Micropropagation and Protoplast Culture in *Paraserianthes falcataria*

Miyuki Chujo, Junji Eizawa, Shinso Yokota, Futoshi Ishiguri, Kazuya Iizuka, Dody Priadi, Nurul Sumiasri, and Nobuo Yoshizawa

Abstract

Paraserianthes falcataria (L.) Nielsen is a fast-growing tree native to Indonesia that has been widely planted throughout the tropics. The growth and wood qualities of *P. falcataria* should be improved to promote the establishment of plantation forests for this species. Tissue culture technique has a potential to be applied for tree breeding programs for *P. falcataria*. The objective of this study is to establish the micropropagation and protoplast culture protocol of *P. falcataria*. In the present study, the respective conditions were investigated for seedling culture, callus induction, protoplast isolation, and protoplast culture. Surface-sterilized seeds were cultured on MS (Murashige and Skoog) medium at 25, 27, and 29°C. After 4 weeks of culture, 27°C gave the best result for average shoot length. Five types of explant (leaflet, petiole, internode, cotyledon, and hypocotyl) obtained from the seedlings were used for callus induction. They were cultured on the MS media containing a combination of 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) at different concentrations. Green nodular callus was obtained from only leaflet. The most effective medium condition for callus induction from leaflet was the MS medium containing 10.0 µM BAP and 10.0 µM 2,4-D. Leaflet of seedlings was used for protoplast isolation. Based on the results of the yield and viability of protoplasts, the best enzymatic condition was as follows: enzyme solution, 1% Cellulase Onozuka RS, 0.5% Pectolyase Y-23, and 1% Driselase; osmoticum, 0.8 M mannitol; treatment temperature and time, 30°C for 4 hrs. Isolated protoplasts were incubated in liquid AA media with a combination of 1-naphthaleneacetic acid and thidiazuron at different concentrations. Although cell wall formation was observed, cell division did not occur.

Key words: callus, fast growing tropical tree, *Paraserianthes falcataria*, protoplast, tissue culture.

Introduction

Paraserianthes falcataria (L.) Nielsen is a woody legume native to the Moluccas, New Guinea, the Bismarck Archipelago, and the Solomon Islands, and one of fast-growing trees. The wood of this species has been used for light-construction materials, veneer, particle board, and pulp.

The growth and wood qualities of *P. falcataria* should be enhanced by tree breeding program to promote the establishment of plantation forests for this species. Because growth characteristics, such as diameter and height, of *P. falcataria* vary among the trees originated from different seed sources, these growth characteristics might be improved by genetic manipulation (Chigira *et al*. 2007). In addition, Ishiguri *et al*. (2007) examined wood qualities, such as basic density and fiber length. A significant difference in the basic density of core wood (from 10 cm to the bark) was observed among the trees, indicating that the selection of a plus tree with high density wood is possible at relatively early stage in this species. Thus, breeding for growth characteristics and wood quality in *P. falcataria* has a strong potential. However, there are only a few tree breeding programs targeting this species (Chigira *et al*. 2007).

Traditional breeding methods are often constrained by the long reproductive cycles and by the difficulty in achieving significant improvements to complex traits such as wood properties, disease and pest control, and tolerance to abiotic stress (Nehra *et al*. 2005). Biotechnology has a potential to solve these problems. However, only a few reports on tissue culture in *P. falcataria* are available (Bon *et al*. 1998; Sukarutiningsih *et al*. 2002; Sumiasri *et al*. 2006). For example, Sukarutiningsih *et al*. (2002) reported that plantlets were successfully regenerated from nodes and cotyledonary nodes obtained from seedlings, when these explants were cultured on a B5 medium with 6-benzylaminopurine (BAP) at 25°C under 16-hours photoperiod. In callus culture, embryogenic callus was successfully obtained from petioles, hypocotyls, and cotyledons taken from seedlings, when they were cultured on MS (Murashige and Skoog 1962) medium with 0.29 µM (0.05 mg/l) indole-3-acetic acid and 9.08 µM (2.0 mg/l) thidiazuron (TDZ) (Sumiasri *et al*. 2005). However, more efficient micropropagation system should be developed in this species.

In the present study, *in vitro* culture of seedlings, callus induction, and protoplast culture were investigated for the establishment of efficient micropropagation protocol in a fast growing tropical tree, *P. falcataria*.

