic cultures were carried out using whole seeds.

Collected cones (Fig. 1B) were disinfected by 15 min immersion in 70% ethanol supplemented with few drops of neutral detergent and then washed in tap water before dissection. Excised seeds (Fig. 1C) were disinfected with 3% (w/v available chlorine) sodium hypochlorite solution for 30 min and then rinsed five times with sterile distilled water.

The explants were cultured in 24-well tissue culture plates (one per well) containing 1 mL of liquid MS medium (Murashige and Skoog's medium) (Murashige and Skoog, 1962), 1/2 MS medium (MS medium with basal salts reduced to half concentration from the standard), MS1/2Gl medium (modified from MS medium replacing half concentration of NH_4NO_3 with 500 mg/L of L-glutamine(filter sterilized)), MSGl medium (modified from MS medium replacing all NH_4NO_3 with 1,000 mg/L of L-glutamine(filter sterilized)), or

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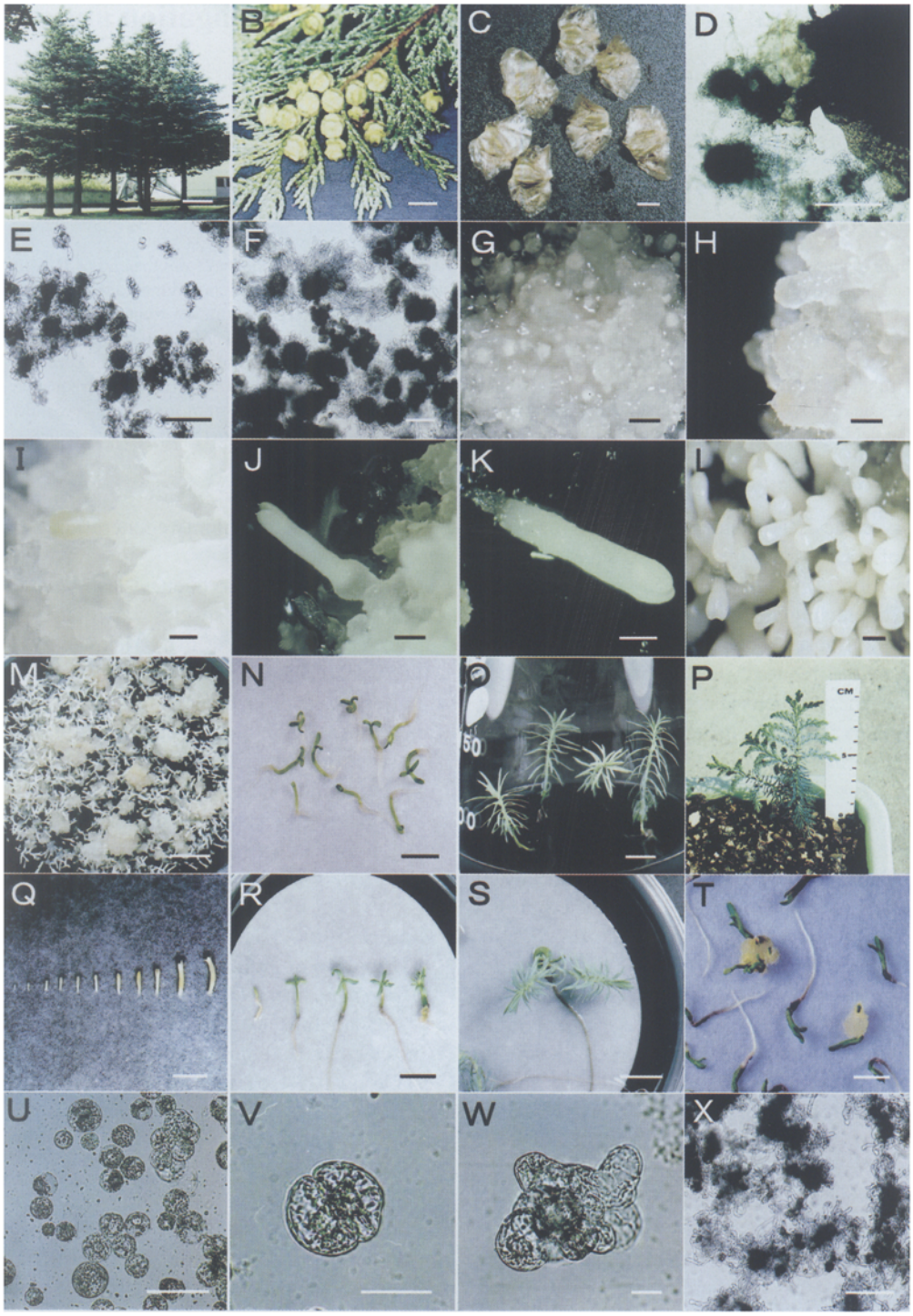

Fig. 1 Somatic embryogenesis in *Chamaecyparis pisifera*. A: Mother trees (bar = 1 m). B: Collected open-pollinated cones (bar = 1 cm). C: Excised immature seeds (bar = 1 mm). D: Embryogenic cell initiation from whole seed in liquid medium (bar = 1 mm). E: Embryogenic cells proliferating in liquid medium (bar = 1 mm). F: Development of embryogenic cells in liquid medium with no plant growth regulators (bar = 1 mm). G–M: Different developmental maturation stages of somatic embryos on solid medium containing ABA, AC, and PEG (bar: G–L = 1 mm; M = 1 cm). N: Germination of somatic embryos (bar = 1 cm). O: Emblings growing *in vitro* (bar = 1 cm). P: Acclimatized embling. Q: Different somatic embryo sizes in function to maturation media (bar = 1 cm). R: Germinated somatic embryos showing (from left to right) 1, 2, 3, 4, and 5 cotyledons (bar = 1 cm). S: Double-shoot embling (bar = 1 cm). T: Secondary embryogenesis and spontaneous plant regeneration on germinated somatic embryos (bar = 1 cm). U: Isolated protoplasts (bar = 100 μm). V: Cell division in a cultured protoplast (bar = 100 μm). W: Colony formation (bar = 100 μm). X: Embryogenic cell proliferation from cultured protoplasts (bar = 1 mm).

MSC (MS medium with 0.1% (w/v) casamino acid) supplemented with different combinations of 2,4-dichlorophenoxyacetic acid (2,4-D)(0, 0.1, 1, 10, 30 μM) and 6-benzylaminopurine (BAP)(0, 0.1, 1, 10 μM). Sucrose content was 10 g/L in initiation medium. The pH of the media was adjusted to 5.8 prior to autoclaving for 15 min at 121°C. Twenty four immature seeds were used for each basic medium treatment. Cultures were kept in darkness at 25 ± 1°C.

