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Protoplast Culture Conditions for Increasing Cell Division in *Betula platyphylla* var. *japonica*

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Mesophyll protoplasts of *Betula platyphylla* var. *japonica* were isolated successfully using a combination of 1% Cellulase Onozuka R-10 and 1% Driselase in 0. 6 M mannitol. For protoplast isolation, loosening leaves with tweezers after enzymatic treatment was tried. This novel method gave a large yield (5×10^7/g fresh weight) of highly viable (more than 80%) protoplasts. Effects of plant growth regulators, plating density, levels of nitrogen, inorganic phosphate and pH on cell division activity in the protoplast culture were examined. An optimal plating density was of 5×10^4 protoplasts/m*l*. Cell division was promoted with phosphate concentrations of 0. 31- 0. 625 mM after one month of culture, whereas its lower and higher levels were rather inhibitory. Active cell division occurred in 1/2 MS medium with 1 μM of NAA and 10 μM of BAP. The presence of ammonium nitrate in the medium largely influenced the viability of protoplasts. Therefore, protoplasts of *B. platyphylla* is considered to require a high level of BAP in addition to the presence of ammonium nitrate for efficient cell division.

Introduction

The use of protoplasts has now become a base-line for the successful genetic improvement of many plant species. Protoplast fusion may be useful for the production of somatic hybrids for the improvement of breeding of forest trees.

Betula platyphylla var. *japonica*, a member of the family Betulaceae, is one of the fast-growing trees attracting attention as a biomass resource in Japan. On the other hand, members of the genus *Alnus* of the same family have an ability of fixing atmospheric nitrogen through a symbiotic association with *Frankia* spp.[1]. Therefore, it would be interesting to produce somatic hybrids by fusion of protoplasts between the *Betula* and *Alnus* species[2]. We tried the isolation and culture of protoplasts of *B. platyphylla* var. *japonica* as the first step in a trial for producing somatic hybrids between these two genera.

In *Betula* species, the isolation of mesophyll protoplasts was first reported by Smith and McCown[3] with *B. platyphylla* var. *szechuanica*, and much later by Ide *et al.*[4] for *B. platyphylla* var. *japonica*. However, active cell division was not observed in either of these experiments. Tremblay[5] succeeded in callus formation from protoplasts of cell suspension cultures of *B. papyrifera*. However, successful callus formation from mesophyll protoplasts of *Betula* species has not yet been reported. Further research input is clearly needed to develop protoplast technology for the *Betula* species.

In the present paper, in order to find an optimal culture condition of protoplasts of *B. platyphylla*

var. *japonica*, several factors including plant growth regulators, nitrogen source, plating densities, inorganic phosphate and pH, were examined.

Materials and Methods

1. *Plant material*

Shoot primordia were induced from petioles of *Betula platyphylla* var. *japonica* using Murashige and Skoog (MS)[6] medium with slight modifications, according to the procedure of Ide[7]. Multiple shoots were subcultured on the same medium as used for induction of shoot primordia, which contains 3.6 μM of 6-benzylaminopurine (BAP), at 25°C under fluorescent illumination of 3,500 lux (16 hr/day). For protoplast isolation, the small leaves (0.5–1.5 cm in length) were collected from two month old plantlets.

2. *Isolation of protoplasts*

Preliminary experiments examined the effects on protoplast isolation of 24 different combinations of Cellulase Onozuka RS, Cellulase Onozuka R-10, Driselase, Pectolyase Y-23, Macerozyme R-10 and Hemicellulase. An enzyme mixture of 1% Cellulase Onozuka R-10 and 1% Driselase in a 0.6 M mannitol solution was chosen for the following experiments. Small leaves collected from the plantlets were floated directly on 15 ml of the enzyme solution without cutting, then incubated at room temperature for 20 hr without shaking. After incubation, they were resuspended in 15 ml of a 0.6 M mannitol solution, and the protoplasts within the leaf tissue were released by gentle loosening the leaf with tweezers. After filtration through a 40 μm nylon mesh, protoplasts were collected by centrifugation at $100\times g$ for 3 min., then washed three times with 0.6 M mannitol solution.

Protoplast yield was measured using a hematocytometer under an inverted microscope. Protoplasts were stained with 0.01% (w/v) fluorescein diacetate (FDA) in a 0.6 M mannitol to examine their viabilities[8].

3. *Culture of protoplasts*

Three basal media; MS, half concentration MS (1/2 MS) and modified MS without ammonium nitrate (MMS), were examined. All media contained 3% sucrose and 0.6 M mannitol.

Hormonal combinations in the media were as follows: 2, 4-dichlorophenoxyacetic acid (2, 4-D; 0, 0.1, 1, 10 μM) or 1-naphthaleneacetic acid (NAA; 0, 0.1, 1, 10 μM) as an auxin, and BAP (0, 0.1, 1, 10 μM) as a cytokinin.