Materials and Methods

In vitro Culture of Seedlings

Seeds of *P. falcataria* were provided from the Indonesian Institute of Sciences. The seeds were soaked in distilled water overnight. After washing with distilled water containing detergent for 1 min, they were rinsed thoroughly with running tap water for 20 min, and then soaked in hot water (80~90°C) for 5 min to break down dormancy of seeds. After that, they were surface-sterilized with 70% ethanol for 1 min. Subsequently surface sterilization was carried out with 3% (v/v) sodium hypochlorite aqueous solution (Kanto Chemical, Japan) containing a few drops of Tween 80 (Wako Pure Chemical Industries, Japan) for 40 min. Then they were washed 3 times with sterile distilled water. Surface-sterilized seeds were cultured on MS (Murashige and Skoog 1962) medium with 3% sucrose and 0.2% gellan gum, whose pH was adjusted to 5.7 ± 0.1. They were cultured under a 16-hour photoperiod (50~70 $\mu mol.m^{-2}.s^{-1}$) at different temperatures (25, 27, and 29°C) for 4 weeks.

Callus Induction and Culture

Five types of explant (leaflet, petiole, internode, cotyledon, and hypocotyl) obtained from the seedlings were used for callus induction. Each explant was cultured in MS media with a combination of a cytokinin (BAP) and an auxin (2,4-dichlorophenoxyacetic acid (2,4-D)) at different concentrations (0, 0.1, 1.0, and 10.0 µM). The explants were cultured under a 16-hour photoperiod (50~70 $\mu mol.m^{-2}.s^{-1}$) at 25°C for 4 weeks.

Protoplast Culture

Leaflet excised from seedlings was used for protoplast isolation. To examine effects of enzyme combinations on protoplast isolation, twenty-four combinations of the enzyme solution were prepared using the following 6 enzymes of the destynated concentrations: 1% Cellulase Onozuka RS (Yakult Pharmaceutical Industry, Japan), 1% Cellulase Onozuka R-10 (Yakult Pharmaceutical Industry, Japan), 1% Driselase 20 (Kyowa Hakko Bio, Japan), 0.5% Pectolyase Y-23 (Kyowa Chemical Products, Japan), 1% Macerozyme R-10 (Yakult Pharmaceutical Industry, Japan), and 1% Hemicellulase (Sigma, U.S.A.). Leaflet, 8 mg in fresh weight, cut in half was floated on 400 µL of the enzyme solution in a 24-well-tissue-culture plate (Falcon, Becton Dickinson, U.S.A.), then incubated stationarily at different temperatures (25, 30, and 35°C) for different duration (4, 8, and 12 h). For examining the effects of the osmotic potential of enzyme solution, the concentration of mannitol or sorbitol was varied from 0.4, 0.6, 0.8, and 1.0 M. After enzyme treatment, the debris was removed off with a 40 µm nylon mesh, and then protoplasts were collected by centrifugation at 100 xg for 5 min. The collected protoplasts were washed 3 times with mannitol or sorbitol solution by centrifugation. Protoplast yield was determined using a hemocytometer (Thoma line, Erma, Japan) under an inverted fluorescence microscope (IX 70, Olympus, Japan). Protoplasts were stained with 0.01% (w/v) fluorescein diacetate (FDA) in mannitol solution to observe their viability (Larkin 1976). The excitation filter and barrier filter were BP 470-490 and BA 515-550, respectively.

The protoplast suspension (5 x 10^4 protoplasts/mL) was cultured in 50 µL of amino acid (AA) liquid medium (Thompson *et al*. 1986; Mimura *et al*. 1997) containing 0.8 M mannitol, 3% sucrose, and a combination of 1-naphtaleneacetic acid (NAA) (0, 0.1, 1.0, 10.0, and 100.0 µM) and TDZ (0, 0.1, 1.0, and 10.0 µM) in a 96-well-tissue-culture plate (Falcon, Becton Dickinson, U.S.A.). Five µg/mL of fluorescent brightener 28 (Sigma, U.S.A.) was added to the medium to visualize cell wall materials under the inverted fluorescence microscope (Hahne *et al.* 1983). The excitation filter and barrier filter were BP 330-385 and BA 420, respectively.