The presence or absence of distinct early stages of embryos characterized by an embryonal head (dense cells) with suspensor system (elongated cells) from the explants was observed under an inverted microscope weekly up to 3 months.

2 Maintenance and proliferation of embryogenic cultures

Embryogenic cells were subcultured using 3.5 mL transferpipette (Sarstedt) in liquid or solid LPm medium (modified from Quorin and Lepoivre's medium)(LP medium)(Aitken-Christie and Thorpe, 1984), containing basal salts reduced to half concentration from the standard, 10 gL^{-1} sucrose, 10 μM 2,4-D and supplemented with 500 mgL^{-1} L-glutamine (filter sterilized). Cultures were kept in darkness at 25 ± 1°C. About 5–10 mg FW per 100 mL flask (containing 30–40 mL of medium) were subcultured every 2 weeks on a rotary shaker at 100 rpm. For solid medium, five embryonal masses (about 10–20 mg FW each) were subcultured per petri dish at intervals of 4 weeks.

3 Maturation of somatic embryos

1) Effect of basal media

Proliferated embryogenic cells were cultured in liquid LPmf medium (LPm medium with no plant growth regulators containing 30 gL^{-1} sucrose) for 1–2 weeks, on a rotary shaker at 100 rpm in darkness at 25 ± 1°C, before plating on maturation media. Maturation media containing basal salts and vitamins from the original MS medium, WP medium (Woody Plant medium)(Lloyd and McCown, 1980), LP medium, SM medium (Embryo Development medium)(Smith, 1996), or EM medium (Embryo Maturation medium) (Maruyama *et al.*, 2000) were tested.

Media were supplemented with 50 gL^{-1} maltose, amino acids according to SM medium, 75 gL^{-1} polyethylene glycol 4,000 (Wako Pure Chem. Ind., Ltd.) (PEG), 2 gL^{-1} activated charcoal (AC), and 100 μM racemic abscisic acid (ABA) (Wako Pure Chem. Ind., Ltd.). Amino acid stock solutions and ABA were filter sterilized and added to the medium after autoclaving. Media were solidified with 5 gL^{-1} gelrite.

For maturation experiments, embryogenic cells cultured for 7–14 days in liquid LPmf medium (Fig. 1F) were collected on 100 μm nylon screen. Then, embryonal masses were resuspended in the same fresh medium and plated on different maturation media. About 100 mg FW of embryonal masses suspended in 2 mL of the same medium were plated on filter paper disks (Advantec No.2, 70 mm in diameter) over 90 × 20 mm petri dishes containing maturation media (30–40 mL per petri dish), sealed with Novix-II film (Iwaki Glass Co., Ltd.) and cultured in darkness at 25 ± 1°C for 6 to 12 weeks. Four petri dishes for each treatment were tested.

2) Effect of kind of sugar

For further experiments, SMm medium (modified from SM medium) containing 40 mgL^{-1} H_3BO_3, 20 mgL^{-1} $MnSO_4 \cdot 4H_2O$, 750 mgL^{-1} KCl, and 75 mgL^{-1} $CaCl_2$, was used as basal maturation medium.

SMm medium containing 30 or 50 gL^{-1} sucrose or maltose, 75 gL^{-1} PEG, and 2 gL^{-1} AC was supplemented with 100 μM ABA, and solidified with 5 gL^{-1} gelrite. The pH of the media was adjusted to 5.8 prior to autoclaving. Three petri dishes for each treatment were tested.

3) Effect of pH of medium

SMm medium containing 50 gL^{-1} maltose, 75 gL^{-1} PEG, and 2 gL^{-1} AC was supplemented with 100 μM ABA and solidified with 5 gL^{-1} gelrite. The pH of the media was adjusted to 4.3, 4.8, 5.3, 5.8, 6.3, 6.8, or 7.3 prior to autoclaving. Three petri dishes for each treatment were tested.

4) Effect of ABA and AC

SMm medium containing 50 gL^{-1} maltose, 75 gL^{-1} PEG, and 0 or 2 gL^{-1} AC was supplemented with 0, 10, 33.3, or 100 μM ABA. The pH of the media was adjusted to 5.8 prior to autoclaving. Media were solidified with 5 gL^{-1} gelrite. Three petri dishes for each treatment were tested.

5) Effect of PEG

SMm medium containing 50 gL^{-1} maltose, 2 gL^{-1} AC, and 0, 50, 75, or 150 gL^{-1} PEG was supplemented with 100 μM ABA and solidified with 5 gL^{-1} gelrite. The pH of the media was adjusted to 5.8 prior to autoclaving. Ten petri dishes for each treatment were tested.

4 Germination and plant conversion

Somatic embryos were collected from maturation media, and transferred to germination media (Table 1). The cultures were kept at 25 ± 1°C under photon flux density of about 65 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps (100 V, 40 W; Toshiba Co.) for 16 h daily. The number of somatic embryos germinated (root emergence) and converted to plantlets (emergence of both root and epicotyl) was recorded after 2 and 4 weeks, respectively. The germinants were transferred to 300 mL Erlenmeyer flasks containing 100 mL of LPmAC fresh medium (modified LP medium containing basal salts reduced to half concentration from the standard, 30 gL^{-1} sucrose, 5 gL^{-1} activated charcoal, and solidified with 12.5 gL^{-1} Wako agar) and kept under the conditions described above. Emblings that showed root and epicotyl growth were transferred into flasks containing vermiculite fertilized with 0.1% (v/v) Hyponex plant food solution (The Hyponex Co., Inc.) containing (w/v): 5.0% N, 4.36% P, and 4.15% K, and cultured for about 4 weeks before *ex vitro* acclimatization.

5 *Ex vitro* acclimatization and growth of emblings

Developed emblings were transplanted into pots filled with vermiculite and/or perlite and acclimatized under different conditions (Table 2). Sixteen to 33 emblings were acclimatized in each treatment. During the first 2 weeks emblings were kept under high relative humidity by covering pots with transparent plastic films and irrigating with tap water. After that, the cover was opened gradually and pots were fertilized with

Table 1 Effect of media on germination and conversion of *Chamaecyparis pisifera* somatic embryos.

