Isolation, Culture and Organogenesis of Leaf Mesophyll Protoplasts of Paper Mulberry (*Broussonetia kazinoki* Sieb.)

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Introduction

Paper mulberry (*Broussonetia kazinoki*) is a small tree plant and cultivated in Japan as an industrial crop for producing Japanese traditional paper, which is characterized by its own feeling and toughness. It is the closest relative of mulberry (*Morus* species) in terms of morphological characters among the *Moraceae* plants. Mulberry, a feed crop for the silkworm, suffers from a dwarf-disease, which sometimes gives serious damage to sericulture. Although efforts of producing disease-resistant plants have been made by conventional sexual breeding of mulberry species, the genetical diversity as for the trait is rather limited, because *Morus* species with complete dwarf-disease resistance has not been found. In this respect a new approach to genetic modification of mulberry through hybridization with *B. kazinoki* has been desired.

Somatic hybridization by protoplast fusion and transformation through gene transfer have become important concerns of most of the plant breeders. Both techniques require protoplast handling; plant regeneration from protoplasts is essential for making practical use of the new technology. Although many attempts to isolate and culture protoplasts of woody plants have been made, successful results concerning regeneration of protoplast-derived plants are quite limited, with two remarkable exceptions of *Citrus*[2,3,9,10] and sandalwood (*Santalum album*)[8]. In *Citrus*, plants were regenerated through embryogenesis from nucellar callus protoplasts. In sandalwood, protoplasts isolated from embryogenic cell suspensions produced embryogenic cell aggregates developing into somatic embryos. Plant regeneration from leaf mesophyll protoplasts of perennial woody plants was first reported in *B. kazinoki*[6]. Shortly after its publication, a similar report was made with wild pear (*Pyrus communis*)[5], in which shoots were regenerated from protoplasts isolated from both field and *in vitro* grown plant materials. These achievements showed potential use of protoplast technology in genetic improvement of woody perennial plants. In this paper the outline of the procedures for the isolation and culture of leaf mesophyll protoplasts of *B. kazinoki*, leading to plant regeneration is described.

Establishment of shoot cultures

Seeds of *B. kazinoki* were surface sterilized in 10% (v/v) sodium hypochlorite solution (active chlorine ca. 10% Cl) for 10 min, followed by three rinses with sterile distilled water. They were aseptically germinated on Murashige and Skoog[4] (MS) medium containing 2% sucrose and solidified with 0.8% agar. It took more than 2 weeks for the seeds to germinate at 25°C and shoot explants with cotyledons and a pair of primary leaves were excised from 40-day-old seedlings and transplanted to MS medium supplemented with 1 mg/*l* benzyl adenine (BA). The proliferated shoots bearing several young leaves and partly developing axillary buds were repeatedly transferred to MS medium with 0.2 mg/*l* BA at intervals of about 30 days. The cultures were maintained at photoperiods of

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12 hr (warm-white fluorescent lamps, 3000–4000 lux) at 25±1.5°C.

Protoplast isolation

Leaves 30–40 mm in length were taken from 30 to 50-day-old shoots (Plate 1). The leaves were cut into 1–2 mm cross sections and incubated in 5 m*l* of enzyme solution (pH 5.5) consisting of 0.5 M mannitol, 0.05% pectolyase Y23 (Seishin Pharmaceutical Co., Ltd., Tokyo, Japan) and 1% cellulase Onozuka R-10 (Yakult Pharmaceutical Industry Co., Ltd., Nishinomiya, Japan) in 20 m*l* Erlenmeyer flasks. The flasks were incubated on a gyratory shaker (75–100 rpm) at 25°C for 3 hr under diffuse light. The enzyme solution was then filtered through a layer of Miracloth (Chicopee Mills, Inc., Milltown, U.S.A.) and released protoplasts were sedimented by centrifugation at 100 g for 2 min. After removing the supernatant with a Pasteur pipette, the protoplasts were resuspended in 5 m*l* of 0.5 M mannitol solution and centrifuged at 100 g for 2 min. The washing procedure was repeated two more times. Following determination of the protoplast density with a hemocytometer and dilution of the suspension to a proper extent, the protoplasts were finally suspended in the culture media. The enzyme solutions and all the media for protoplast cultures were sterilized with Millipore filters (0.45 μm). The yield of protoplasts was $1\text{–}2\times10^6$ per 100 mg fresh weight of the leaf material. *B. kazinoki* protoplasts had a diameter ranging from 10 to 20 μm and contained a small number of relatively large chloroplasts (Plate 2).