Results and Discussion

***In vitro* Culture of Seedlings**

Table 1 shows shoot length, number of leaf, and fresh weight of seedlings cultured under different temperatures for 4 weeks. The best result for shoot length (88.5 mm per shoot), leaf number (4.8 leaves per shoot), and fresh weight of seedling (0.40 g per seedling) was obtained at the temperature of 27°C. Especially in average value of shoot length, significant differences were found among the temperatures. However, significant differences were not observed in average number of leaf and average fresh weight among temperatures. Whitening of leaves was observed in plantlets cultured at 29°C (Figure 1). Successful culture of some tropical tree species is very sensitive to temperature (Bonga and von Aderkas 1992). For example, Rahman and Blake (1988) reported that optimal temperature and photoperiod for shoot proliferation were 30°C and 12 h, when nodes from seedlings of *Artocarpus heterophyllus* were cultured under different temperatures (20, 25, 30, and 35°C) with different photoperiods (0, 8, 12, and 16 h). In *Leucaena leucocephala* which is a tropical tree legume, shoots from seedlings and adult trees multiplied at a rate of 6- to 7-fold every three weeks at the optimal temperature of 30°C (Dhawan and Bhojwani 1985). In the present study, seedlings cultured at 27°C gave the best result, suggesting that 27°C is the optimal temperature for culture of *P. falcataria*.

Callus Induction and Culture

Table 2 shows the formation rate and color of callus induced from 5 types of explant in MS media with a combination of BAP and 2,4-D at different concentrations after 4 weeks of culture. When petiole, internode, and hypocotyl were used as explants, high formation rates of callus (more than 80%) were obtained with many PGR conditions. On the other hand, leaflets produced calli with 100% formation rate only in two PGR conditions (10.0 µM BAP + 0.1 µM 2,4-D and 10.0 µM BAP + 10.0 µM 2,4-D). Cotyledon explants showed very low performance for callus induction under any PGR conditions. High concentrations of BAP and 2,4-D were effective for callus proliferation in all explants except for cotyledon. Especially, in leaflet, petiole, internode, and hypocotyl, the combination of 10.0 µM BAP and 10.0 µM 2,4-D was the most effective. Formation of green color callus was observed in leaflet explants cultured on the media with all the PGR combinations, except for 2,4-D alone. Nodular callus was produced from leaflets in a combination of 10.0 µM BAP and 10.0 µM 2,4-D (Figure 2). Shoot differentiation has been achieved from nodular callus in woody legumes, such as *Acacia sinuata* (Vengadesan *et al*. 2000). As the results, leaflet-derived callus proliferated in the MS medium containing 10.0 µM BAP and 10.0 µM 2,4-D is considered to have a possibility for shoot regeneration in *P. falcataria*.

Table 1. Effects of temperature on *in vitro* growth of seedlings.

Temperature (°C)	Shoot length (mm)	Number of leaf	Fresh weight (g)
25	59.2 ± 10.6[c]	4.5 ± 0.8[a]	0.29 ± 0.13[a]
27	88.5 ± 9.3[a]	4.8 ± 0.9[a]	0.40 ± 0.12[a]
29	75.0 ± 11.1[b]	3.8 ± 0.6[a]	0.30 ± 0.07[a]

Note: Values (average ± standard deviation) with different letters are statistically significant according to Tukey's multiple comparison test. Data were recorded after 4 weeks of culture. MS medium was used as a basal medium without any PGRs. Number of sample = 10.

Table 2. Formation rate and color of callus from various explants cultured with combinations of BAP and 2,4-D.

Explant	2,4-D (µM)	BAP (µM) 0			0.1			1.0			10.0		
		FR (%)	P	C	FR (%)	P	C	FR (%)	P	C	FR (%)	P	C
Leaflet	0	0	-	-	10	+	G	30	+	G	50	+	G
	0.1	0	-	-	40	+	G	20	+	G	100	++	G
	1.0	0	-	-	40	+	G	90	++	G	60	+++	G
	10.0	0	-	-	20	+	G	30	+	G	100	+++	G
Petiole	0	0	-	Y	100	+	Y	100	+	Y	100	+	Y
	0.1	0	-	Y	100	++	Y	100	++	Y	100	+	Y
	1.0	70	+	Y	100	+	Y	100	++	Y	100	+++	Y
	10.0	80	+	Y	100	+	Y	100	++	Y	100	+++	Y
Internode	0	0	-	-	0	-	W	100	+	Y	100	+	Y
	0.1	100	+	Y	100	++	Y	100	++	Y	100	++	Y
	1.0	100	++	Y	100	++	Y	100	+++	YG	100	+++	Y
	10.0	100	++	Y	100	++	Y	100	+++	YG	100	+++	YG
Cotyledon	0	0	-	-	0	-	-	10	+	W	0	-	-
	0.1	0	-	-	20	+	W	0	-	-	10	+	YG
	1.0	10	+	W	10	+	YG	20	+	YG	20	+	YG
	10.0	10	+	YG	0	-	-	10	+	W	10	+	Y
Hypocotyl	0	10	+	W	40	+	W	30	+	W	20	+	W
	0.1	100	+	Y	100	+	Y	100	++	Y	100	++	Y
	1.0	100	+	Y	100	++	YG	100	++	YG	100	+++	YG
	10.0	100	+	Y	100	++	YG	100	+++	YG	100	+++	YG

