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Tissue Culture Note

In vitro culture of various genotypes of male sterile Japanese cedar (*Cryptomeria japonica* D. Don)

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Abstract The pollen allergy problem of Japanese cedar has been a nation-wide problem in spring time in Japan. It is said that about 20% of people suffer Japanese cedar pollinosis in urban areas. New projects substituting Japanese cedar forest stands with low-pollen or sterile male Japanese cedar trees started from 2008. The propagation of male sterile Japanese cedar genotypes is one of the most important steps in that project. We developed a micropropagation technique using an *in vitro* culture of male sterile Japanese cedar genotypes. Leafy shoot segments were used for explants. There was a genotypic difference in tissue culture response. Among the cytokinin tested, zeatin and 6-benzylaminopurine (BAP) were relatively effective for bud induction. More buds were induced in the medium containing zeatin than that containing BAP with the male sterile genotype Fukushima 5. Shoots developed from buds on the medium 1/2 LP containing 5 g l^{-1} activated charcoal. Shoots produced roots on a medium containing 23.8 μM (5 mg l^{-1}) 4-chloroindole-3-acetic acid and 0.044 μM (0.01 mg l^{-1}) BAP. Rooted plantlets were successfully habituated and cultured in pots outside. The *in vitro* cultured materials will be used for the micropropagation of male sterile Japanese cedar in order to reduce the pollen in the air.

Key words: *Cryptomeria japonica*, *in vitro* culture, male sterile, micropropagation.

Japanese cedar (*Cryptomeria japonica* D. Don) is an important conifer tree for industrial plantation in Japan. There are also many ornamental varieties of Japanese cedar like 'Spiralis', 'Cristata' and 'Albospicata'. Forty five percent of man-made forests in Japan are comprised of Japanese cedar. However, pollen allergy problems caused by Japanese cedar are serious these days. For a solution to pollinosis, the propagation of non-pollen male sterile clones by tissue culture is considered one option. A micropropagation using tissue culture techniques needs smaller explants than the conventional cutting method and thus enables fast propagation even from limited male sterile materials. A complete male sterile Japanese cedar was found in Toyama prefecture, Japan (Taira et al. 1993). The male sterile gene is of Mendelian inheritance and the homo type expresses a non-pollen character. Until the year 2009, more than 24 individual genotypes of pollen sterile Japanese cedar have been found in Toyama, Niigata, Fukushima, Aomori, Kanagawa, Ibaraki and Mie prefectures in Japan (Ueuma et al. 2009). Here we describe the screening of initial, subculture and rooting media for the tissue cultures of various male sterile Japanese cedar genotypes. So far, there is a report about the micropropagation of one male sterile Sugi genotype 'Soushun' (Tsubomura and Taniguchi 2008). However, they used *in vitro* scion stocks which produce a relatively low number of vigorous shoots (2.1 per plant) for the micropropagation of 'Soushun'. It is also necessary to propagate other male sterile genotypes for increasing biodiversity in the forest. We examined the culture conditions for the repeated organ culture of axillary buds as the basis of more efficient micropropagation using other male sterile genotypes of Japanese cedar.

Twelve original male sterile genotypes of Japanese cedar and 3 male sterile hybrids produced by crossing between individuals which possessed sterile gene as hetero allele (Table 1) were used. Surface sterilization of leafy shoots from 2–3 years old cuttings in spring and early summer was tried using several chemicals like benzalkonium chloride, sodium hypochloride, ethyl alcohol and hydrogen peroxide. For the initial culture,

Abbreviations: BAP, 6-benzylaminopurine; 4-Cl-IAA, 4-chloroindole-3-acetic acid; IBA, indole-3-butyric acid; NAA, naphthaleneacetic acid; PVPP, polyvinylpolypyrrolidone; RIM, root induction medium; WPM, woody plant medium

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1/2 LP (Aitken-Christie and Thorpe 1984) or CD (Campbell and Durzan 1975) (both containing 10 μM BAP, kinetin, thidiazuron or zeatin and 20 g l^{-1} sucrose) medium was compared after 1 month of cultivation. Culture tubes (18 mm i.d.×160 mm) containing 15 ml of 8 g l^{-1} agar solidified medium were used for initial culture. For the subculture and elongation of induced axillary buds, 1/2 LP medium containing 5 g l^{-1} activated charcoal and 15 g l^{-1} sucrose solidified with 12 g l^{-1} agar was used. Shoot explants with induced buds were subcultured whole. For rooting, about 3 cm long shoots were dissected and cultured on RIM (Abo El-Nil and Milton 1982 *United States Patent* No. 4353186) medium containing 23.8 μM 4-Cl-IAA or 24.6 μM IBA and 0.044～0.44 μM BAP and other media with 15 g l^{-1} sucrose solidified with 8 g l^{-1} agar as listed in Table 5.

