CHAPTER 5

MICROPROPAGATION OF *PINUS ARMANDII* VAR. *AMAMIANA*

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1. INTRODUCTION

In the survey conducted by the Environmental Agency on endangered species in Japan in the year 2000, 1665 species were listed as endangered one among 7087 vascular plants (Environmental Agency of Japan 2000). Collection for ornamental use, natural succession, and deforestation are the three major causes for threatening the species. To recover the endangered species, propagation of such plants for *ex situ* or *in situ* conservation is important. Among them, micropropagation by tissue culture is considered effective and useful method.

There were several reports on micropropagation of endangered trees (Okochi et al., 2003; Sugii & Lamoureux, 2004). Here we describe protocols for micropropagation of an endangered five needle pine, *Pinus armandii* Franch. var. *amamiana* (Koidz.) Hatusima for preserving it *ex situ,* and supply plants for rehabilitation.

Pinus armandii var. *amamiana* is an endangered tree inhabiting only in the south western islands of Japan, Yakushima and Tanegashima (Yahara et al., 1987). Recent survey showed that there are only about 2000 trees remaining in the both islands. Pine wilt disease by nematodes is suggested as one of the causes of the decline of this species (Akiba & Nakamura, 2005). Because of its decreasing number in recent years in the field populations, it was claimed as IB(EN) in the new Japanese Red List (Environmental Agency of Japan 2000) which denotes a high possibility of extinction in the near future. Five needle pine group which include this species are widely used as timber resources and ornamental bonsai trees in the world.

S.M. Jain and H. Häggman (eds.), Protocols for Micropropagation of Woody Trees and Fruits, 41–50.

2. EXPERIMENTAL PROTOCOL

2.1. Organ Culture

2.1.1. Materials

1. Mature seeds collected from late August to early September from remaining trees (Figure 1A) of *Pinus armandii* var. *amamiana* in Yakushima island.
2. Laminar-flow chamber, Petri dishes, forceps, scalpel, pipettes.
3. Ethanol, sterile distilled water, culture tubes, culture flasks.
4. Dissecting microscope.
5. Media (see Table 1).

2.1.2. Methods

The regeneration method can be divided into three main steps: initial culture, shoot elongation, and rooting.

Initial culture. For elimination of empty seeds caused by inbreeding depression, only submerged seeds in 100% ethanol were used for further experiments in mature seeds.

1. Remove the seeds from the cones.
2. Sterilize the seeds with 70% ethanol for 3 min.
3. Wash the seeds two times with distilled water.
4. Mature embryos were excised from the seeds.
5. Transfer the sterile embryos on to the induction medium in the test tubes. Mainly a half strength DCR (Gupta & Durzan, 1985) medium with different concentration of plant growth regulators (2 or 10 μM BAP plus 0.1 μM NAA, 0.4, 2 and 10 μM BAP) was used. Culture tubes (18 mm i.d. × 160 mm) containing 15 ml of agar solidified media were used for initial culture, and 200 ml culture flasks containing 70 ml agar-solidified medium were used for subculture.

Shoot elongation

1. Regenerated buds with embryos are transferred to shoot elongation medium containing 2g/l activated charcoal (Figure 1B) (Table 1).
2. Elongated shoots are divided and further subcultured on the same medium for 1–2 months interval.

Rooting

1. Shoots more than 1.5 cm length are cut and transferred to the rooting medium.
2. For rooting of shoots, RIM medium (Abo El-Nil & Milton, 1982) containning indole butyric acid (IBA) was used.
3. Rooted shoots (Figure 1C) are subcultured to the hormone free florialite® (Nisshinbo, Japan) medium containing 0.1% hyponex®.

