Ann. For. Res. 51: 5-10, 2008

ANNALS OF FOREST RESEARCH
www.e-afr.com

Micropropagation of an endangered species *Pinus armandii* var. *armamiana*

K. Ishii, Y. Hosoi, E. Maruyama, S.-I. Kanetani

Ishii K., Hosoi Y. Maruyama E., Kanetan S.-I., 2008. Micropropagation of an endangered species *Pinus armandii* var. *amamiana* Ann. For. Res. 51:5-10

Abstract. For micropropagation via organ culture, mature embryos were excised from the seeds of *Pinus armandii*. Franch. var. *amamiana* (Koidz.) Hatusima, an endangered species only inhabiting the south west islands of Japan. Adventitious buds were induced on the surface of the embryo on 1/2 DCR medium containing BAP, and they grew shoots after subculturing to medium containing activated charcoal or a low concentration of thidiazuron. From the elongated shoots, root primordia and roots were induced in medium containing IBA as an auxine. We found that a low concentration of zeatin or BAP added to the medium was beneficial for plant regeneration of mature embryos of this species. For micropropagation via somatic embryogenesis, embryogenic cell suspensions were induced from a mature and immature seed of *P. armandii* var. *amamiana* on MS liquid medium supplemented with 1 μM 2, 4-D and 3 μM BAP. The suspensions were incubated in the dark at 25^0. Induced suspension cells were transferred to ammonium free MS liquid medium supplemented with 1 μM 2, 4-D, 3 μM BAP and 30m M L-glutamine and subcultured every 2 weeks. In the other set of the experiment, the induction rate of somatic embryogenesis was high with ammonium free half strength MS medium. 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% PEG (MW6000), 30m M L-glutamine and 6% maltose. The cultures were incubated under a 16h light/8h dark photoperiod. After 1-2 months of culture, differentiation of embryos progressed and cotyledonary embryos were obtained. These embryos were transferred on ammonium free MS solid medium under 16 h photoperiod. After 2-3 weeks plantlets with roots and green cotyledons were obtained. Plantlets were transplanted to vermiculite containing modified MS liquid medium in 200 ml culture flasks, then out planted after habituation procedure
Keywords: micropropagation, *Pinus armandii* var. *amamiana*, conifer, somatic embryogenesis

Authors. Katsuaki Ishii (katsuaki@ffpri.affrc.go.jp), Yoshihisa Hosoi, Emilio Maruyama, Sei-ichi Kanetan -Forestry and Forest Products Research Institute, Matsunosato 1, Tsukuba, Ibaraki 305-8687, Japan

Introduction

In a survey conducted by the Environmental Agency on endangered species in Japan in the year 2000, 1665 species were listed as endangered ones among 7087 vascular plants (Environmental Agency of Japan 2000). Collection for ornamental use, natural succession, and deforestation are the three major causes threatening the species. To recover the endangered species, propagation of plants for *ex situ* or *in situ* conservation is

important. Among them, micropropagation by tissue culture is considered an effective and useful method.

There were several reports on micro-propagation of endangered trees (Okochi et al. 2003, Sugii & Lamoureux 2004). Here, we report micropropagation of an endangered tree, *Pinus armandii* Franch. var. *amamiana* (Koidz.) Hatusima, in order to preserve them *ex situ* and to supply plants for rehabilitation. *P. armandii* var. *amamiana* is an endangered tree inhabiting only the south western islands of Japan, Yakushima and Tanegashima (Yahara et al. 1987). A recent survey showed that there are only about 2000 trees remaining on both islands (fig.1). 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 numbers in recent years in the natural populations, it was declared as endangered (Environmental Agency of Japan 2000), which denotes a high possibility of extinction in the near future. Part of this report was published elsewhere (Ishii et al. 2005).

Material and methods

Mature and immature seeds were collected from early July to early September from the remaining trees of *P. armandii* var. *amamiana* in Yakushima Island. For separation of empty seeds caused by inbreeding depression, only submerged seeds in 100% ethanol were used for experiments in the case of mature seeds.