Media	Basal salts [1]	Sucrose (gL^{-1})	Glucose (gL^{-1})	AC (gL^{-1})	Agar (gL^{-1})	Gelrite (gL^{-1})	Germination[2] %	(SE)	Conversion[2] %	(SE)
LPS1	1/2 LP	10	0	0	8	0	100	(0.0) [a]	91	(1.9) [a]
LPS2	1/2 LP	20	0	0	8	0	99	(1.0) [a]	91	(4.1) [a]
LPS3	1/2 LP	30	0	0	8	0	96	(2.8) [a]	92	(1.6) [a]
LPS2AC2	1/2 LP	20	0	2	12	0	99	(1.0) [a]	93	(2.5) [a]
LPS2AC5	1/2 LP	20	0	5	12	0	98	(1.2) [a]	92	(3.7) [a]
LPS3AC5	1/2 LP	30	0	5	12	0	97	(1.0) [a]	92	(1.6) [a]
LPG	1/2 LP	0	30	0	0	5	56	(4.9) [c]	53	(5.3) [b]
SMG	SM-GER	0	30	0	0	5	19	(3.4) [e]	19	(3.4) [d]
WPMAC2	WPM	20	0	2	0	5	100	(0.0) [a]	90	(3.8) [a]
Water (−)	Without	0	0	0	8	0	72	(5.9) [b]	16	(4.3) [d]
Water (+)	Without	20	0	0	8	0	96	(1.6) [a]	4	(1.6) [de]
Vermiculite	Nm	0	0	0	0	0	80	(8.6) [b]	30	(7.4) [c]
Perlite	Nm	0	0	0	0	0	40	(8.6) [d]	10	(3.5) [de]

[1] 1/2LP, half strength Quorin and Lepoivre's medium (Aitken-Christie and Thorpe, 1984); SM-GER, Embryo germination medium (Smith, 1996); WPM, Woody plant medium (Lloyd and McCown, 1980); Nm, modified Nagao's medium (Maruyama *et al.*, 2000). [2] Means followed by same letter are not significantly different at $p < 0.05$; (SE), standard error; One hundred to 500 somatic embryos were used for each treatment.

Table 2 Effect of substrate and acclimatization conditions on survival and growth of regenerated *Chamaecyparis pisifera* emblings.

Environment	Acclimatization conditions [1] T (°C)	RH (%)	Light	Substrate [2] (ratio)	Total No.	Survival (%)	Growth[3] (cm)	(SE)
Glass-house	20	No controled	Natural	V	30	100	2.50	(0.12)[bc]
Glass-house	20	No controled	Natural	P	33	75.8	2.38	(0.12)[bc]
Glass-house	20	No controled	Natural	V:P (1:1)	30	100	2.74	(0.11)[b]
Phytotron	20/15	75	Natural	V:P (1:1)	16	100	4.76	(0.28)[a]
Growth cabinet	25–35	No controled	White cool	V:P (1:1)	16	93.8	2.28	(0.16)[c]

[1] Phytotron, alternating temperature of 20°C for 16 h and 15°C for 8 h; Growth cabinet, temperature ranging from 25 to 35°C under photon flux density of 35 μmol m^{-2} s^{-1} with a 16 h photoperiod provided by cool white fluorescent lamps (100 V, 20 W; Toshiba Co.). [2] V, vermiculite; P, perlite. [3] Means followed by same letter are not significantly different at $p < 0.05$; (SE), standard error; Data were collected after 2 months culture.

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. Covers were removed completely about 4 weeks after transplanting.

6 Statistical analysis

Standard errors of means were calculated according to Snedecor (1957), and statistically significant mean differences are determined by the Least Significant Difference (LSD) test at 95% probability level. Numbers of the cultures are described in each Table or Figure.

7 Isolation and culture of protoplast

Protoplasts were isolated from suspension cultures maintained for about 1 year. Cells from 14 to 21-day-old embryogenic suspension cultures were collected on 100 μm nylon Falcon cell strainer (Becton Dickinson & Co.) and incubated in an enzyme solution containing different combinations of 1% (w/v) Cellulase Onozuka RS (Yakult Honsha Co., Ltd.) (RS), 0.1% (w/v) Pectolyase Y-23 (Kikkoman Corporation) (P), 1% (w/v) Macerozyme R-10 (Yakult Honsha Co., Ltd.) (M), 1% (w/v) Cellulase Onozuka R-10 (Yakult Honsha Co., Ltd.) (R-10), and 0.4 or 0.6 M mannitol for 5 h at 25°C. The digest was filtered through a 40 μm cell strainer and then centrifuged at $100 \times g$ for 3 min. The supernatant was removed and the protoplasts were resuspended in 0.4 or 0.6 M mannitol solution and centrifuged once more. The yield of protoplasts was counted with a haemacytometer and their viability was verified using fluorescein diacetate (FDA). Protoplasts were cultured at a density of 1×10^2, 5×10^2, 1×10^3, 5×10^3, 1×10^4, and 5×10^4 mL^{-1} in 96-well tissue culture plates containing 50 μL of 0.6 M mannitol proliferation media supplemented with various combinations of 2,4-D (0.1, 0.3, 1, 3, 10, and 20 μM) and BAP (0, 0.1, 0.3, 1 μM). Plates containing protoplast cultures in wells and sterile distilled water in outspaces among wells (to maintain relative humidity) were sealed with Novix-II film and kept at 28 ± 1°C in darkness. The plating efficiency percentages (number of colonies/initial plating density × 100) were determined after 6 weeks of culturing.

Results

1 Initiation, maintenance and proliferation of embryogenic cells

The initiation of somatic embryogenesis was observed in tested media supplemented with different combinations of

2,4-D plus BAP (data not shown). Embryogenic cells characterized by an embryonal head (dense cytoplasm cells) with suspensor system (elongated cells) appeared after 2–4 weeks of culture (Fig. 1D) and about 45 days after transferring of whole seeds to induction media the initiation frequencies varied from 12.5 to 33.3%.

The results of experiments for somatic embryogenesis initiation, where relatively small variations were achieved, indicated that the medium was not the most critical factor for embryogenic cell induction when explants were collected from late June to early July. Among the factors influencing the somatic embryogenesis initiation the appropriate developmental stage of zygotic embryos seems to be the most critical one. 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.*, 1994c; Jain *et al.*, 1995; Zoglauer *et al.*, 1995; Klimaszewska *et al.*, 1997; Lelu *et al.*, 1999; Kim *et al.*, 1999). However, due to the difficulty of determining the precise time of fertilization in open-pollinated cones and the the fact that the variation in the zygotic embryo development depends on weather and location, the criteria for explant collection for embryogenic cell induction can not be easily generalized. In addition, variation in developmental stage of embryos may be observed among trees and also within the same tree, including even variation inside the some cone. In the case of Sawara, most immature embryos seemed at the late embryogeny stage from late June to early July. Probably, the observation of developmental stage of individual embryos is the most appropriate method to determine the optimal time for embryo selection.