Table 1. *Media for organ culture of Pinus armandii var. amamiana.*

Chemicals (mg/l)	Initial culture (1/2 DCR)	Shoot elongation (1/2 DCR + AC)	Rooting (RIM)
NH_4NO_3	200	200	
KNO_3	170	170	187.5
$Ca(NO_3)_2 \times 4H_2O$		152	
$MgSO_4 \times 7H_2O$	185	185	200
$CaCl_2 \times 2H_2O$	42.5	42.5	
$NaH_2PO_4 \times H_2O$	138		
KH_2PO_4	85	85	170
K_2SO_4		860	
Na_2SO_4		200	
KCl		65	
$FeSO_4 \times 7H_2O$	13.9	13.9	27.8
Na_2EDTA	18.65	18.65	37.3
$MnSO_4 \times 4H_2O$	11.15	11.15	5
$ZnSO_4 \times 7H_2O$	4.3	4.3	0.5
H_3BO_3	3.1	3.1	10
KI	0.415	0.415	1
$Na_2MoO_4 \times 2H_2O$	0.125	0.125	0.1
$CuSO_4 \times 5H_2O$	0.0125	0.0125	0.1
$CoCl_2 \times 6H_2O$	0.0125	0.0125	0.1
$NiCl_2$	0.0125	0.0125	
Myo-inositol	100	100	100
Nicotinic acid	0.5	0.5	5
Pyridoxine HCl	0.1	0.1	0.5
Thiamine HCl	1	1	5
Glutamine	100		
Glycine	1	1	
Coumarin		0.0146	
Riboflavin		0.2	
BAP	0.045–2.25		
NAA	0–0.0186		
IBA		1–3	
Activated charcoal	2000		
Sucrose	15000	15000	15000
Agar	8000	9000	8000

All culture conditions were conducted at 25°C under a 16-h photoperiod (fluorescent light, 70 $\mu Mm^{-2}s^{-1}$). Regenerated and habituated plantlets from organ culture of mature embryos were transferred to the nursery field made of black soil loam (Figure 1D).

Figure 1. *Micropropagation of Pinus armandii var. amamiana by organ culture. A) Remaining Pinus armandii var. amamiana in Yakushima island. B) Multiple shoots on the shoot elongation medium. C) Rooting of the shoot. D) Field grown plantlets obtained by organ culture of Pinus armandii var. amamiana.*

2.2. Somatic Embryogenesis

2.2.1. Materials

1. Mature and immature seeds collected from early July to early September from remaining trees of *Pinus armandii* var. *amamiana* in Yakushima island.
2. Laminar-flow chamber, Petri dishes, forceps, scalpel, pipettes.
3. Ethanol, sterile distilled water, multi well plate, plastic Petri dish, culture flasks.
4. Dissecting microscope. Inverted microscope.
5. Media (see Table 2).

2.2.2. Methods

The regeneration method can be divided into four main steps: initiation of embryogenic cultures, proliferation of embryogenic cultures, maturation of somatic embryos, and germination.

Initiation of embryogenic cultures

1. For propagation via somatic embryos, embryogenic cell suspensions were induced from immature and mature seeds on modified 1/2MS (Murashige & Skoog, 1962) or 1/2EM (Maruyama et al., 2000) medium supplemented with different concentration of 2,4-D and BAP.
2. Disinfect cones by 15 min immersion in 70% ethanol containing few drops of neutral detergent and then wash in tap water before dissection.
3. Disinfect excised seeds with 3% (w/v chlorine) sodium hypochlorite solution for 30 min then rinse five times with sterile distilled water.
4. For induction of embryogenic cells, culture whole seed explants in 24-well tissue culture plates (one per well) containing induction medium as shown in Table 2.
5. Seal culture plates with Novix-II® (Iwaki Co. Tokyo) film and incubate in the dark at 25°C.
6. The presence or absence of distinct early stage of somatic embryo characterized by embryonal head (dense cells) with suspensor system (elongated cells) from the explant is observed weekly under the inverted microscope, up to 3 months.

Proliferation of embryogenic culture

1. Induced suspension cells were transferred to ammonium free (just by omitting ammonium nitrate) 1/2MS liquid medium supplemented with 2,4-D, BAP and L-glutamine and subcultured every 2–3 weeks.
2. For continuously proliferation routines, subculture embryogenic cells to fresh medium using transfer pipette (about 0.5 ml suspension culture in 30–40 ml fresh medium) and incubate in 100 ml flask on rotatory shaker at 100 rpm in the dark.

