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Isolation and Culture of Mesophyll Protoplasts from *In vitro* Subcultured Japanese White Birch

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I. Introduction

Protoplast culture has many probabilities for genetic improvement of woody plants such as somatic hybridization, soma clonal plants formation and genetic engineering (DURZAN, 1982). Protoplast research of woody plants was advanced recently especially on isolation and culture. Successful plantlet regenerations from protoplasts were reported on some species of *Populus, Citrus, Broussonetia* etc. (McCOWN and RUSSELL, 1987; SAITO, 1989).

In *genus Betula*, SMITH and McCOWN (1982/3) reported cell divisions of mesophyll protoplasts which isolated from shoot culture of Asian white birch (*B. platyphylla* var. *szechuanica*). And callus formation from protoplasts which isolated from cell suspension culture of paper birch (*B. papyrifera*) (TREMBLAY, 1988) were reported. But there is no report on plantlet regeneration from birch protoplasts.

During these five years, studies on *in vitro* propagation of *Betula* in Japan has been remarkably progressed. Japanese white birch (*B. platyphylla* var. *japonica*) (SAITO and IDE, 1985ab; SATO *et al.*, 1986; IDE, 1987b), Japanese cherry birch (*B. grossa*) (IDE, 1987a) and monarch birch (*B. maximowicziana*) (KATAYOSE and TAMAI, 1988; IDE and YAMAMOTO, 1990) were successfully regenerated in glass vessels. However there are no references on their protoplast isolation.

This paper deals with the isolation and the culture of protoplasts of Japanese white birch.

II. Materials and Methods

For the isolation of protoplasts, fully developed leaves of plantlets which were aseptically cultured in culture tubes were used. The plantlets were originated from shoot primordia developed on a petiole of mature tree (SAITO and IDE, 1985b) and were intermittently subcultured by *in vitro* cuttings of shoot tips on modified MS meduim (MURASHIGE and SKOOG, 1962) with 0.02 mg/*l* of NAA(α-naphtylacetic acid) and 0.5 mg/*l* of IBA (indolebutylic acid) (IDE, 1987b) (Table 1). They were cultured at 25℃ under fluorescent light at an intensity of about 5,000 lx for a 16 hr photoperiod.

Enzyme solution, sucrose solution and wash solution were prepared for the isolation and purification of protoplasts (Tabel 2). These solutions were filter sterilized by passing through a 0.22 μm membrane filter. Enzyme solution was centrifuged at 500$\times g$ for 15 min before the filter sterilization.

Two types of modified MS medium (MURASHIGE and SKOOG, 1962): A and B were prepared with the concentration of 3 mg/*l* of 2, 4-D(2, 4-dichlorophenoxyacetic acid) (Table 3) for the culture of protoplast isolation. Medium A contained only glucose and medium B contained both of glucose and sorbitol, respectively. They were also filter-sterilized through a 0.22 μm membrane filter.

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Table 1. Medium for subculturing of birch plantlets* (mg/*l*)

NH_4NO_3	1,650	$CoCl_2 \cdot 6H_2O$	0.025
KNO_3	1,580	FeEDTA**	5.6
$Ca(NO_3)_2 \cdot 4H_2O$	740	Nicotinic acid	0.5
KH_2PO_4	170	Thiamine HCl	0.1
K_2SO_4	280	Pyridoxine HCl	0.1
$MgSO_4 \cdot 7H_2O$	370	L-lysine	100
$MnSO_4 \cdot 4H_2O$	22.3	*myo*-inositol	100
$ZnSO_4 \cdot 7H_2O$	8.6	NAA***	0.02
$CuSO_4 \cdot 5H_2O$	0.025	IBA****	0.5
H_3BO_3	6.2	Sucrose	15,000
KI	0.83	Agar	8,000
$Na_2MoO_4 \cdot 2H_2O$	0.25		

* Modified from MURASHIGE and SKOOG's medium (1962) pH was adjusted at 5.8 by KOH and HCl
** Ferric monosodium ethylenediaminetetraacetic acid
*** α-naphtylacetic acid
**** Indolebutylic acid

Table 2. Solutions for protoplast isolation and purification* (mg)

Components	Solutions		
	Enzyme	Wash	Sucrose
Isotonic solution[1]			
Mannitol	11,000	11,000	
Sucrose			21,000
H_3BO_3	2		
$CaCl_2$		80	80
Potassium citrate	162		
Enzyme and others[2]			
Pectolyase Y-23[3]	100		
Cellulase 'Onozuka' R-10[4]	2000		
Driselase[5]	1000		
Potassium dextran sulfate[6]	100		
Dextran T-40[7]			8,000
Antibiotics[2]			
Ampicillin	40		
Carbenicillin (Disodium salt)	3		
Gentamicin sulfate	3		

* pH were adjusted at 5.6 by NaOH and HCl
[1] Dissolve in water and bring to a final volume of 100 m*l*
[2] Dissolve in 100 m*l* of isotonic solution
[3] Seishin pharmaceutical Co., Japan
[4] Yakult pharmaceutical Co., Japan
[5] Kyowa hakko kogyo Co. Japan
[6] Meito sangyo Co., Japan
[7] Pharmacia Co., Sweden