In order to examine the effects of the medium pH and inorganic phosphate on the protoplast culture responses, two media were prepared: 1/2 MS medium with 1 μM NAA and 10 μM BAP. The medium pH was adjusted from 3.5 to 5.8 using KOH or HCl before autoclaving. Inorganic phosphate was added to the medium to give final concentrations of 0.125 to 2.5 mM.

In all experiments, cell density was adjusted to 5×10^3–1×10^5/ml by adding 10 to 30 μl of the concentrated protoplast suspension to a 0.3 ml of medium in each well of a 24-well culture plate (Falcon). Ten to 15 ml of autoclaved ultrapure water was dispensed between the wells to maintain the humidity and the plate was tightly sealed with Parafilm®. Protoplasts were cultured in the dark, at 28°C, using a CO_2 incubator without gas supply.

4. *Measurement of visually intact protoplasts*

In this experiment, enlarged protoplasts or those showing a spherical shape, were defined as visually intact protoplasts (VIP), according to the method of Russell and McCown[9], and the number of them was measured in an area of 1.5×1.0 mm^2 in each culture well under an inverted microscope. Measurements were conducted with triplicates in two wells every two-week-culture.

Increase rate of protoplasts per originally plated protoplasts was calculated, as follows :

$$\text{Increase rate} = \frac{\text{Number of increased protoplasts}}{\text{Number of originally plated protoplasts}}$$

Results and Discussion

1. *Isolation of protoplasts*

In general, selection of digesting enzymes is very important to obtain a high viability and yield of protoplasts. For *B. platyphylla*, several enzyme combinations were tested preliminarily to isolate mesophyll protoplasts(data not shown). Both Macerozyme R-10 and Hemicellulase were not effective in combination with Cellulase Onozuka R-10 or RS. Pectolyase Y-23 plus Cellulases gave many debris, and particularly with Cellulase Onozuka RS it frequently gave brown, inactive protoplasts without any FDA fluorescence. Out of the 24 enzymatic combinations examined, a mixture of 1% Cellulase Onozuka R-10 and 1% Driselase was chosen for *B. platyphylla*, since it gave the most active protoplasts and coupled with a relatively high yield of them.

In *B. platyphylla*, standard procedure, cutting leaves before enzymatic treatment, yielded a small number of protoplasts and large quantities of cell debris. Hence, loosening leaves gently with tweezers after incubation in the enzyme solution was tried for *B. platyphylla*. This strategy not only gave high yields of protoplasts but also made washing of them easy, because of the small amount of debris.

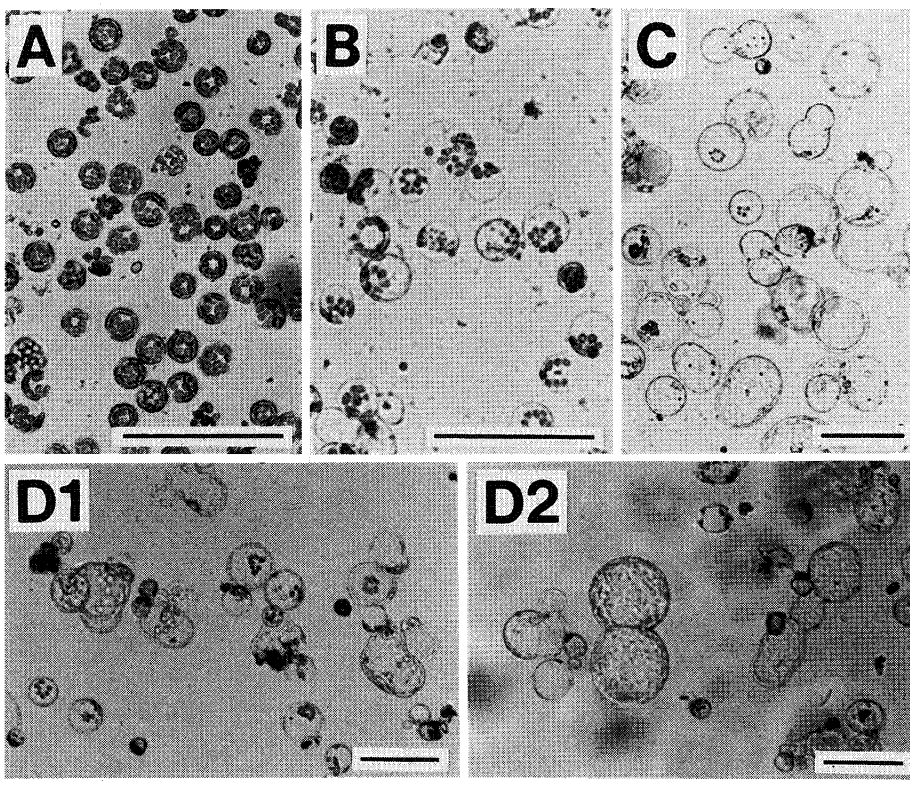

Fig. 1 Culture of protoplasts of *Betula platyphylla*.
A: Protoplasts immediately after isolation.
B: Enlarged protoplasts after one week of culture.
C: Protoplasts showing non-spherical shape after two weeks of culture.
D: Divided cells after one month of culture. Bar: 100 μm.