Note: Callus proliferation (P): -, no callusing; +, low; ++, moderate; +++, high. Callus color (C): G, green; YG, yellowish green; Y, yellow, W, white. FR, formation rate. Data were recorded after 4 weeks of culture. MS medium was used as a basal medium.

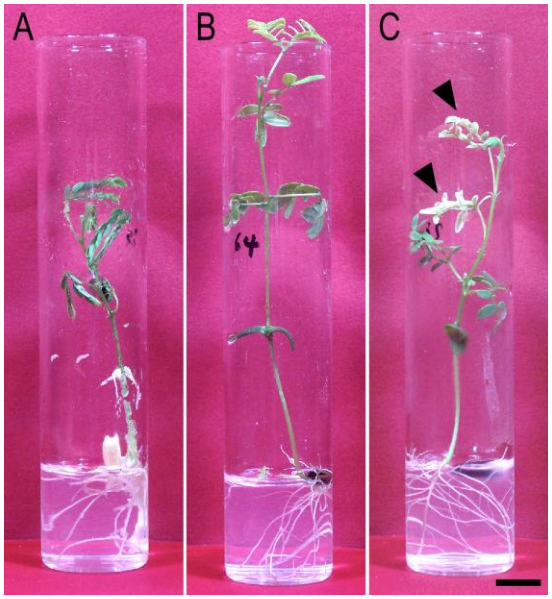

Figure.1. *In vitro* seedlings cultured at different temperatures.

Note: All photographs were taken at 4 weeks after culture. A, 25°C; B, 27°C; C, 29°C. Arrowheads indicate whitening of leaves. Culture was carried out of 4 weeks on MS medium without plant growth regulators. Scale bar = 1 cm.

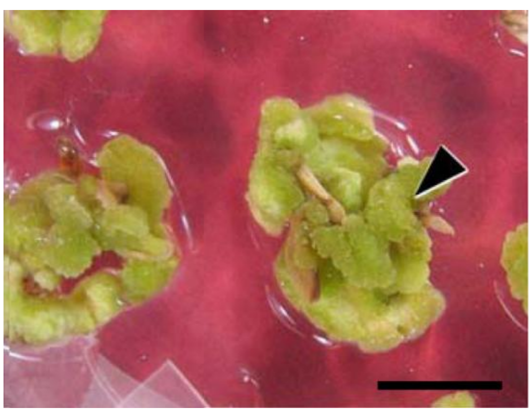

Figure. 2. Leaflet-derived callus proliferated on MS medium with 10 μM BAP and 10 μM 2,4-D.

Note: Arrowhead indicates nodular structure of callus. Culture was carried out of 4 weeks. Scale bar = 5 mm.

Protoplast Culture

Enzyme combination suitable for protoplast isolation from leaflets was selected as the first step for experiments. The combinations of Driselase and Pectolyase were effective for protoplast isolation in combination with Cellulase Onozuka R-10 or RS (data not shown). In addition, combinations of Driselase, Pectolyase, and Hemicellulase showed almost the same results. The highest yield of protoplasts (1.9 x 10^7 protoplasts/g FW) was obtained with a combination of Cellulase Onozuka R-10, Driselase, and Pectolyase. However, this combination gave relatively low viability of protoplasts (26.2%). On the other hand, two enzyme combinations containing Cellulase Onozuka RS produced more viable protoplasts (about 60% viability). Viability of protoplasts obtained with a combination of Cellulase Onozuka RS, Driselase, and Pectolyase (63.1%) was higher than that of a combination of Cellulase Onozuka RS, Driselase, Pectolyase, and Hemicellulase (59.9%). McCown and Russell (1987) suggested that high viability and purity (absence of debris) are required for protoplast culture. It is considered, therefore, that a combination of Cellulase Onozuka RS, Driselase, and Pectolyase is the most suitable for protoplast isolation from leaflets in *P. falcataria*.