Plate 1. An *in vitro* grown shoot of *B. kazinoki* used for isolating leaf mesophyll protoplasts

Protoplast culture

Protoplasts were cultured in 1.5 m*l* of liquid medium in 35×10 mm Falcon plastic petri dishes, which were sealed with Parafilm and placed in a humidified plastic container. Exploration of the most suitable medium for *B. kazinoki* protoplast culture was performed with respect to inorganic salts of the basal medium, sugar, osmoticum and growth regulators.

The most striking factors affecting sustained divisions of the protoplasts were the level of NH_4NO_3 content and the kind of sugar. When MS medium of the original formula was used, cell wall regeneration and occasional cell divisions occurred several times, but the cell aggregates soon turned brown without colony formation. Sustained divisions of protoplasts leading to colony formation were brought about only when the amount of NH_4NO_3 was reduced to less than 825 mg/*l* (Table 1), the optimum being 200–400 mg/*l*. Similar results that protoplasts were successfully cultured in reduced

Table 1. Effect of NH_4NO_3 on colony formation from *Broussonetia kazinoki* protoplasts after 8 weeks in culture

NH_4NO_3 Conc.[a]	Degree of colony formation[b]				
	Repetition 1	2	3	4	5
0 mg/*l*	++	−	+	−	++
100	+++	+	−	+++	+++
200	+++	++	+++	+++	+++
410	++	++	+++	++	+++
825	+	++	+	++	+++
1650	−	−	−	−	+

a) Other major salts were composed of 950 mg/*l* KNO_3, 370 mg/*l* $MgSO_4\cdot7H_2O$, 220 mg/*l* $CaCl_2\cdot2H_2O$ and 170 mg/*l* KH_2PO_4.

b) Visual estimation: − = non; + = poor; ++ = good; +++ = very good.

Table 2. Effect of sugars on colony formation from *Broussonetia kazinoki* protoplasts after 8 weeks in culture

Sugar	Degree of colony formation[a)]				
	Repetition 1	2	3	4	5
Sucrose 2%	−	−	−	−	−
Fructose 2%	−	−	−	−	+
Glucose 2%	+++	+++	+++	++	+++

a) The same as in Table 1.

Table 3. Effect of mannitol concentration of the culture medium on colony formation from *Broussonetia kazinoki* protoplasts after 6 weeks in culture

Mannitol Conc.	Degree of colony formation[a)]			
	Repetition 1	2	3	4
0.25 M	−	−	−	−
0.30 M	−	+++	+	++
0.35 M	−	+++	+++	+++
0.40 M	+	+++	+++	++
0.45 M	−	+++	+++	++
0.50 M	−	++	++	+

a) The same as in Table 1.

Table 4. Modified MS medium for *Broussonetia kazinoki* protoplast culture

Constituent	mg/*l*
NH_4NO_3	200
KNO_3	1,900
$MgSO_4 \cdot 7H_2O$	370
$CaCl_2 \cdot 2H_2O$	220
KH_2PO_4	170
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 4H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Na_2EDTA	37.25
$FeSO_4 \cdot 7H_2O$	27.85
Thiamine · HCl	1
Pyridoxine · HCl	0.5
Nicotinic acid	0.5
myo-Inositol	100
Glucose	10,000
Mannitol	72,900
NAA	0.2
BA	0.1