In many conifer species, embryogenic cultures have been successfully initiated, culturing intact megagametophytes as well as embryos isolated from immature seeds. In Sawara cypress, we obtained relatively high initiation frequency using whole seed explants in liquid medium. This method offers the potential for less handling in preparing explants for somatic embryogenesis initiation, particularly for species with small seeds.

Culture conditions for maintenance and proliferation of embryogenic cells were determined using different media and combinations of 2,4-D and BAP (data not shown). The maintenance and proliferation of Sawara cypress embryogenic cells was possible in several media such as LPm, MSm, 1/2 EM, SM2, and MSG3 medium containing a combination of 2,4-D (1–10 μM) plus BAP (0–3 μM). The principal characteristic of these media was the reduction in concentration of inorganic components from the standard and the addition of L-glutamine as an organic nitrogen source (Maruyama *et al.*, 2000). The positive effect of organic nitrogen sources in the medium on the maintenance and proliferation of embryogenic cells has 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; Jain *et al.*, 1995; Zoglauer *et al.*, 1995; Smith, 1996; Klimaszewska and Smith, 1997; Lelu *et al.*, 1999).

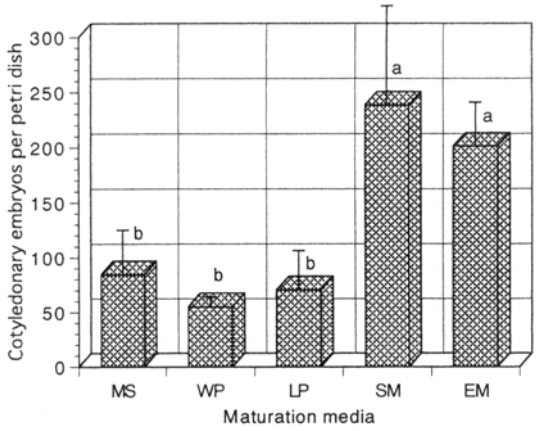

Fig. 2 Effect of media on maturation of *Chamaecyparis pisifera* somatic embryos. Bars indicate the standard error. Means followed by same letter are not significantly different at $p < 0.05$. Four petri dishes for each treatment were used.

In general, the presence or absence of BAP in media was not a critical factor for maintenance and proliferation of tested embryogenic cells. Therefore, for further subcultures LPm medium supplemented with 10 μM 2,4-D alone was used for rapid proliferation (Fig. 1E). The same medium solidified with 3 gL^{-1} gelrite was used for maintenance of the cultures. Low density of subculture (5–10 mg FW per flask) was beneficial for good condition maintenance of embryogenic cells in suspension culture. In contrast, when embryogenic cells were cultured at high density the development of suspensor system was drastically suppressed. A similar response was reported for Japanese cedar (Maruyama *et al.*, 2000).

2 Maturation of somatic embryos

1) Effect of basal media

Embryonal masses developed gradually showing early stages of somatic embryos going to mature stage (Fig. 1G-I). Initial formation of cotyledonary embryos (Fig. 1J) was observed about 4 weeks after transfer of embryonal masses to the maturation media, and was evident at 6 weeks of culturing (Fig. 1L). Normally, maturation experiments were recorded up to 12 weeks of culturing; however, in some cases we monitored the maturation process of Sawara cypress and verified that this might be continuous up to more than 28 weeks *via* secondary embryogenesis (data not shown). The results after 2 months of culturing are shown in Fig. 2. The best results were obtained in SM and EM medium with the average number of mature cotyledonary embryos collected per petri dish being 238 and 201, respectively. This result suggests a beneficial effect of the addition of filter-sterilized organic nitrogen combined with a low nitrate content in the medium (SM and EM medium) on the maturation of Sawara cypress somatic embryos. In contrast, the number of cotyledonary embryos collected in media with a high nitrate content (MS, LP, and WP medium) decreased notably. The superior effects of filter-sterilized organic nitrogen supply on maturation of somatic embryos have been reported not only by an increase of the embryo number but also of the percentage of mature

cotyledonary embryos (Zoglauer *et al.*, 1995). Although statistical difference was not found between SM and EM medium regarding the average number of mature cotyledonary embryos produced and then plant conversion, for further experiments on embryo maturation SM medium was used.

2) Effect of kind of sugar

Figure 3 shows the result of effect of kind of sugar on maturation of somatic embryos. At the concentrations of sugar tested, the best results were achieved by using maltose as carbohydrate source. Although 30 and 50 gL^{-1} did not result in a statistical difference in terms of cotyledonary embryos per petri dish, the highest embryo maturation frequency resulted from the medium containing 50 gL^{-1} maltose with 372 mature embryos as the average number. 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. Medium containing maltose as carbohydrate source and PEG as osmoticum was reported as an effective combination to enhance somatic embryo maturation in 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 morphology of cotyledonary embryos induced on the medium with sucrose or maltose was not too different from each other. The principal difference was in regard to the embryo maturation efficiency (Fig. 3).

On the other hand, results achieved in this experiment indicated that SMm medium enhanced the production of mature somatic embryos in comparison with SM medium. Although exhaustive experiments to elucidate the effect of supplying mineral components in SMm medium were not carried out, the results suggested a positive effect of KCl and boron supply on embryo maturation of Sawara cypress. The average number of cotyledonary embryos collected from SM medium (Fig. 2) was 238, whereas 372 mature embryos was obtained from SMm medium (Fig. 3). The beneficial effect of KCl on embryo maturation in *Pinus taeda* is reported by Li *et al.* (1997) which may be related to an osmotic effect on maturation medium. Zoglauer *et al.* (1995) and Behrendt and Zoglauer (1996) report the role of boron in suspensor development in embryogenic cultures of *Larix decidua*.