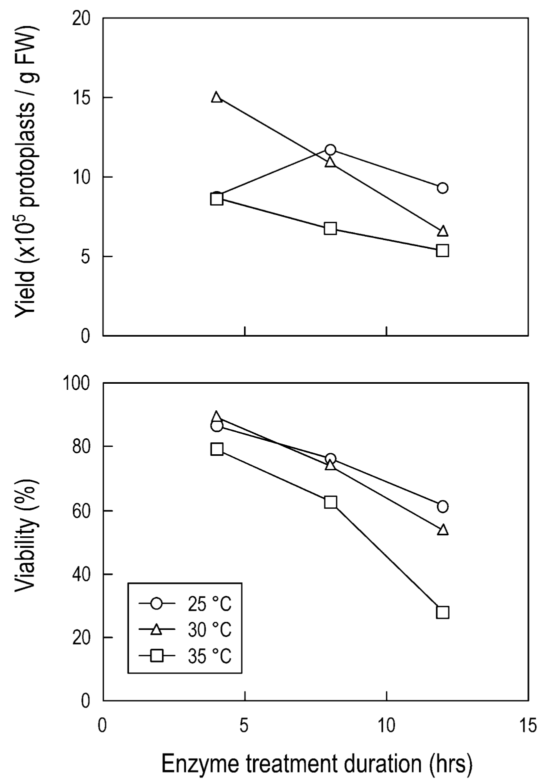

Figure 3. Effects of enzyme treatment temperature and duration on the yield and viability of protoplasts isolated from leaflets.

Note: Protoplasts were isolated with Cellulase Onozuka RS, Driselase, and Pectolyase containing 0.6 M monnitol as osmoticum.

Figure 3 shows the yield and viability of protoplasts isolated from leaflets treated with a combination of Cellulase Onozuka RS, Driselase, and Pectolyase under different temperatures and durations. As the results, both the yield and viability decreased with increase in the duration of enzyme treatment at all temperatures tested here. The highest yield (1.5 x 10^6 protoplasts/g FW) and viability (89.5%) of protoplasts were obtained at 30°C for 4 hrs. In general, the yield of protoplasts depends on temperature of enzyme solution during protoplast isolation (Kim *et al*. 2005). Grèzes *et al*. (1994) reported that the highest yield was obtained at 30°C, when protoplasts were isolated from suspension cultures of *Coffea arabica* using a combination of 3 enzymes (Cellulase Onozuka R-10, Driselase, and Macerozyme R-10) at different temperatures (25, 30, and 38°C). In *Acacia mangium* which belongs to legume, protoplasts derived from various explants were obtained with a combination of Cellulase Onozuka RS, Driselase, and Pectolyase Y-23 for 2 to 3 hrs (Saito *et al*. 1993). The results were quite similar to those in the present study in terms of enzyme combination and treatment duration.

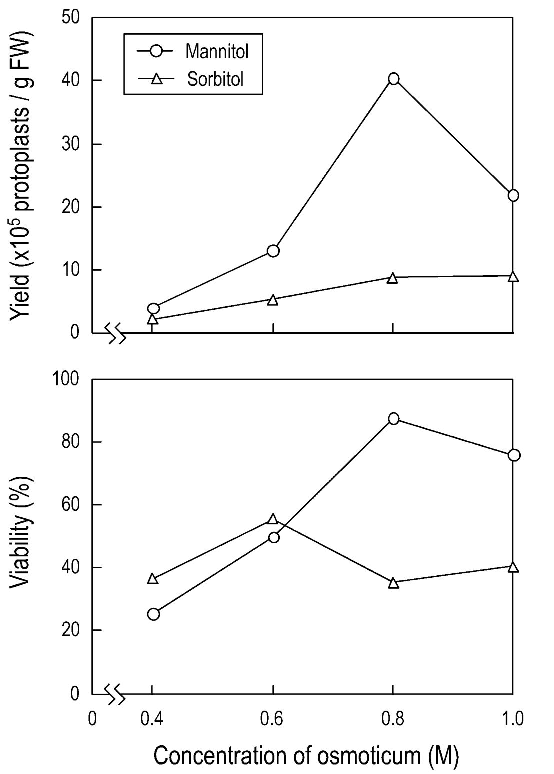

Figure 4. Effects of type and concentration of osmoticum on the yield and viability of protoplasts.
Note: Protoplasts were isolated with Cellulase Onozuka RS, Driselase, and Pectolyase at 30°C.