Figure 1 A survival tree of *P. armandii* var. *amami ana* in Yakushima Island

Mature embryos were excised from the seeds, and were cultured *in vitro* under different tissue culture conditions. Mainly a half strength DCR (Gupta & Durzan 1985) medium with different concentrations of plant growth regulators (2 μM or 10 μM BAP plus 0.1 μM NAA, 0.4 μM, 2 μM and 10 μM BAP) was used. Culture tubes (18 mm i.d. x 160mm) containing 15ml of agar solidified media were used for initial culture, and 200ml culture flasks containing 70ml agar-solidified medium were used for subculture. For shoot elongation, media containing 5g/l activated charcoal or 0.36 to 9μM thidiazuron were used. For rooting of shoots, RIM medium (Abo El-Nil & Milton 1982) containing indole butyric acid (IBA) was used. Culture condition was at 70 $\mu Mm^{-2}s^{-1}$ fluorescent light under daily 16/8h light-dark photoperiods at 25^0. Regenerated and habituated plantlets from organ culture of mature embryos were transferred to the nursery field made of black soil loam.

For propagation via somatic embryogenesis, embryogenic cell suspensions were induced from immature and mature seeds on modified MS (Murashige & Skoog 1962) or 1/2 EM (Maruyama 2000) medium supplemented with different concentration of 2, 4-D and BAP. The cultures were incubated in the dark at 25^0. Induced suspension cells were transferred to ammonium free MS liquid medium supplemented with 2, 4-D, BAP and L-glutamine and subcultured every 2-3 weeks. In order to obtain mature somatic embryos, the suspension cells were transferred to an ammonium free MS medium supplemented with 10 μM abscisic acid (ABA), 0.2% activated charcoal, 10% polyethylene glycol (PEG, MW 6000), 30m M L-glutamine and 6% maltose. The cultures were incubated under daily 16/8h light-dark photoperiods of fluorescent lamp at 25^0. Mature cotyledonary embryos were transferred on ammonium free MS agar-solidified medium under a 16h photoperiod for germination. Plantlets were transferred to vermiculite containing modified MS (ammonium and sugar free) liquid medium in 200ml culture flasks, then out planted after habituation procedure of 2 weeks in 100% moisture content.

Results and discussion

Organ culture

Adventitious buds were induced on the surface of the mature embryos on 1/2 DCR medium containing 0.4μM μ2 μM BAP (table 1, fig. 2), and they grew shoots after subculturing to medium containing 5g/l activated charcoal or 0.36 μM to 9 μM thidiazuron. 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 1). From the elongated shoots, root primordia and roots were induced in RIM medium containing 4.9 μM to 14.8 μM IBA (fig. 3). Regenerated plantlets were in the pots with the florialite O, R containing 0.1% hyponex O, R for 2 weeks under 100% humidity, then 13 plantlets were planted out successfully to the field (Ishii et al. 2004) (figure 4). Survival rate of the plantlets was 92% after one year in the field.

Table 1 Effects of plant growth regulators (PGR) on induction of adventitious bud forma tion from mature seed embryos of *Pinus armandii* var. *amamiana*

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

Figure 2 Adventitious buds on mature embryos of *P. armandii* var. *amamiana*

Somatic embryogenesis

Embryogenic cell suspensions were induced better from immature seeds of *P. 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 2). However, it seems that effects of plant growth regulator combinations were not so determinative because somatic embryogenic cells were also obtained in other combinations. Physiological and genetic state of immature embryos might be also important for somatic embryogenesis. Induced suspension cells were subcultured successfully every 2-3 weeks (fig. 5). There are many

Figure 3 Root formation from shoots of *P. armandii* var. *amamiana.*

Figure 4 Field grown plantlets obtained by organ culture of *P. armandii* var. *amamiana*

1995, Von Aderkas et al. 2005). Like other *Pinus* species, ABA, activated charcoal and PEG were effective for maturation of somatic embryos of *P. armandii* var. *amamiana*. Phytohormone and osmotic potential of the medium might be also the key factors for the embryo maturation with this pine. After 1 to 2 months culture on maturation medium, differentiation of embryos progressed and cotyledonary embryos were obtained (fig. 6). Further transplanting of somatic embryos to ammonium free MS solidified medium for 3 weeks was necessary for developing plantlets with roots and green cotyledons. Plantlets transplanted to vermiculite in 200ml culture flasks survived (fig.7).