Protoplasts obtained from leaves of *B. platyphylla* are shown in **Fig. 1-A**. They contained many chloroplasts and ranged from 10 to 30 μm in diameter, in line with data of Ide *et al.*[4]. The protoplast viability exceeded 80%. The average yield of protoplasts was of 5×10^7/g fresh weight of leaves. This value was greater than those previously reported for *Betula* spp.[3-5,10].

2. *Culture of protoplasts*

(1) *Effects of plant growth regulators*

All of the hormonal combinations examined promoted the enlarging of protoplasts after one week of culture (**Fig. 1-B**). Thereafter, the protoplasts further grew and became non-spherical during two weeks of culture (**Fig. 1-C**). This fact suggests that cell wall formation had commenced, as previously pointed out by Smith and McCown[3]. Chloroplasts were no longer observed at this stage. After one month of culture, active cell division occurred in the media containing high concentration of BAP (1 and 10 μM) in combination with NAA or 2, 4-D (**Fig. 1-D**). In *B. platyphylla*, NAA and 2, 4-D showed similar effects on cell division. A hormonal combination of NAA (1 μM) and BAP (10 μM) appeared most effective for culture of mesophyll protoplasts among the several media, judging from both visual intactness (52.0%) and increase rate (2.8) of protoplasts (**Table 1**). These results suggest that there is a possibility of the successful callus formation from mesophyll protoplasts in *B. platyphylla* by applying a BAP concentration higher than 10 μM. Therefore, the use of another cytokinins with higher activity than BAP should also be investigated for further promoting cell division.

(2) *Effects of plating densities*

Plating densities lower than 2×10^4/m*l* were less effective for cell division, and those of more than 7×10^4/m*l* caused cell aggregation without cell division. A plating density of 5×10^4/m*l* gave active growth and division of protoplasts. This was similar to that of *Populus alba*[11].

(3) *Effects of nitrogen*

The 1/2 MS medium gave relatively good results for both the percentage of VIP and the total number of protoplasts after two weeks of culture (**Fig. 2**). After one month of culture most of the protoplasts remained visually intact in both MS and 1/2 MS. Half-strength MS medium yielded a slightly greater number of protoplasts than did full-strength MS medium, although almost no differences in the percentage of VIP were recognized between the media. Of interest is that VIP significantly decreased during the culture in MMS medium. This indicates that ammonium ion is necessary for the culture of protoplasts in *B. platyphylla*, contrasting the case of *P. alba*[11]. In *P.*

Table 1. Effects of concentrations of plant growth regulators on the visual intactness and increase rate of protoplasts of *B. platyphylla* after one month of culture.

		NAA (μM)		
		0.1	1	10
BAP (μM)	0.1	*	36.7±7.7 (1.3±0.3)	43.2±11.1 (1.5± 0.3)
	1	38.8±11.8 (1.4± 0.2)	50.7±7.4 (1.8±0.3)	54.5± 7.5 (2.1± 0.4)
	10	39.8±10.6 (2.0± 0.5)	52.0±3.0 (2.8±0.6)	41.5±12.3 (2.1± 0.5)

Percentage of visually intact protoplasts (%); mean±S. D.

Values in parenthesis indicate increase rate of protoplasts; mean±S. D.

* No measurement.