Twenty leaves were excised from the plantlets by scissors and were cut into fine strips about 2–3 mm wide with a sterilized razor on a sterilized filter paper. About 1 *g* of leaf

Table 3. Medium for protoplasts culture* (mg/*l*)

NH_4NO_3	1,650	$CoCl_2 \cdot 6H_2O$	0.025
KNO_3	1,900	FeEDTA**	28
$CaCl_2 \cdot 2H_2O$	440	Nicotinic acid	0.5
KH_2PO_4	170	Thiamine HCl	0.1
$MgSO_4 \cdot 7H_2O$	370	Pyridoxine HCl	0.5
$MnSO_4 \cdot 4H_2O$	22.3	*myo*-inositol	100
$ZnSO_4 \cdot 7H_2O$	8.6	Glutamine	370
$CuSO_4 \cdot 5H_2O$	0.025	2, 4-D***	3
H_3BO_3	6.2	Mannitol	5,500
KI	0.83	Glucose****	
$Na_2MoO_4 \cdot 2H_2O$	0.25	Sorbitol****	

* Modified from MURSHIGE and SKOOG's medium (1962) pH was adjusted at 5.6 by NaOH and HCl

** Ferric monosodium ethylenediaminetetraacetic acid

*** 2, 4-dichlorophenoxyacetic acid

**** Two different concentration were tested;
medium A: Glucose 5,500 mg/*l*
medium B: Glucose 2,750 mg/*l* and Sorbitol 2,750 mg/*l*

strips were stirred in a 100 m*l* Erlenmeyer flask containing about 20 m*l* of enzyme solution. The flask was shaken reciprocally with 80 turn/min for 2 hr in a water bath incubator at 30℃ to dissolve cell walls of the tissue.

After the incubation, the enzyme solution was filtered through a 100 μm nylon mesh and centrifuged at 100×*g* for 5 min to collect protoplasts. After removing the supernatant the pellet consisting of protoplasts and debris was resuspended in 10 m*l* of wash solution and centrifuged at 100×*g* for 5 min. Washing procedure was repeated twice.

There after the pellet were resuspended in 8 m*l* of sucrose solution and 2 m*l* of wash solution was laid as an upper layer. The suspension was centrifuged at 300×*g* for 10 min. Protoplasts floating between the two solutions were pipetted and were resuspended in wash solution. And washing procedure was repeated.

The protoplasts pellet was suspended in 10 m*l* of culture medium and numbers of yielded protoplasts was counted in a hemacytometer. The protoplast suspension was centrifuged at 100×*g* for 5 min and supernatant was removed. The protoplast pellet was resuspended in culture medium, and the density of the protoplasts was adjusted at about 2 $\times 10^6$/m*l*. One and half m*l* of protoplast suspension was poured in 35 mm×10 mm plastic petri dish and sealed with para-film (American Can Co., U.S.A.). Cultures were maintained at 25℃ in darkness.

III. Results and Discussions

The procedure of protoplast isolation was almost same as the method used in *Populus sieboldii* (SAITO *et al.*, 1987), but the preincubation in enzyme solution for releasing broken cells was eliminated, as this procedure had no effect on the final yield of protoplasts in this experiment. There were numerous light debris observed just after the digestion of leaf tissue. Therefore, the washing procedure was conducted twice before collecting protoplasts.

A few different enzyme compositions were tested preliminarily, and the most effective composition used in this experiment was the one modified from the enzyme solution used for the protoplasts isolation from 'konara' (*Quercus serrata*) callus culture (KOYAMA *et al.*,

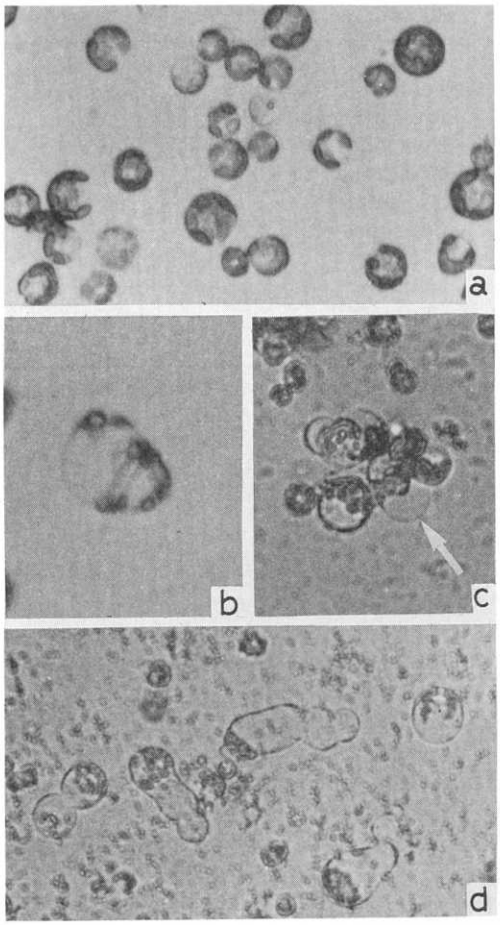

Fig. 1. Protoplasts and their buddings.
a: Purified mesophyll protoplasts which isolated from *in vitro* cultured plantlets of Japanese white birch.
b: First cell budding observed after 24 hr of protoplast culture in medium A.
c: Cell budding among agrregated cells after 9 days of culture in medium A.
d: Active cell buddings observed after 22 days of culture in medium A.