3) Effect of pH of medium

Most of the studies on somatic embryogenesis in conifer trees have reported maturation media adjusted to 5.6 to 6.1 (Jain *et al.*, 1995). However optimal response often occurs at a pH different from that (Bonga and von Aderkas, 1992). So, in an attempt to determine the optimal pH condition of maturation medium for Sawara cypress we tested pH ranges from 4.3 to 7.3 (Fig. 4). The best response was achieved on medium with pH adjusted to 5.8 collecting on average 359 mature cotyledonary embryos per petri dish, and drastically reduced to 69 embryos at pH 4.3. In general, higher pH (5.8 to 7.3) resulted in more mature embryos produced than at lower pH (5.3 to 4.3). This response may be related to a hardening of medium when the pH is increased which can result in reduced vitrification of the tissues grown on it (Selby *et al.*, 1989). However, precipitation of some media minerals is a reported problem at higher pH (Dalton *et al.*, 1983). The pH of the medium also affects ammonium metabolism. An increase above pH 6 resulted in an accumulation of ammonium, glutamine, glutamate, and asparagine in cell cultures of *Acer pseudoplatanus* (Goodchild and Givan, 1990). Smith and Krikorian (1990) reported that a drop in pH from 5.6 to 4.0 during carrot embryogenesis resulted in the accumulation of embryos in the preglobular stage, and when these cultures were placed on medium at higher pH (6.0–7.0) the embryos resumed development. More embryogenic cultures of *Picea abies* were obtained at pH 6.5–7.5 than at pH 5.0–6.0 (von Arnold, 1987). Most of the studies on somatic embryogene-

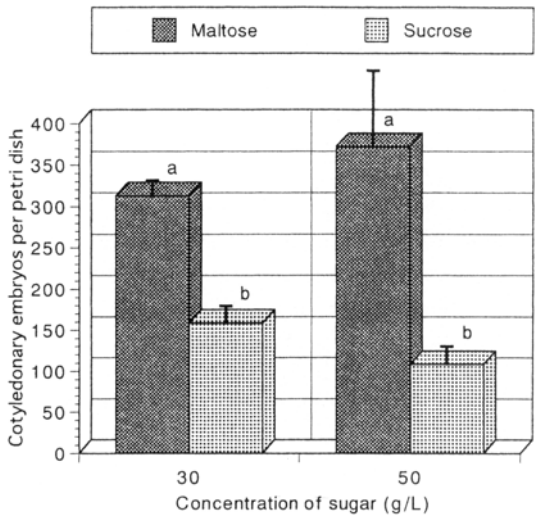

Fig. 3 Effect of kind of sugar on maturation of *Chamaecyparis pisifera* somatic embryos. Bars indicate the standard error. Means followed by same letter are not significantly different at $p < 0.05$. Three petri dishes for each treatment were used.

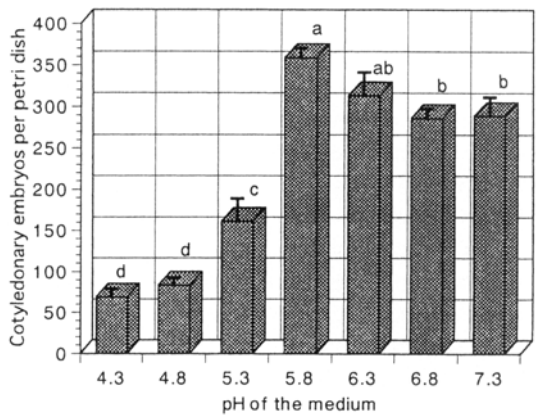

Fig. 4 Effect of pH of medium on maturation of *Chamaecyparis pisifera* somatic embryos. Bars indicate the standard error. Means followed by same letter are not significantly different at $p < 0.05$. Three petri dishes for each treatment were used.

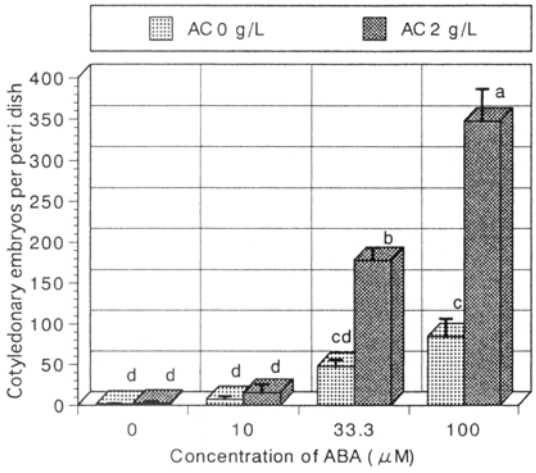

Fig. 5 Effect of ABA and AC on maturation of *Chamaecyparis pisifera* somatic embryos. Bars indicate the standard error. Means followed by same letter are not significantly different at $p < 0.05$. Three petri dishes for each treatment were used.

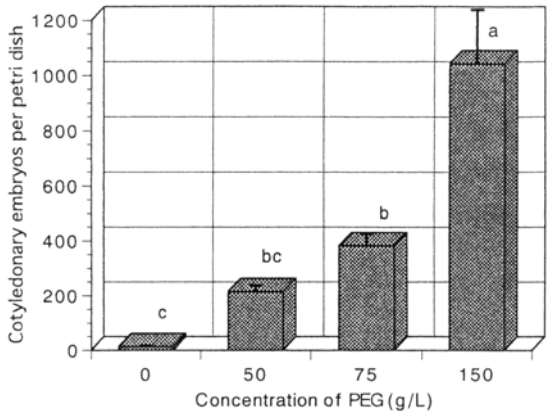

Fig. 6 Effect of PEG on maturation of *Chamaecyparis pisifera* somatic embryos. Bars indicate the standard error. Means followed by same letter are not significantly different at $p < 0.05$. Ten petri dishes for each treatment were used.

sis in conifer trees have reported maturation media adjusted to 5.6 to 6.1 (Jain *et al.*, 1995).

4) Effect of ABA and AC

Figure 5 shows the beneficial effect of increased ABA content in the media supplemented with AC on embryo maturation. The best result was achieved with 100 μM ABA in the presence of AC, obtaining an average number of 348 cotyledonary embryos per petri dish. Higher concentrations of ABA resulted in higher numbers of mature embryos. A similar result is reported in *Pinus strobus* (Klimaszewska and Smith, 1997), *Picea glauca-engelmannii* complex (Roberts *et al.*, 1990b), and *P. glauca* (Dunstan *et al.*, 1991).

Addition of AC to media notably enhanced the maturation efficiency. About 4-fold enhancement was 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. Gupta *et al.* (1995) reported that the quality of cotyledonary embryos of Douglas-fir (*Pseudotsuga menziesii*) further improved by combination of ABA, AC, and PEG.