Table 1. Male sterile Japanese cedar genotypes used in the present study

Genotype	Type	Discovered prefecture
Aomori 1	Urasugi	Aomori
Fukushima 1	Urasugi	Fukushima
Fukushima 2	Urasugi	Fukushima
Fukushima 5	Urasugi	Fukushima
Shindai 1	Urasugi	Niigata
Shindai 3	Urasugi	Niigata
Shindai 5	Urasugi	Niigata
Shindai 8	Urasugi	Niigata
Toyama 1	Urasugi	Toyama
Toyama 3	Urasugi	Toyama
Tahara 1	Omotesugi	Kanagawa
Mie 1	Omotesugi	Mie
F1 Ryowa 6	Urasugi	Toyama
F1 Hayatsuki 1	Urasugi	Toyama
F1 Obara 13	Urasugi	Toyama

Generally speaking, Urasugi type is along the Japan Sea while Omotesugi type is along the Pacific Ocean.

Two hundred ml culture flasks containing 70 ml medium were used for subculture and rooting during the 2 months of cultivation. Regenerated plantlets were cultured in florialite® (Iwaki Co., Japan) with 60 ml of 0.1% Hyponex medium in plant boxes. Each plant box contained 1 regenerated plantlet. All culture media were adjusted to pH 5.8 before autoclaving. The culture condition was at 25°C constant temperature under 16 h photoperiod of 70 μM m^{-2} s^{-1} using a fluorescent lamp.

Sequential surface sterilization using 0.1% benzalkonium chloride for 15～20 min., 1% sodium hypochroride for 10 min., 70% ethyl alcohol for 2 min. and 5% hydrogen peroxide for 10 min. then washed well with sterile water was effective for eliminating microorganisms from the surface of explants. Axillary buds were induced from 2 cm length dissected shoot explants of male sterile Japanese cedar genotypes, Aomori 1, Fukushima 1, 2, 5, Shindai 1, 3, 5, 8, Toyama 1, 3, Tahara 1, Mie 1, F_1 Hayatsuki 1, F_1 Ryowa 6 and F_1 Obara 13. In the case of Mie 1, bud induction was observed only in the 1/2 LP or CD medium containing BAP or zeatin (Table 2). Among the cytokinin tested, zeatin was relatively effective for the bud induction of Fukushima 2 and 5 while BAP was effective with Aomori 1 (Table 2). More buds were induced in the medium containing zeatin than that containing BAP with male sterile genotype Fukushima 5 from which explants were collected on June 20th 2007 (Table 2, Figure 1A). In the subculture medium, zeatin was also more effective for further shoot elongation than BAP (Table 3). Shoots were developed from buds on the medium 1/2 LP containing 5 g l^{-1} activated charcoal (Figure 1B). Shoots were rooted on the RIM medium containing 23.8 μM 4-Cl-IAA and 0.044 μM BAP (Table 4, Figure 1C, D). However, we need to improve the rooting percentage for

Table 2. Effects of basal medium and cytokinin on bud induction from leafy shoot explants in male sterile Japanese cedar Aomori 1(A), Fukushima 5(F) and Mie 1(M)

Basic medium	Cytokinin (10 μM)	genotype	No. of explants inducing buds (%)	No. of buds induced per explant±SE
12LP	BAP	A	4 (40)	1.75 ± 0.48^{c}
1/2LP	BAP	F	8 (80)	4.8 ± 0.7^{b}
1/2LP	BAP	M	4 (40)	4.0 ± 0.4^{b}
1/2LP	zeatin	A	2 (20)	1 ± 0^{d}
1/2LP	zeatin	F	8 (80)	6.4 ± 0.9^{a}
1/2LP	zeatin	M	4 (40)	2.8 ± 0.4^{c}
1/2LP	kinetin	A	5 (50)	1.4 ± 0.38^{c}
1/2LP	thidiazuron	A	0 (0)	0^{e}
1/2LP	thidiazuron	M	0 (0)	0^{e}
CD	BAP	A	5 (50)	2.4 ± 0.88^{c}
CD	BAP	M	3 (30)	1.7 ± 0.5^{c}
CD	zeatin	A	3 (30)	1.33 ± 0.33^{cd}
CD	zeatin	M	4 (40)	3.5 ± 0.8^{b}
CD	kinetin	A	5 (50)	2.2 ± 0.35^{c}
CD	kinetin	M	0 (0)	0^{e}
CD	thidiazuron	A	0 (0)	0^{e}
CD	thidiazuron	M	0 (0)	0^{e}

N=10. $^{a-e}$ Responses with the same letter are not significantly different at P=0.05 by Duncan's new multiple range test.