Effects of osmoticum on the yield and viability of protoplasts were examined to obtain the better yield and viability of protoplasts (Figure 4). The yield and viability of protoplasts isolated using mannitol or sorbitol as an osmoticum showed similar tendency within the concentration from 0.4 to 0.6 M. When osmoticum concentration was varied from 0.8 to 1.0 M, mannitol gave higher yield and viability of protoplasts than sorbitol. The highest yield (4.1 x 10^6 protoplasts/g FW) and viability (87.6%) of protoplasts were obtained with 0.8 M mannitol as an osmoticum. Use of 0.6 M mannitol as an osmoticum also was the most effective for protoplast

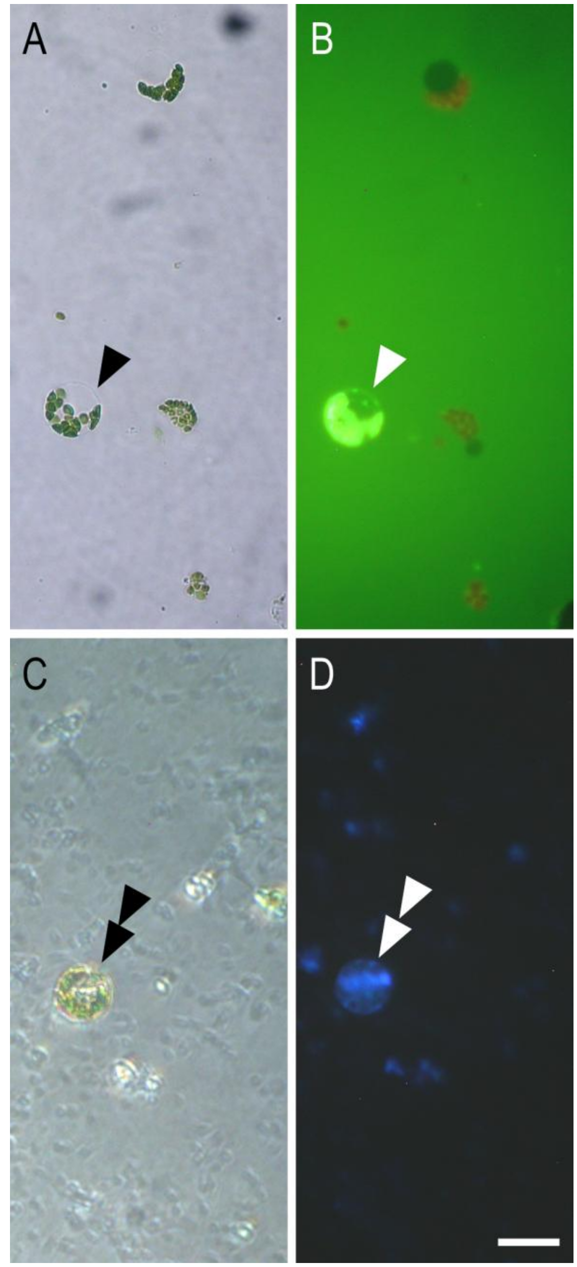

Figure 5. Isolated protoplasts with the combination of Cellulase Onozuka RS, Driselase, and Pectolyase containing 0.8 M monnitol for 4 hours at 30°C.
Note: A and C, Light microphotographs of protoplasts from leaflets; B, fluorescence microphotograph of protoplasts stained with FDA; D, fluorescence microphotograph of protoplasts stained with fluorescent brightener 28. The white and black arrowheads in A and B, and double arrowheads in C and D indicate the same protoplast. Scale bar = 40 µm.

isolation in *Populus alba* (Sasamoto *et al*. 1989), and *Betula platyphylla* (Wakita *et al*. 1996). On the other hand, in *Kandelia obovata*, suitable osmoticum for protoplast isolation was 0.8 M mannitol, and was 0.6 M mannitol for protoplast culture (Kaai *et al*. 2008). In the present study, as shown in Figure 4, relatively high osmotic concentration (0.8 M mannitol) gave the highest yield and viability of protoplasts in *P. falcataria*, suggesting that optimal osmotic condition is different among plant species.