ABA-free media or supplemented with a low concentration (10 μM) failed to stimulate appropriate embryo maturation producing only a few mature cotyledonary embryos. 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 showed arrested development, evident in the proliferation of embryogenic cells. Lelu *et al.* (1999) have reported that much higher numbers of mature embryos of *Pinus sylvestris* and *P. pinaster* were produced and that the development of cotyledonary somatic embryos *versus* abnormal, shooty ones was enhanced with addition of 60 μM ABA in comparison with media without ABA. Somatic embryos of hybrid larch (*Larix* × *leptoeuropaea*) developed normally on medium supplemented with 60 μM ABA, but abnormally on medium with no ABA (Gutmann *et al.*, 1996). Most of the studies on somatic embryogenesis in conifers have reported that ABA is a very important hormone in embryo development and that the number and quality of embryos produced was very inferior in the absence of ABA (Durzan and Gupta, 1987; von Arnold and Hakman, 1988; Hakman and von Arnold, 1988; Attree and Fowke, 1993; Dunstan *et al.*, 1998).

Several authors have suggested that the role of ABA in somatic embryogenesis is the inhibition of cleavage polyembryony with consequent development of individual somatic embryos (Durzan and Gupta, 1987; Boulay *et al.*, 1988; Krogstrup *et al.*, 1988; Gupta *et al.*, 1991), the stimulation of the accumulation of nutrients, lipids, proteins, and carbohydrates (Hakman and von Arnold, 1988), and the suppression of precocious germination (Roberts *et al.*, 1990b). 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 reported extensively in the compilation of Jain *et al.* (1995).

5) Effect of PEG

As shown in Fig. 6, the addition of PEG stimulated mature embryo production. Higher concentrations of PEG in the medium resulted in 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 with elongated suspensors extending from them (stage 1 somatic embryos).

Although in recent years several studies have reported that the maturation of somatic embryos is promoted by the addition of ABA to media solidified with a high concentration of gel-

Table 3 Effect of maturation media on size, cotyledon number, germination, and conversion of *Chamaecyparis pisifera* somatic embryos.

Maturation media[1]	Germination media[1]	Size range (mm)	Total No.	1 Cotyledon[2] % (SE)	2 Cotyledons[2] % (SE)	3 Cotyledons[2] % (SE)	4 Cotyledons[2] % (SE)	5 Cotyledons[2] % (SE)	Germination[2] % (SE)	Conversion[2] % (SE)
DM5My2.5AC	LPS2AC2	3–13	250 (25 × 10)	0.4 (0.4)[a]	64.4 (4.7)[a]	29.6 (5.5)[a]	5.2 (1.8)[a]	0.4 (0.4)[a]	96 (1.3)[a]	91.6 (2.1)[a]
DM5AC	LPS2AC2	3–10	100 (25 × 4)	0 (0.0)[a]	67 (6.2)[a]	29 (5.7)[a]	4 (1.6)[a]	0 (0.0)[a]	97 (1.9)[a]	92 (1.6)[a]
DM5P5AC	LPS2AC2	2–8	500 (25 × 20)	0.2 (0.2)[a]	65.8 (2.8)[a]	29.8 (2.3)[a]	4 (0.9)[a]	0.2 (0.2)[a]	98 (0.7)[a]	92.8 (1.4)[a]
DM5P7.5AC	LPS2AC2	2–6	500 (25 × 20)	0.8 (0.5)[a]	63.8 (4.5)[a]	28.6 (3.8)[a]	6.4 (1.8)[a]	0.4 (0.3)[a]	97 (1.0)[a]	93 (1.3)[a]
DM5P15AC	LPS2AC2	1–3	500 (25 × 20)	0.6 (0.3)[a]	66 (3.2)[a]	28 (3.1)[a]	4.8 (1.0)[a]	0.6 (0.3)[a]	96.8 (1.0)[a]	92 (1.4)[a]
Total	LPS2AC2	1–13	1850 (25 × 74)	0.5 (0.2)[a]	65.2 (1.8)[a]	28.9 (1.6)[a]	5 (0.7)[a]	0.4 (0.1)[a]	97.1 (0.5)[a]	92.4 (0.7)[a]

[1] DM5My2.5AC: SMm medium containing in gL^{-1}: maltose 50, AC 2, gelrite 5, myo-inositol 25, and 100 μM ABA ; DM5AC: SMm medium containing in gL^{-1}: maltose 50, AC 2, gelrite 5, and 100 μM ABA; DM5P5AC: SMm medium containing in gL^{-1}: maltose 50, AC 2, gelrite 5, PEG 50, and 100 μM ABA; DM5P7.5AC: SMm medium containing in gL^{-1}: maltose 50, AC 2, gelrite 5, PEG 75, and 100 μM ABA; DM5P15AC: SMm medium containing in gL^{-1}: maltose 50, AC 2, gelrite 5, PEG 150, and 100 μM ABA; LPS2AC2: Half strength LP medium containing in gL^{-1}: sucrose 20, AC 2, Wako agar 12. [2] Means followed by same letter are not significantly different at $p < 0.05$; (SE), standard error.

lan 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 a routine method for stimulating somatic embryo maturation in many gymnosperms. In contrast, some authors have reported that PEG promotes maturation but inhibits further development of *Picea glauca* (Kong and Yeung, 1995) and *P. abies* somatic embryos (Bozhkov and von Arnold, 1998). Results of our experiments indicated that the positive effect of PEG on maturation did not inhibit further development of Sawara cypress somatic embryos. Almost all mature cotyledonary embryos germinated and developed into normal plants. 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.*, 1990b; Attree *et al.*, 1992; Misra *et al.*, 1993), and to a tolerance to water loss (Attree *et al.*, 1991).

In the present study, morphological difference among somatic embryos obtained on media supplemented with different concentrations of PEG was restricted to the size of them. Higher concentrations of PEG resulted in smaller embryos (Table 3). The smaller size of PEG-treated embryos was reported also for *Larix laricina* (Klimaszewska *et al.*, 1997) and *Picea abies* (Find, 1997). Iraki *et al.* (1989) reported that small cell size is a typical symptom of PEG-induced osmotic stress. Low external osmotic potential may have led to alterations in cell wall composition, decreasing the ratio of cellulose to hemicellulose. This results in decreased cell wall tensile strength and a 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). However, in our work the subsequent development of PEG-treated embryos did not different to untreated ones. Germination frequencies and plant conversion rates were similar on different PEG-treated media (Table 3).

The addition of myo-inositol to medium in exchange for PEG as osmoticum was also effective in inducing maturation. About 100–200 cotyledonary embryos per petri dish were collected after about 2 months of culturing (data not shown). The most notable characteristic of somatic embryos matured on medium containing myo-inositol as osmotic agent was the size of them. Contrary to the results achieved with PEG-media, embryos were of bigger size. We obtained the biggest embryo size when myo-inositol was supplemented in maturation media (Table 3). Gupta and Pullman (1991) reported embryo maturation treating embryogenic cells with a high concentration of myo-inositol and ABA in the medium but without mention of embryo size.