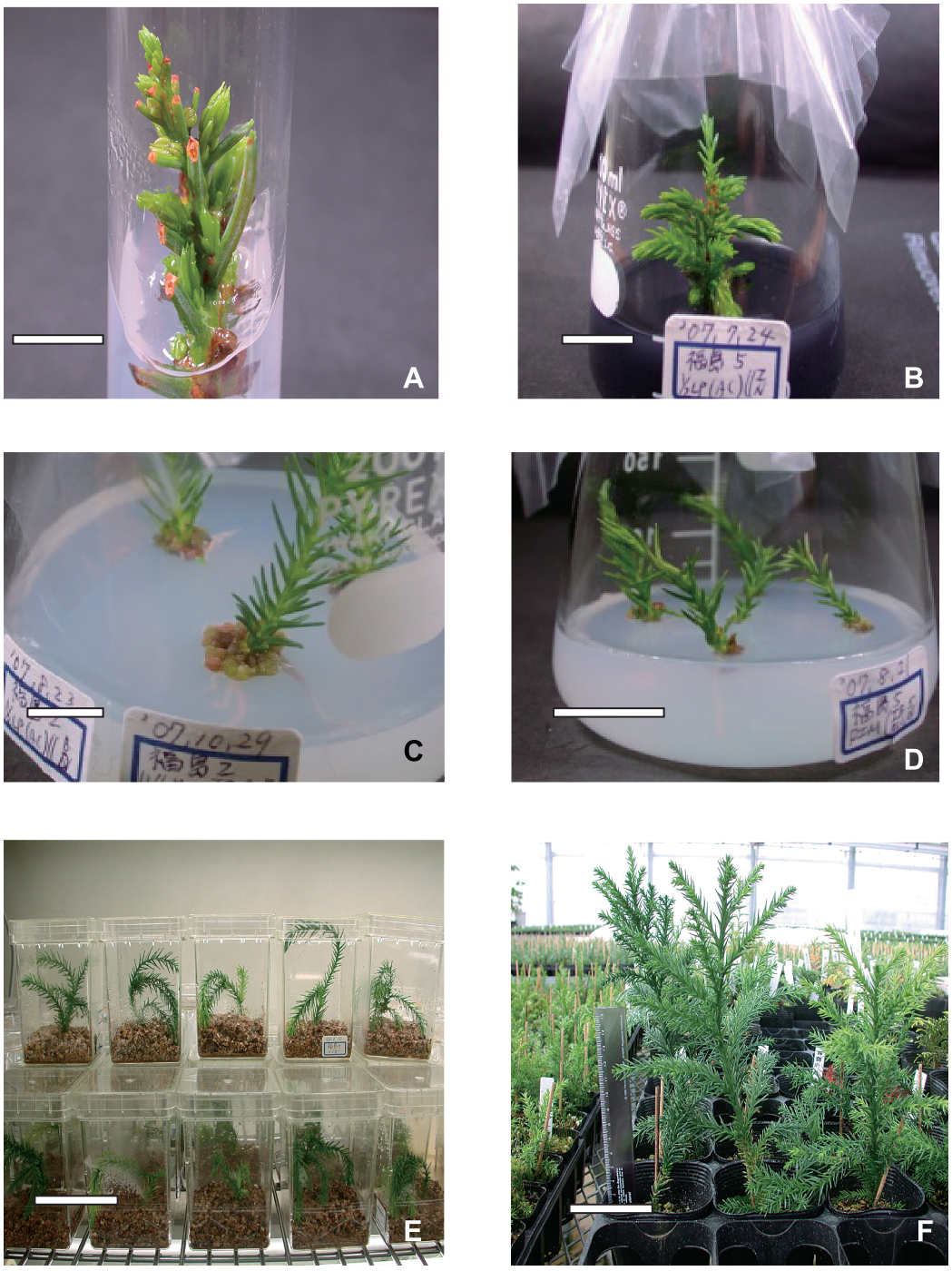

Figure 1. *In vitro* regeneration of male sterile Japanese cedar. (A) Induction of axillary buds from explants. (Fukushima 5) bar; 1 cm, (B) Shoot elongation from buds. (Fukushima 5) bar; 2 cm, (C) Rooting of the shoots. (Fukushima 2) bar; 1 cm, (D) Rooting of the shoots. (Fukushima 5) bar; 2 cm, (E) Regenerated plantlets of male sterile Japanese cedar in the plant boxes (Fukushima 5) bar; 5 cm, (F) Regenerated Japanese cedar after habituation. bar; 5 cm.