Cell wall formation was observed in all media used after 6 hrs of culture (Figure 5). Cell division, however, did not occur even after 2 months of culture. Cell wall was regenerated in protoplasts derived from somatic embryos of *Coffea canephora* after 1 day of culture, and cell division was observed within 2 days of culture (Schöpke *et al*. 1987). Saito *et al*. (1987) also reported that cell wall was regenerated on the 1st day of culture, and the first cell division was observed on the 1st day of culture in mesophyll protoplasts of *Populus sieboldii* MIQ. On the other hand, in protoplasts isolated from leaves of *B. platyphylla*, cell division was observed after 1 month of culture (Wakita *et al*. 1996). McCown and Russell (1987) suggests that the slow developmental sequence may be a result of the highly differentiated nature of the original cell or merely the consequence of stress factors still present in the culture system that particularly influences a subpopulation of the original cells. In the present study, delay of development of protoplast-derived cells might occur. Further research is still needed for protoplast culture in *P. falcataria*.

Conclusions

In the present study, *in vitro* culture of seedlings, callus induction, and protoplast culture were investigated for the establishment of efficient micropropagation protocol in a fast growing tropical tree, *P. falcataria*. Seedlings cultured at 27°C gave the best result, suggesting that culture temperature of 27°C is the optimal for *P. falcataria* culture. Leaflet-derived callus proliferated in the MS medium containing 10.0 µM BAP and 10.0 µM 2,4-D is considered to have a possibility for shoot regeneration in *P. falcataria*. A combination of Cellulase Onozuka RS, Driselase, and Pectolyase is the most suitable for protoplast isolation from leaflets in *P. falcataria*. Relatively high osmotic concentration (0.8 M mannitol) gave the highest yield and viability of protoplasts in *P. falcataria.*

Acknowledgements

The authors would like to thank Prof. Dr. Hamako Sasamoto, Faculty of Environment and Information Sciences, Yokohama National University, for providing her kind guidance.

References

Bon, M.C.; D. Bonal; D.K. Goh; O. Monteuuis. 1998. Influence of Different Macronutrient Solutions and Growth Regulators on Micropropagation of Juvenile *Acacia mangium* and *Paraserianthes falcataria* explants. Plant Cell, Tissue and Organ Culture 53: 171-177.

Bonga, J.M.; P. von Aderkas. 1992. *In Vitro* Culture of Trees. Kluwer Academic Publishers, Dordrecht Boston London, Pp. 236.

Chigira, O.; K. Matsune; Y. Ogawa; S. Kurinobu. 2007. Family Variation in Two Years Old Seedling Seed Orchard of *Paraserianthes falcataria* in East Java, Indonesia. Kyushu Journal of Forest Research 60: 106-108.

Dhawan, V.; S.S. Bhojwani. 1985. *In Vitro* Vegetative Propagation of *Leucaena leucocephala* (Lam.) de Wit. Plant Cell Reports 4: 315-318.

Grèzes, J.; D. Thomas; B. Thomasset. 1994. Factors Influencing Protoplast Isolation from *Coffea arabica* Cells. Plant Cell, Tissue and Organ Culture 36: 91-97.

Hahne, G.; W. Herth; F. Hoffmann. 1983. Wall Formation and Cell Division in Fluorescence-Labelled Plant Protoplasts. Protoplasma 115: 217-221.

Ishiguri, F.; J. Eizawa; Y. Saito; K. Iizuka; S. Yokota; D. Priadi; N. Sumiasri; N. Yoshizawa. 2007. Variation in the Wood Properties of *Paraserianthes falcataria* Planted in Indonesia. IAWA Journal 28: 339-348.

Kaai, F.; Y. Kawana; H. Sasamoto. 2008. The Relation Between Recalcitrancy of A Mangrove Plant, *Kandelia obovata*, and High Endogenous Level of Abscisic Acid. Plant Cell, Tissue and Organ Culture 94: 125-130.

Kim, J.B.; J.E.M. Bergervoet; C.J.J.M. Raemakers; E. Jacobsen; R.G.F. Visser. 2005. Isolation of Protoplasts, and Culture and Regeneration into Plants in *Alstroemeria*. *In Vitro* Cellular and Developmental Biology - Plant 41: 505-510.

Larkin, P.J. 1976. Purification and Viability Determinations of Plant Protoplasts. Planta 128: 213-216.

McCown, B.H.; J.A. Russell. 1987. Protoplast Culture of Hardwoods. In: Bonga, J.M.; D.J. Durzan (Eds.), Cell and Tissue Culture in Forestry Volume 2 Specific Principles and Methods: Growth and Developments. Martinus Nijhoff Publishers, Dordrecht Boston Lancaster, P. 16-30.