Table 2. *Media for somatic embryo culture of Pinus armandii var. amamiana.*

Chemicals (mg/l)	Induction & proliferation (ammonia free 1/2 MS)	Maturation (1/2 DCR + AC)	Germination (ammonia free MS)
KNO_3	950	950	1900
$MgSO_4 \times 7H_2O$	185	185	370
$CaCl_2 \times 2H_2O$	220	220	440
KH_2PO_4	85	85	170
$FeSO_4 \times 7H_2O$	13.9	13.9	27.8
Na_2EDTA	18.65	18.65	37.3
$MnSO_4 \times 4H_2O$	22.3	22.3	22.3
$ZnSO_4 \times 7H_2O$	8.6	8.6	8.6
H_3BO_3	6.2	6.2	6.2
KI	0.83	0.83	0.83
$Na_2MoO_4 \times 2H_2O$	0.25	0.25	0.25
$CuSO_4 \times 5H_2O$	0.025	0.025	0.025
$CoCl_2 \times 6H_2O$	0.025	0.0125	0.025
Myo-inositol	100	100	100
Nicotinic acid	0.5	0.5	5
Pyridoxine HCl	0.5	0.5	0.5
Thiamine HCl	0.1	0.1	5
Glutamine		1 500	100
Glycine	2	2	
ABA		13.22	
PEG (M.W. 6,000)		100000	
Maltose		60000	
BAP	0.675		
2,4-D	0.663		
IBA		1–3	
Activated charcoal		2000	
Sucrose	15000	30000	
agar		8000	
gelrite	3000		

Maturation of somatic embryos

1. In order to develop somatic embryos, the suspension cells were transferred to ammonium free MS medium supplemented with 10 μM ABA, 0.2% activated charcoal, 10% polyethylene glycol (PEG, MW 6000), 30 mM L-glutamine and 6% maltose.
2. Collect embryogenic cells on 100 μm nylon screen.
3. Resuspend embryogenic cells in fresh medium (about 500 mg FW per 10 ml cell suspension medium).

4. Dispense as 2 ml aliquots on filter paper disk over each Petri dish containing maturation medium as specified in Table 2.
5. Seal Petri dish and culture in the dark.

Germination

1. Collect somatic embryos from maturation medium and transfer to filter paper disk over each Petri dish containing germination medium as described in Table 2.
2. Obtained cotyledonary embryos were transferred on ammonium free MS agar-solidified medium in culture flasks under a 16 h photoperiod.
3. Plantlets were transferred to vermiculite containing modified MS (ammonium and sugar free) liquid medium in 200 ml culture flasks, then out planted after habituation procedure of 2 weeks in 100% moisture content.
4. The cultures were incubated under daily 16/8 h light-dark photoperiods of fluorescent lamp at 25°C.

3. CONCLUDING COMMENTS

3.1. Organ Culture

Adventitious buds were induced on the surface of the mature embryos on 1/2 DCR medium containing 0.4 μM to 2 μM BAP (Table 3), and they grew to shoots after subculturing to medium containing 2 g/l activated charcoal. Cotyledon development was observed in the medium containing 0.1 μM NAA and green callus was prevalent at the higher concentrations of BAP in the medium in the initial culture (Table 3). From the elongated shoots, root primordia and roots were induced in RIM medium containing 4.9 to 14.8 μM IBA. Regenerated plantlets were in the pots with the florialite® containing 0.1 % hyponex® for 2 weeks under 100% humidity, then 13 plantlets were planted out successfully to the field (Ishii et al., 2004) (Figure 1D). Survival rate of the plantlets was 92% after 1 year in the field condition.

3.2. Somatic Embryogenesis

Embryogenic cell suspensions were induced better from immature seeds of *Pinus armandii* var. *amamiana* on modified MS (half strength in major elements and ammonium free) liquid medium supplemented with 3 μM 2,4-D and 3 μM BAP (Table 4). However, it seems that effects of hormonal combination was not so determinative because somatic embryogenic cells were also obtained in other combinations. Physiological and genetic conditions of immature embryos might be also important for somatic embryogenesis. Induced suspension cells were subcultured successfully every 2–3 weeks (Figure 2A). After 1 to 2 months culture on maturation

Table 3. *Effects of plant growth regulators (PGR) on culture of mature embryos from seeds of Pinus armandii var. amamiana.*

PGR	μM	No. of responded embryos/No. of embryos (%)		
		Adventitious buds	Green callus	Cotyledon development
BAP	2			
NAA	0.1	2/20 (10)	4/20 (20)	6/20 (30)
BAP	10			
NAA	0.1	0/20 (0)	14/20 (70)	2/20 (10)
BAP	0.4	6/10 (60)	2/10 (20)	0/10 (0)
BAP	2	4/10 (40)	4/10 (40)	0/10 (0)
BAP	10	0/10 (0)	6/10 (60)	0/10 (0)

Ten to twenty mature embryos were used for each treatment.

medium, differentiation of embryos progressed and cotyledonary embryos were obtained (Figure 2B). Transplanting of somatic embryos further to ammonium free MS solidified medium for 3 weeks was necessary for developing plantlets with roots and green cotyledons. Plantlets transplanted to vermiculite in 200 ml culture flasks survived (Figure 2C).