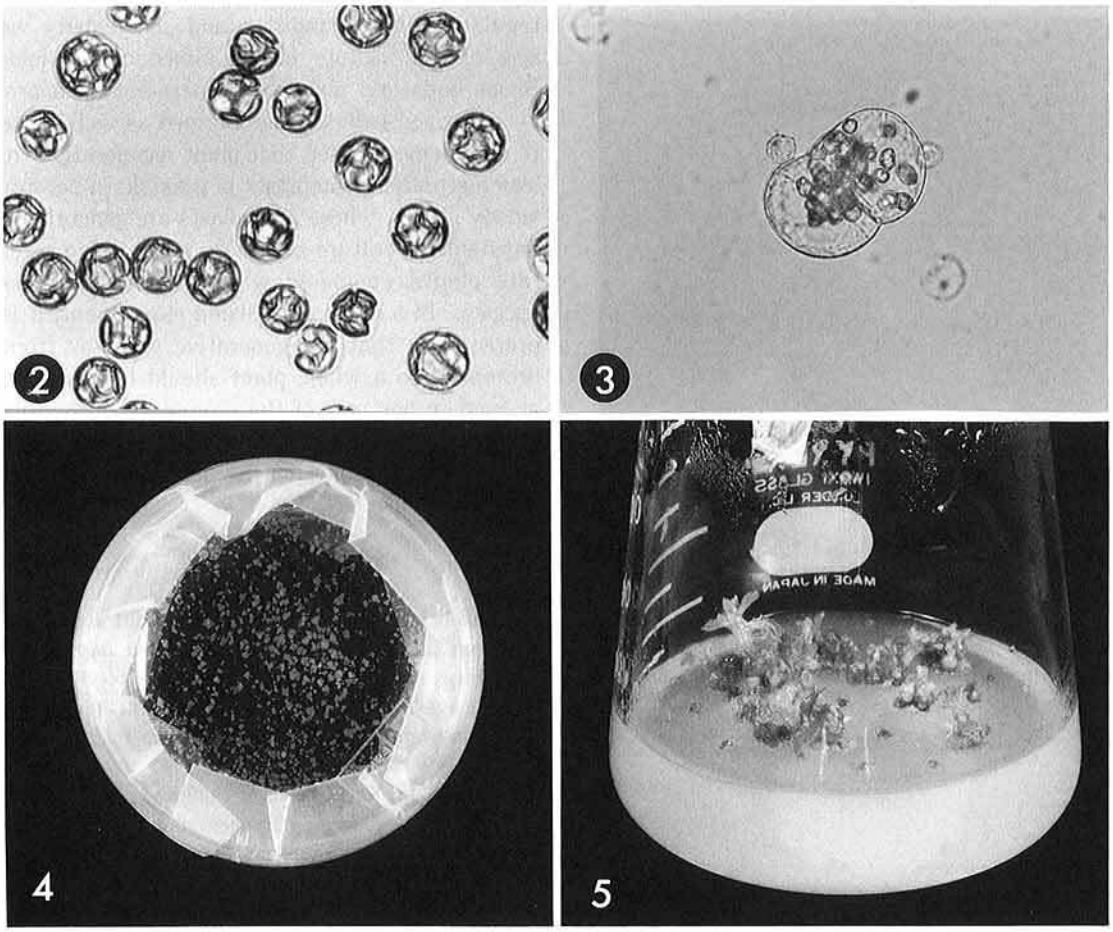
pH 5.6

NH_4NO_3-containing medium have been reported in potato[1], *Asteraceae*[7], and Japanese butterbur[11]. As for the kind of sugar, glucose was superior to sucrose (Table 2). In the presence of glucose, starch grains accumulated in the cells throughout the early stage of the development of cell aggregates. Sucrose or fructose induced several times of cell divisions, but daughter cells were cytoplasmic-poor without starch accumulation and finally ceased to divide. Both auxin and cytokinin were essentially required for the protoplasts to divide. α-Naphthaleneacetic acid (NAA) appeared to give more reproducible results than 2,4-dichlorophenoxyacetic acid (2,4-D); in the medium containing 2,4-D, no divisions of the protoplasts or early browning of cell aggregates sometimes occurred. The optimal concentrations of NAA and BA were 0.1–1 and 0.1 mg/*l*, respectively. Division of protoplasts and colony formation were induced at relatively wide ranges of osmoticum level (0.25–0.5 M mannitol), but the best callus growth was observed when 0.35–0.4 M mannitol was used as an osmotic stabilizer (Table 3). From these results modified MS medium (Table 4) was adopted for *B. kazinoki* protoplast culture. The viability of the protoplasts was quickly lost in the light, so that all the cultures were kept in the dark.