Mimura, T.; M. Mimura; S. Washitani-Nemoto; K. Sakano; T. Shimmen; S. Siripatanadilok. 1997. Efficient Callus Initiation from Leaf of Mangrove Plant, *Bruguiera sexangula* in Amino Acid Medium: Effect of NaCl on Callus Initiation. Journal of Plant Research 110: 25-29.

Murashige, T.; F. Skoog. 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarium 15: 473-497.

Nehra, N.S.; M.R. Becwar; W.H. Rottmann; L. Pearson; K. Chowdhury; S. Chang; H.D. Wilde; R.J. Kodrzycki; C.

Zhang; K.C. Gause; D.W. Parks; M.A. Hinchee. 2005. Forest Biotechnology: Innovative Methods, Emerging Opportunities. *In Vitro* Cellular and Developmental Biology - Plant 41: 701-717.

Rahman, M.A.; J. Blake. 1988. Factors Affecting *In Vitro* Proliferation and Rooting of Shoots of Jackfruit (*Artocarpus heterophyllus* Lam.). Plant Cell, Tissue and Organ Culture 13: 179-187.

Saito, A.; Y. Hosoi; K. Ishii; T. Sato. 1987. Callus Formation from Protoplasts of Mesophyll Cells of *Populus* Plantlets. Journal of The Japanese Forestry Society 69: 472-477.

Saito, Y.; Y. Ide; K. Kojima; S. Sasaki. 1993. Isolation of Protoplasts form Various Tissues of *Acacia mangium* Cultured *In Vitro*. Bulletin of The Tokyo University Forests 90: 17-21.

Sasamoto, H.; Y. Hosoi; K. Ishii; T. Sato; A. Saito. 1989. Factors Affecting the Formation of Calluls from Leaf Protoplasts of *Populus alba*. Journal of Japanese Forestry Society 71: 449-455.

Schöpke, C.; L.E. Müller; H.W. Kohlenbach. 1987. Somatic Embryogenesis and Regeneration of Plantlets in Plotoplast Cultures from Somatic Embryos of Coffee (*Coffea canephora* P. *ex* Fr.). Plant Cell, Tissue and Organ Culture 8: 243-248.

Sukarutiningsih; Y. Saito; Y. Ide. 2002. *In Vitro* Plantlet Regeneration of *Paraserianthes falcataria* (L.) Nielsen. Bulletin of The Tokyo University Forests 107: 21-28.

Sumiasri, N.; D. Priadi; S. Yokota; N. Yoshizawa. 2006. Tissue Culture of Fast Growing Tropical Trees in Indonesia: Mangium (*Acacia Mangium* Wild) and Sengon (*Paraserianthes falcataria* (L) Nielsen). In: Imamura, Y.; T. Umezawa; T. Hata (Eds.) Sustainable Development and Utilization of Tropical Forest Resources. Research Institute for Sustainable Humanosphere, Kyoto University, Kyoto, P. 123-130.

Thompson, J.A.; R. Abdullah; E.C. Cocking. 1986. Protoplast Culture of Rice (*Oryza sativa* L.) Using Media Solidified with Agarose. Plant Science 47: 123-133.

Vengadesan, G.; A. Ganapathi; R.P. Anand; V.R. Anbazhagan. 2000. *In Vitro* Organogenesis and Plant Formation in *Acacia sinuata*. Plant Cell, Tissue and Organ Culture 61: 23-28.

Wakita, Y.; H. Sasamoto; S. Yokota; N. Yoshizawa. 1996. Plantlet Regeneration form Mesophyll Protoplasts of *Betula platyphylla* var. *japonica*. Plant Cell Reports 16: 50-53.

Miyuki Chujo, Junji Eizawa, Shinso Yokota, Futoshi Ishiguri, Kazuya Iizuka, and Nobuo Yoshizawa*
Faculty of Agriculture, Utsunomiya University,
Utsunomiya 321-8505, Japan

Corresponding author*
Tel./Fax. : +81-28-649-5541
E-mail : nobuoy@cc.utsunomiya-u.ac.jp

Dody Priadi and Nurul Sumiasri
Research Center for Biotechnology,
Indonesian Research Institute of Sciences,
Cibinong, Indonesia
Tel. : +62-21-8754587
Fax. : +62-21-8754588
E-mail : d_priadi2002@yahoo.com