Embryogenic cells were also induced from mature seeds of *Pinus armandii* var. *amamiana* on 1/2 EM medium (Maruyama et al., 2000) containing 10 μM 2,4-D and 5 μM BAP. The supplement of L-glutamine into media enhanced embryo maturation and prevented somatic embryos from browning (Hosoi & Ishii, 2001). Forty seven regenerated plantlets showed normal growth in the greenhouse (Figure 2D).

For *ex situ* conservation of endangered *Pinus armandii* var. *amamiana, in vitro* culture methods will help propagate rootstocks for grafting or seedlings from seed orchard (Ishii et al., 2005). *In vitro* culture itself might be used as the *ex situ* conservation method.

Table 4. *Effects of combination of 2,4-D and BAP on induction rate of somatic embryo forming cells from immature embryos of Pinus armandii var. amamiana.*

BAP (μM)	2,4-D (μM)			
	0.3	1	3	10
0	*0/20 (0)	2/18 (11.1)	2/18 (11.1)	2/14 (14.3)
1	2/18 (11.1)	2/19 (10.5)	2/19 (10.5)	1/13 (7.7)
3	3/17 (17.6)	2/19 (10.5)	4/19 (21.1)	1/12 (8.3)

Twelve to twenty immature embryos were used for each treatment. * No. of embryos inducing somatic embryos/No. of embryos (%)

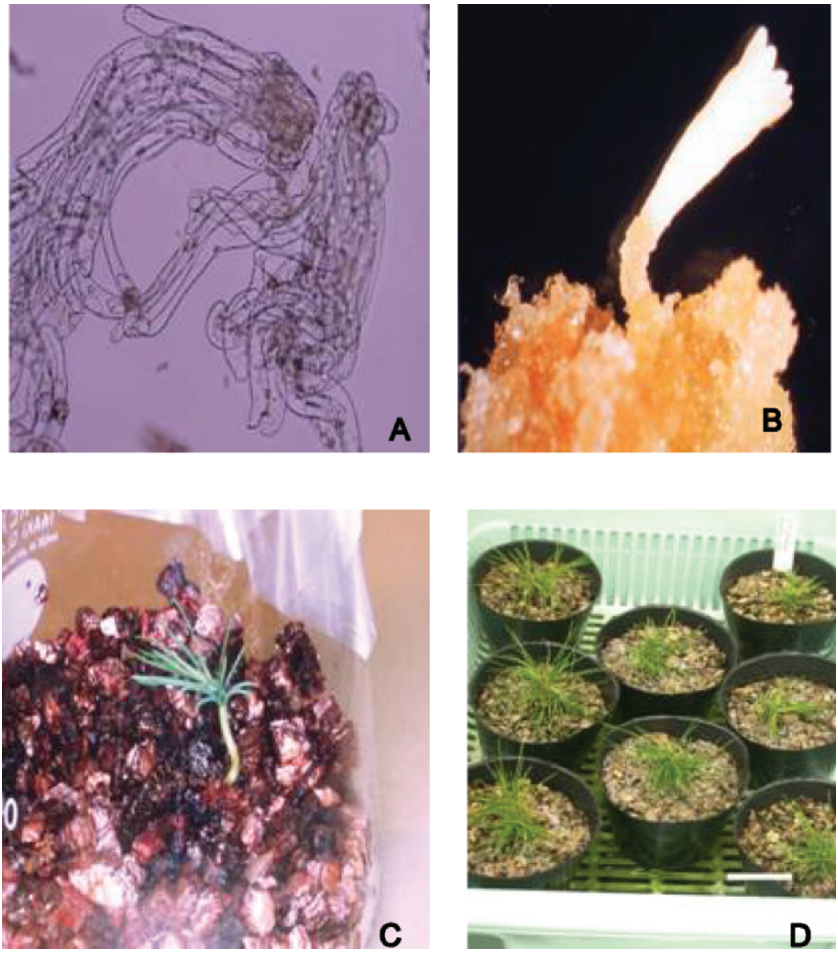

Figure 2. *Micropropagation of Pinus armandii var. amamiana by somatic embryogenesis. A) Suspension culture of somatic embryogenic cells of Pinus armandii var. amamiana. B) Maturation of somatic embryo of Pinus armandii var. amamiana. C) Regenerated plantlet of Pinus armandii var. amamiana from somatic embryo. D) Habituated plantlets of Pinus armandii var. amamiana from somatic embryos.*

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