3 Germination and plant conversion

Table 1 shows the effect of media on germination and conversion of Sawara cypress somatic embryos. The start of germination was observed 5–10 days after transfer to germination media. The number of germinants (Fig. 1N) and then converted into emblings (Fig. 1O) was very high when basal salts and sucrose were supplemented in the germination media. The percentage of germination and plant conversion ranged from 96 to 100% and from 90 to 93%, respectively. Contrary to expectations, when glucose (30 gL^{-1}) and gelrite (5 gL^{-1}) were supplemented, the percentage of germination was low on LPG medium (56%) and drastically reduced on SMG medium (19%). Cotyledonary embryos showed arrested development and eventually generate secondary embryogenesis. This result was contradictory to that achieved by Smith (1996) for germination of *Pinus radiata* somatic embryos. He reported that for somatic embryos from new cell lines, roots appeared as early as 10 days after transfer to SMG medium, and that over 4,000 plants from 50 clones germinated in this medium were transferred to soil in the greenhouse, nursery beds, or in field trials. In our results, greater differences were observed among germination on media with sucrose and glucose. However, a negative effect of glucose on plant conversion was not found. Plant conversion was observed in almost all germinants from LPG and SMG medium (Table 1). On other media (Water, Vermiculite, and Perlite) the germination and plant conversion varied from 40 to 96% and 4 to 30%, respectively. These results suggest that despite germination being observed at a relatively high frequency, both basal salts and sucrose are neces-

sary for subsequently high plant conversion rate of Sawara cypress somatic embryos.

4 *Ex vitro* acclimatization and growth of emblings

Regenerated emblings were acclimatized successfully under conditions described above (Fig. 1P). The results of survival rates and growth 2 months after transplanting are shown in Table 2. The best result in terms of survival and growth was achieved in Phytotron conditions using a mixture of vermiculite and perlite (1:1, ratio by volume) as substratum. An alternating temperature of 25°C (16 h) and 20°C (8 h) at a relative humidity of 75% enhanced the growth of Sawara cypress emblings. Perlite alone was the worst substratum for survival and growth (Glass-house conditions). However, when perlite was mixed with vermiculite the survival and growth was enhanced in comparison also with a pure vermiculite substratum. This response may be related to the relation between water retention (vermiculite) and aeration for root system (perlite).

5 Isolation and culture of protoplasts

A simple and reliable method for isolation and culture of protoplasts from embryogenic cell cultures of Sawara cypress was developed (Fig. 1U–X). Trial and error in preliminary experiments using different combinations of enzymes, concentration of mannitol, and combinations of plant growth regulators (all data not shown) determined isolation and culture conditions. The enzyme solution containing 1% (w/v) Cellulase Onozuka RS, 0.1% (w/v) Pectolyase Y-23, and 0.6 M mannitol resulted in the best isolation efficiency. No great differences were achieved using Cellulase Onozuka RS or R-10. Pectolyase Y-23 was the critical enzyme to enhance isolation efficiency (Fig. 7). Similar responses were achieved in protoplast isolation from embryogenic cultures of *Cryptomeria japonica* (Maruyama *et al.*, 2000)

The viability examination by fluorescein diacetate staining showed that up to more than 90% of protoplasts were survivors after isolation (Fig. 8). This result indicated that the combinations of Cellulase Onozuka RS or R-10 (1%) and Pectolyase Y-23 (0.1%) in the presence of 0.6 M mannitol as osmotic agent were effective for protoplast isolation from embryogenic cells of Sawara cypress.

Isolated protoplasts were cultured in media supplemented with different combinations of 2,4-D and BAP at a density of 5×10^3 mL^{-1} determined in other experiments as appropriate for Sawara embryogenic cells (data not shown). The plating efficiency percentages are shown in Fig. 9. Colony formation was obtained for almost all combinations of plant growth regulators, but with most efficiency at a concentration of 3–10 μM 2,4-D plus 0–0.1 μM BAP. The presence of BAP in the medium sometimes enhanced the plating efficiency percentage but was not essential for the colony formation. Thus, the best result was obtained in medium supplemented with 10 μM 2,4-D alone.

Establishment of the protoplast technique represents an important tool in fundamental research on the synthesis of cell walls, membrane transports, and the cytoskeleton in relation to the cell cycle and division (Klimaszewska, 1995). In addition, the single cell culture system represents a powerful tool for studies on the physiology of different cell types, analysis of differentiation programs, genetic manipulation of plant cells, and cell-cell interactions (Spangenberg *et al.*, 1986; Schweiger *et al.*, 1987).

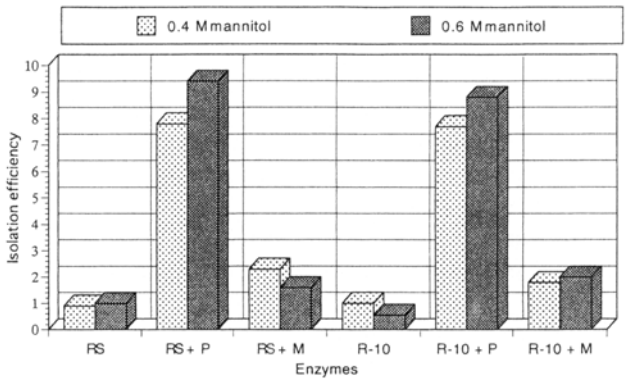

Fig. 7 Effect of enzyme combinations and mannitol concentration on isolation efficiency of *Chamaecyparis pisifera* protoplasts. Isolation frequency: protoplast numbers divided by protoplast numbers at RS-0.6 M mannitol treatment.

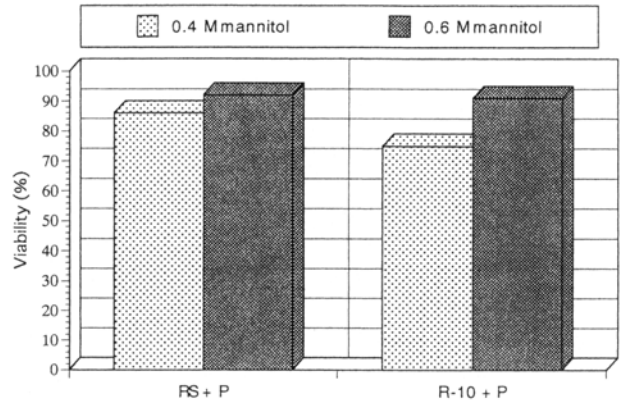

Fig. 8 Effect of enzyme combinations and mannitol concentration on viability of *Chamaecyparis pisifera* protoplasts.