the practical micropropagation of male sterile Japanese cedar. So far, we obtained regenerated plantlets in the cases of Aomori 1, Fukushima 2, 5, Shindai 1, 5, 8, Toyama 3, Tahara 1, Mie 1, F_1 Ryowa 6 and F_1 Hayatsuki 1. Apparently, there is a genotypic difference in response to tissue culture conditions. Regenerated plantlets cultured in the plant boxes grew slowly (Figure 1E). They were habituated in 100% humidity conditions for 2 weeks (Figure 1F) then cultured successfully outside. *In vitro* cultured materials will be used for the micropropagation of male sterile Japanese cedar in order to reduce its pollen in the air. There may be three applications of *in vitro* culture to the biotechnology of male sterile Japanese cedar. First, the direct application

Table 3. Effects of cytokinins in the subcultured medium on the number of elongated shoots from axillary buds of male sterile Japanese cedar Fukushima 5 after further subcultured to the elongation medium

Cytokinin (10 μM)	No. of shoots/ explant±SE	Ave. length of shoots (mm)±SE
zeatin	10.4 ± 1.5[a]	12.2 ± 1.7[a]
BAP	1.6 ± 0.6[b]	8.3 ± 2.8[a]

N=5. After 1 month culture on 1/2LP containing 5 g activated charcoal (shoot elongation medium).

[a,b] Responses with the same letter are not significantly different at P=0.05.

Table 4. Effects of different medium on rooting of male sterile Japanese cedar Fukushima 5

Basic medium	PGR (M)	Rooting %±SE	Root length (mm)±SE
RIM	IBA (24.6), BAP (0.044)	23.3 ± 11[ab]	30 ± 0[a]
RIM	IBA (4.4)	10 ± 0[b]	15 ± 0[a]
RIM	IBA (0.99)	0 ± 0[c]	0 ± 0[b]
RIM	IAA (28.6), BAP (0.044)	0 ± 0[c]	0 ± 0[b]
RIM	IAA (28.6), BAP (0.44)	7 ± 4[bc]	5 ± 0[ab]
RIM	4-Cl-IAA (23.8), BAP (0.044)	46 ± 10[a]	26.4 ± 3.1[a]
RIM	4-Cl-IAA (23.8), BAP (0.44)	36 ± 12[a]	27 ± 8.5[a]
White	IBA (2.46), NAA (0.32)	0 ± 0[c]	0 ± 0[b]
White	IBA (4.9)	0 ± 0[c]	0 ± 0[b]
White	IBA (4.9), PVPP (500 ppm)	16.7 ± 13[b]	22.5 ± 2.5[a]
White	IBA (4.9), PVP (500 ppm)	8.3 ± 0[b]	20 ± 3[a]
White	IBA (0.99)	0 ± 0[c]	0 ± 0[b]
WS	IBA (14.8), NAA (0.54)	20 ± 11[ab]	2 ± 0[b]
CD	IBA (14.8), NAA (0.54)	10 ± 8[b]	15 ± 3[a]
1/4WPM	IBA (9.85)	17 ± 10[b]	3.2 ± 0.46[b]

N=15. [a–c] Responses with the same letter are not significantly different at P=0.05 by Duncan's new multiple range test.

White (White 1943), WS (Wolter and Skoog 1966), WPM (Lloyd and McCown 1980).

of *in vitro* culture for micropropagation using organ cultures. Second, the indirect application of *in vitro* culture for the production of scion stock plants for cutting. In this case, the promotion of juvenility by rejuvenation *in vitro* culture may be beneficial for further microcutting propagation. Third, *in vitro* culture may be used for genetic engineering to produce complete sterile breeding materials which produce no male or female flowers. In this study, we developed the *in vitro* culture regeneration method using axillary bud induction on leafy shoots from many different genotypes of male sterile Japanese cedar for the first time.

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