Under appropriate nutritional and environmental conditions, the protoplasts increased in size and reformed cell walls in 3–7 days, as judged by a change in protoplast shape. First cell division of the protoplast was observed mostly between 10–20 days in culture (Plate 3). However, these developmental responses of the protoplast depended on the cell density; the optimum was 2×10^4/m*l* (Table 5). Sustained cell divisions gave rise to colonies in 4 weeks, which developed into small calli macroscopically visible in another 4 weeks (Plate 4).

Plant regeneration from protoplast-derived callus

When small calli 0.2–1 mm in diameter were transferred to 30 m*l* of modified MS medium supplemented with 0.1 mg/*l* NAA and 1 mg/*l* BA and without mannitol, the inocula grew in size, turned green and regenerated shoot buds in 20–30 days (Plate 5). Transfer of the bud forming callus to MS medium



Plates 2–5. Development of *B. kazinoki* protoplasts cultured in modified MS medium containing 1% glucose, 0.4 M mannitol, 0.2 mg/*l* NAA and 0.1 mg/*l* BA

2: Freshly isolated protoplasts

3: First division of a protoplast after 14 days in culture

4: Protoplast-derived callus macroscopically visible after 8 weeks in culture

5: Shoot bud formation from callus 4 weeks after transfer to regeneration medium

Table 5. Effect of cell density on the developmental response of *Broussonetia kazinoki* protoplasts after 3 weeks in culture

Cell density	% Protoplast[a)]				
	Unchanged	Cell wall forming	Dividing	Dead	Total
5×10^3/m*l*	15.5	2.8	0	81.7	100
10^4/m*l*	2.0	0.1	2.0	84.9	100
2×10^4/m*l*	3.1	5.1	19.5	72.3	100
5×10^4/m*l*	18.2	5.2	1.5	75.5	100
10^5/m*l*	32.2	0.6	0	67.2	100
2×10^5/m*l*	26.7	0	0	73.3	100

a) The number was visually determined under an inverted microscope by observing 100—500 protoplasts in each treatment.

Plate 6. A whole plant regenerated from a *B. kazinoki* protoplast

containing 0.1–1 mg/*l* BA produced a great many small shoots, most of which were stunted but some showed normal elongation. Otherwise, the bud forming callus was further transferred to MS medium containing 0.1 mg/*l* BA to enhance shoot elongation. The shoots reaching sufficient length were then rooted on MS medium with 0.05 mg/*l* NAA, and finally a potted whole plant was obtained (Plate 6).

Conclusion

A reliable culture system of *B. kazinoki* leaf mesophyll protoplast was achieved by employing *in vitro* shoot cultures as a source material and modified MS medium with reduced amount of NH_4NO_3 and addition of glucose in place of sucrose. Growth regulators, light condition and cell density were also critical factors for sustained cell divisions. Shoot buds and plantlet regeneration from protoplast-derived callus were obtained as well. These results demonstrated that plant regeneration from leaf mesophyll protoplasts is possible in perennial woody plants, whose protoplasts are generally less amenable to culture and more reluctant to regenerate plants, compared with those of herbaceous species. In a protoplast fusion experiment, it is a prerequisite that a regenerative pathway from a protoplast to a whole plant should be established at least in one part of the protoplasts to be fused. In this respect, the result with *B. kazinoki* is encouraging us to develop the protoplast technology in *Moraceae* plants.

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(Received for publication, October 2, 1986)