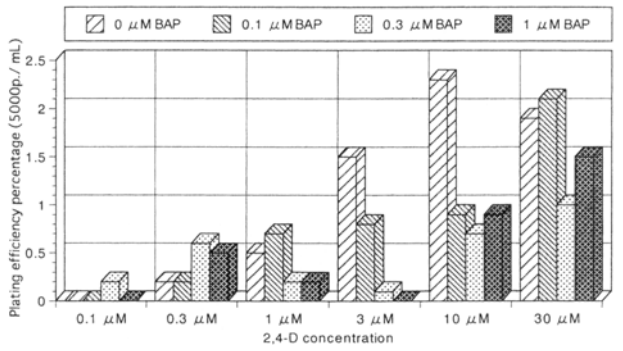

Fig. 9 Effect of plant growth regulator combinations on the plating efficiency percentages of *Chamaecyparis pisifera* protoplasts.

Discussion

Induction of embryogenic cells from immature seeds of Sawara cypress occurred at a relatively high frequency when material was collected from the end of June to early July. These results indicating that in this period the explant was highly responsive to inducing somatic embryogenesis. In conifer trees the most frequently used explants for embryogenic culture initiation are the isolated zygotic embryos and the whole megagametophytes, but with the disadvantage that much more time is required for explant dissection. Because of the small size (about 2 mm long) of Sawara cypress seeds, embryogenic cell induction on whole seeds facilitated the handling in preparing explants for somatic embryogenesis initiation.

Embryogenic cultures were maintained and proliferated for more than a year without decreasing in capacity to develop cotyledonary embryos. The presence of a filter-sterilized organic nitrogen source in medium combined with a reduction of the nitrate content increased the proliferation rate and the number of mature cotyledonary embryos of Sawara cypress. In contrast, Zoglauer *et al.* (1995) reported that continuously subcultured embryogenic suspensions of *Larix decidua* on organic nitrogen-supplemented medium resulted in a dramatic decrease in the number of mature embryos obtained. Jalonen and von Arnold (1991) demonstrated a dependence of embryo maturation on the type of embryo morphology during proliferation.

High embryo maturation efficiency was obtained when embryogenic cells were cultured on media supplemented with a combination of maltose, PEG, ABA, and AC. More than one thousand mature cotyledonary embryos were produced from about 100 mg (FW) of inoculum on maturation medium supplemented with 150 gL^{-1} PEG (Fig. 1M and 6). Some successful embryo maturation of conifer trees reported an average of up to 109 cotyledonary embryos per gram for *Pinus taeda* (Li *et al.*, 1997), 262 for *Pinus sylvestris* (Lelu *et al.*, 1999), 295 for *P. strobus* (Klimaszewska and Smith, 1997), 460 for *Larix laricina* (Klimaszewska *et al.*, 1997), about 1,250 for *Larix decidua* (Zoglauer *et al.*, 1995), about 575 for *Picea glauca* (Kong and Yeung, 1995), and 437 for *Picea abies* (Bozhkov and von Arnold, 1998). The maturation efficiency achieved with Sawara cypress in our laboratory represents an average of more than ten thousand cotyledonary embryos per gram (FW) of embryogenic cells, which is very much higher than that reported to date. In addition to the highly synchronized and high yielding maturation procedure, the subsequently high germination and plant conversion frequencies attained demonstrated the high quality of somatic embryos produced. Somatic embryos readily germinated after transfer to plant growth regulator-free media without application of any partial drying treatment as has been reported necessary to promote germination of some *Picea* and *Pinus* species (Roberts *et al.*, 1990a; Roberts *et al.*, 1991; Kong and Yeung, 1992; Jain *et al.*, 1995; Kong and Yeung, 1995). Over ten thousand plants were produced and about 1% of them has been monitored in the field.

Maturation frequency and the quality of embryos produced are the most important criteria for the optimization of a somatic embryogenesis protocol. The high quality of embryo is related to its morphology and the ability to produce normal plants. In experiments with Sawara cypress, we obtained high quality somatic embryos with size ranging from 1 to 13 mm long (Fig. 1Q) (embryos less than 1 mm long were not counted). PEG-treated embryos were of smaller size compared to those matured on media without PEG. However, the embryo size was not found to be an influential factor in germination or subsequent plant conversion. Cotyledonary embryos germinated and converted into plants at high frequencies independent of their size (Table 3). In contrast to our results, Bozhkov and von Arnold (1998) determined that the morphology of mature somatic embryos of *Picea abies* is changed after PEG-treatment (smaller, irregularly shaped embryos; smaller cell size; larger root caps with intercellular spaces in pericolumn; degraded quiescent center), which could decrease further growth of the embryos. Relatively short embryos are generally considered low quality, which may lead to low efficiency of embryo germination and then plant establishment (Liao and Amerson, 1995). High quality somatic embryos, which have the ability to produce normal plants like the zygotics, are commonly longer than they are wide and generally have a radial symmetry (Fig. 1K). Normally, the zygotic embryos of Sawara cypress have two cotyledons. However, in the somatics, we obtained a range from 1 to 5 cotyledons (Fig. 1R). Somatic embryos having 2 or 3 cotyledons were the most frequent with about 65 and 29%, respectively (Table 3). No statistical differences were found among maturation media regarding the effect on cotyledon number, germination frequency and plant conversion. Double-shoot emblings (Fig. 1S) were observed with a frequency of 1 to 2% of the total germinants. The specific reason was not explored in this study.

Secondary embryogenesis with spontaneous plant regeneration was observed sporadically in mature cotyledonary embryos on germination media (Fig. 1T). Repetitive embryogenesis with subsequently plant conversion is a desirable protocol for genetic transformation.

In this study, a simple procedure for protoplast culture was established as a first step to develop an efficient technique for individual culture of a single protoplast. This technique offers significant advantages for improvement of the tissue culture system by identification and selection of desirable cells, as well as for genetic manipulation through direct gene transfer such as microinjection and electroporation. To our knowledge this is the first report on somatic embryogenesis of Sawara cypress including protoplast culture.

In this study, we have achieved a stable and efficient plant regeneration system for propagation, conservation and genetic transformation of Sawara cypress. In addition, this report may also provide useful information for somatic embryogenesis in other conifer tree species.

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