1988). Except the enzyme composition, we followed SAITO and others (1987) in another components of enzyme solution with some modifications.

The leaf tissue was almost perfectly digested and approximately 6×10^6 protoplasts were yielded from about 1 *g* leaf tissue (Fig. 1a). SMITH and MCCOWN (1982/3) achieved the yields of $3\text{–}4\times10^6$ protoplasts from 1 *g* of leaf tissue of Asian white birch cultures. TREMBLAY (1988) obtained $1\text{–}2.7\times10^6$ protoplasts from 1 *g* of cell suspension cultures of paper birch. The yield of protoplasts in our experiment was same order as the yields of these two reports. The enzyme solution and the procedure used here were effective for the isolation of mesophyll protoplasts from plantlets of Japanese white birch which cultured in *in vitro*.

Diameters of protoplasts ranged from 10 μm to 40 μm. This variation of protoplast size was wider than protoplasts of *P. sieboldii* (SAITO *et al.*, 1987) and *Q. serrata* (KOYAMA *et al.*, 1988). Protoplasts in these reports were isolated from newly expanded young leaves in *P. sieboldii* and from upper layer of callus cultures in *Q. serrata*. Perfectly expanded leaves including old ones were used in the present experiment. Young leaves and upper layer of callus cultures provide comparatively uniform protoplasts in size, whereas expanded leaves provide protoplasts with wider size variation because their tissue was consisted of fully developed cells.

Throughout the culture period, there was no remarkable difference in protoplast behaviors such as cell division, budding and mortality between two culture media.

Viability of protoplasts were not examined here, but our observations suggest that first cell buddings occurs 24 hr after culture of protoplasts (Fig. 1b).

After two days of culture, a considerable number of cells were aggregated. Cell aggregation tends to cause the death of cells in *Q. serrata* (KOYAMA *et al.*, 1988). Similarly our experiment shows the death of cells in a few days after cells were aggregated. In spite of detrimental effects of cell-aggregation, cell buddings were observed among aggregated cells (Fig. 1c) in some cases.

Among the cells which did not aggregate cell buddings continued after a week of culture. At about 3 weeks of culture, the most active cell buddings were observed in both culture medium (Fig. 1d). However cells ceased their budding and died in the following week. After one month of protoplasts culture, almost entire of cells died and the cultures were abandoned.

For the active cell division, plating density of protoplasts effects seriously. In paper birch, between 5×10^4 to 1×10^5 protoplasts/m*l* (TREMBLAY, 1988) and in *P. alba*, $5\text{–}7\times10^4$ protoplasts/m*l* (SASAMOTO, 1989) gave best results for cell division and callus formation. In the present experiment the density of protoplasts was adjusted at about 2×10^6. This density is considerably higher than the case of paper birch and *P. alba*. More research is needed to define an appropriate culture density for inducing cell divisions.

This is the initial step of plantlet regeneration of Japanese white birch from protoplasts. Although the further experiments are needed, the method in the present experiments is appropriate for the isolation of sound mesophyll protoplasts from leaf tissues of *in vitro* cultured Japanese white birch plantlets. While the cell divisions were not induced, active cell buddings were shown in the present experiments. This indicates that there is some possibilities for cell divisions from mesophyll protoplasts of Japanese white birch.

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Summary

Mesophyll protoplasts were successfully isolated from leaf tissue of *in vitro* cultured Japanese white birch (*Betula platyphylla* var. *japonica*) plantlets which were originated from shoot primordia developed on petiole culture of mature tree. Approximately 6×10^6 protoplasts were isolated from about 1 *g* of leaf tissue by enzyme solution. The protoplasts were cultured in liquid modified MS (MURASHIGE and SKOOG, 1962) medium with 3 mg/*l* of 2,4-D(2,4-dichlorophenoxyacetic acid) at the density of about 2×10^6. Although the certain cell divisions were not observed, active cell buddings were observed after three weeks of protoplasts culture.

Key words: Japanese white birch, Mesophyll protoplast, Isolation, Cell budding

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* Japanese with an English summary

** Only in Japanese; the title are tentative translations from original Japanese titles by the authors of this paper.

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試験管内で継代培養されたシラカンバ幼植物体からの葉肉プロトプラストの単離と培養

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要　　旨

成木の葉柄培養によって得されたシラカンバ幼植物体の葉の組織から，酵素液を用いてプロトプラストを単離することができた。プロトプラストの収量は葉1gあたり約6×10^6個であった。単離されたプロトプラストを2, 4-Dを3 mg/*l*含む，一部改変したMS液体培地に2×10^6個の密度で培養した。その結果，確かな細胞分裂は観察されなかったが，培養3週間後には活発なバディング現象が観察された。

キーワード： シラカンバ，葉肉プロトプラスト，単離，バディング