Embryogenic cells were also induced from mature seeds of *P. armandii* var. *amamiana* on 1/2 EM medium (Maruyama et al. 2000) containing 10 μM 2, 4-D and 5 μM BAP. The supplement L-glutamine into media enhanced embryo maturation and prevented somatic embryos from browning (Hosoi and Ishii 2001). Forty-seven regenerated plantlets showed normal growth in the greenhouse (fig. 8).

For *ex situ* conservation of endangered *Pinus armandii* var. *amamiana,* the only practical method so far was grafting the scion buds from *in situ* mother trees onto exotic five needle pine like *Pinus strobus* for establishing clone bank or seed orchard. However, it requires management of exotic pine rootstock is essential. *In vitro* culture methods will help propagate rootstocks for grafting on the same domestic pine species or seedlings from seed orchard. *In vitro* culture itself might be used as the *ex situ* conservation method in conjunction with cryopreservation.

Table 2 Effects of combinations of 2, 4-D and BAP on induction rate of somatic embryogenesis from immature zygotic embryos of *Pinus armandii* var. *amami ana.*

BAP	2,4- D (ĕM)			
(ĕ M)	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	3/14 (17.6)	2/19 (10.5)	4/19 (21.1)	1/12 (8.3)

Figure 5 Suspension culture of somatic embryogenic cells of *P. armandii* var. *amamiana*

Acknowledgements

This study was partly supported by a grant provided by Ministry of the Environment, government of Japan, for a research project

Figure 6 Maturation of somatic embryo of *P. armandii* var. *amamiana*

Figure 7 Regenerated plantlet of *P. armandii* var. *amamiana* from somatic embryo

Figure 8 Habituated plantlets of *P. armandii* var. *amamiana* from somatic embryos

'Research on Conservation of Endemic Tree Species and Genetic Diversity in Ecosystem of Yakushima Island'. We thank Kagoshima Prefectural Forestry Experiment Station for supplying plant materials and NPO Yattanetai for their kind support in collecting seeds.

References

Abo El-Nil, M.M. & Milton, W. 1982. Method for asexual reproduction of coniferous trees. United States Patent, No. 4353186.

Akiba, M. & Nakamura, K. 2005. Susceptibility of adult trees of the endangered species *Pinus armandii* var. *amamiana* to pine wilt disease in the field. J. For. Res. 10:3-7.

Environmental Agency of Japan. 2000. Threatened wildlife of Japan, Red Data Book 2nd edition, vol. 8. Vascular Plants 8:660.

Gupta, P.K. & Durzan, D.J. 1985. Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). Plant Cell Rep. 4:177-179.

Hosoi, Y. & Ishii, K. 2001. Somatic embryogenesis and plantlet regeneration in *Pinus armandii* var. *amamiana*. In: Morohoshi, N. and Komamine, A. (eds.). Molecular Breeding of Woody Plants, Elsevier Science B.V. Amsterdam - Tokyo, 313-318.

Ishii, K., Hosoi, Y., Maruyama, E., Kanetani, S. & Koyama, T. 2004. Plant regeneration from mature embryos of endangered species *Pinus armandii* Franch. var. *amamiana* (Koidz.) Hatusima. Journal of Society of High Technology in Agriculture 16:71-79.

Ishii, K., Maruyama, E., Hosoi, Y. & Kanetani, S. 2005. In vitro propagation of three endangered species in Japanese forests. Propagation of Ornamental Plants 5:173-178.

Jain, S.M., Gupta, P.K. & Newton, R.J. 1995. Somatic embryogenesis in woody plants vol. 3 - Gymnosperms. Kluwer Academic Publishers 388 pp.

Maruyama, E., Tanaka, T., Hosoi, Y., Ishii, K. & Morohoshi, N. 2000. Embryogenic cell culture, protoplast regeneration, cryopreservation, biolistic gene transfer and plant regeneration in Japanese cedar (*Cryptomeria japonica* D. Don). Plant Biotechnology. 17:281-296.

Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15:473-497.

Okochi, I., Tanaka, N., Makino, S. & Yamashita, N. 2003. Restoration and conservation of island forest ecosystems in the Ogasawaras. Bio-refor proceedings of Yogyakarta workshop 117-118.

Sugii, N. & Lamoureux, C. 2004. Tissue culture as a conservation method. An empirical view from Hawaii. In: Guerrant Jr. et al. (eds.) *Ex Situ* Plant Conservation - Supporting Specific Survival in the Wild. Island Press 189-205.

Von Aderkas, P., Coulter, A., White, L., Wagner, R., Robb, J., Rise, M., Temmel, N., Maceacheron, I., Park, Y.S. & Bonga, J. 2005. Somatic embryogenesis via nodules in *Pinus strobes* L. and *Pinus banksiana* Lamb. - Dead ends and new beginnings. Propagation of Ornamental Plants 5:3-15.

Yaharta, T., Ohba, H., Murata, J. & Iwatsuki, K. 1987. Taxonomic review of vascular plants endemic to Yakushima Island, Japan. J. Fac. Sci. Univ. Tokyo III 14: 69-111.

Rezumat. Ishii K., Hosoi Y., Maruyama E., Kanetan S.-I., 2008. Înmulţirea in vitro a unei specii periclitate: *Pinus armandii* var. *amamiana*. Ann. For. Res. 51:5-10

În ideea micro propagării prin cultura de organe în vitro au fost prelevaţi embrioni din seminţe de *Pinus armandii* Franch. var. *amamiana* (Koidz.) Hatusima; aceasta este o specie periclitată răspândită doar în sud vestul insulelor japoneze. Pe suprafaţa embrionilor însămânţaţi pe mediul de cultură de tipul mDCR conţinând BAP au fost induşi muguri adventivi care după ce au fost repicaţi pe un mediu conţinând cărbune activat sau o concentraţie scăzută de thidiazuron au format lujeri. Pe mediul conţinând IBA ca auxină, din lujerii alungiţi au fost induse primordii sau rădăcini.

S-a observat că zeatina sau BAP în concentraţii mici au avut efect pozitiv asupra regenerării plantelor din embrioni somatici. Pentru mictopropagarea prin embriogeneză somatică a speciei, *P. armandii* var. *amamiana*, au fost induse suspensii embriogene din seminţe imature şi mature, pe mediu MS (Murashige-Skoog, 1968) lichid, suplimentat cu 1 mM 2, 4-D şi 3 mM BAP. Suspensiile au fost incubate în întuneric, la 250C. Suspensiile au fost

menţinute în cultură pe mediu MS lichid modificat (lipsit de amoniu), suplimentat cu 1mM 2, 4-D, 3 mM BAP şi 30m M L-glutamină, prin subculturi efectuate la intervale de două săptămâni. Într-un alt set de experimente, a fost obţinută o rată ridicată a inducţiei embriogene utilizând mediu MS 1/2 concentrare şi lipsit de amoniu. În scopul dezvoltării embrionilor somatici, suspensiile celulare au fost transferate pe mediu MS lipsit de amoniu şi suplimentat cu 10 m M ABA, 0.2% cărbune activ, 10% PEG (MW6000), 30m M L-glutamină şi 6% maltoză. Culturile au fost incubate la lumină (fotoperioada de 16 ore lumină / 8 ore întuneric). După 1-2 luni de cultură, a început diferenţierea embrionilor şi au fost observaţi primii embrioni cotiledonari. Aceşti embrioni au fost separaţi şi cultivaţi pe mediu MS solidificat, lipsit de amoniu, într-o fotoperioadă de 16 ore lumină/8 ore întuneric. După 2 - 3 săptămâni au fost obţinute plantule verzi, cu cotiledoane şi rădăcini embrionare. Acestea au fost transferate în tuburi de 200 ml cu vermiculit îmbibat cu mediu MS lichid modificat, şi apoi plantate în exterior pentru aclimatizare.

Cuvinte cheie: micropropagare, *Pinus armandii* var. *amamiana*, conifere, embriogeneza somatică.

(Tradus de I. Blada)