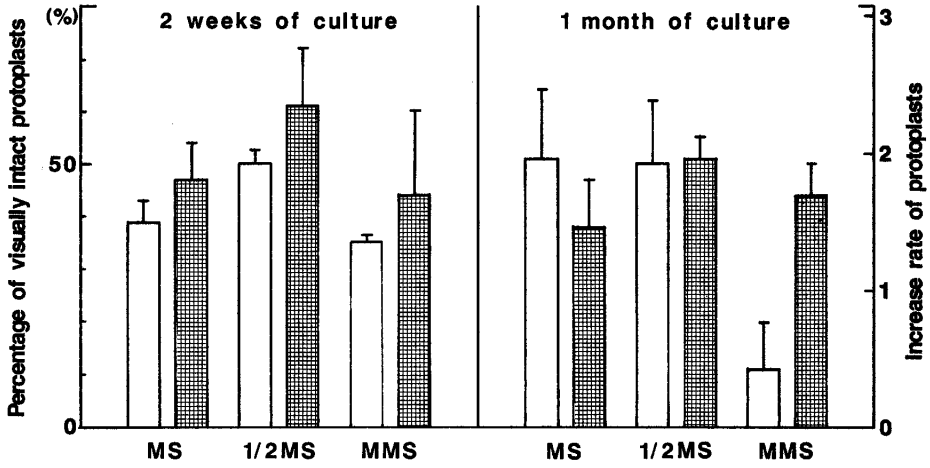

Fig. 2 Effects of basal medium on the visual intactness and increase rate of protoplasts of *B. platyphylla*.
1/2 MS: half concentration of MS.
MMS: modified MS without ammonium nitrate.
White columns: Percentage of visually intact protoplasts.
Dotted columns: Increase rate of protoplasts.

alba, elimination of nitrogen from the medium is needed for promoting cell division. It is concluded that in *B. platyphylla*, ammonium nitrate in medium largely influences the viability of protoplasts and its necessity is genotype-dependent, as already shown for various other woody and herbaceous species.

(4) *Effects of inorganic phosphate*

Considering that the original MS medium contains 1.25 mM inorganic phosphate, this was added to the medium with concentration levels from 0.125 to 2.5 mM. The results are shown in **Fig. 3**. In *B. platyphylla*, relatively high percentages of VIP were obtained with intermediate phosphate concentrations (0.31 and 0.625 mM) after one month of culture, whereas the lowest (0.125 mM) and highest (2.5 mM) levels were rather inhibitory. This also was in line with the increase rate of

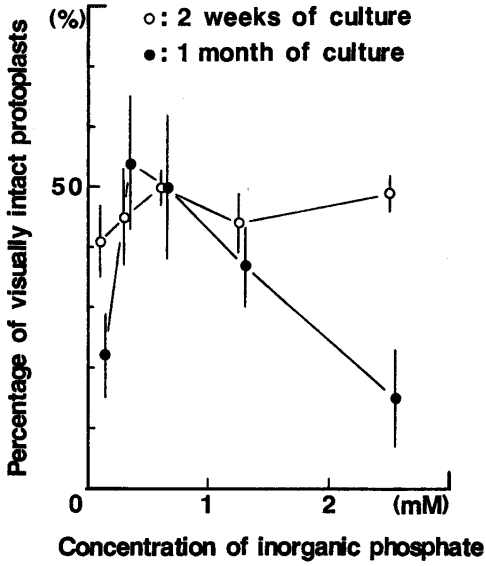

Fig. 3 Effects of concentration of inorganic phosphate on the percentage of visually intact protoplasts of *B. platyphylla*.

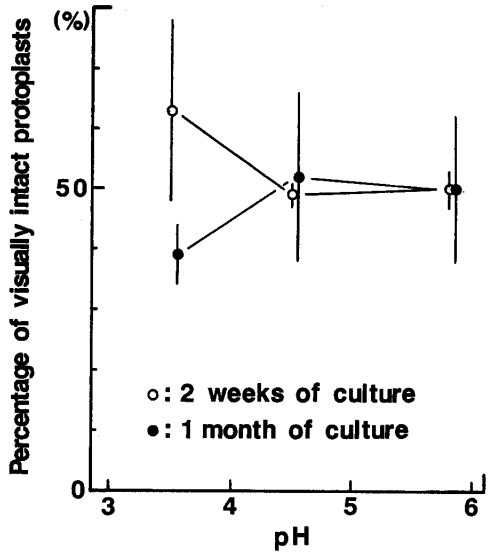

Fig. 4 Effects of medium pH on the percentage of visually intact protoplasts. The medium pH was adjusted before autoclaving.

protoplasts (data not shown).

(5) *Effects of medium pH*

Medium pH decreased about 10% after autoclaving, which was observed in the preliminary experiments. The pH decreases were as follows: 3.0 for 3.5, 4.0 for 4.5 and 5.2 for 5.8. As shown in **Fig. 4**, however, no great differences in the percentage of VIP were observed among the pH ranges tested in this experiment throughout one month of culture. This fact indicates that protoplasts can grow in the medium with wide ranges of pH. This is in contrast with the case of various other woody plants, in which protoplast response to the medium pH seem to be severe.

In conclusion, for *B. platyphylla*, 1/2 MS medium containing 1 μM of NAA and a BAP concentration higher than 10 μM appeared effective for cell division. In addition, the presence of ammonium nitrate also appeared necessary for increasing cell division efficiency. Further improvement of culture conditions for the successful callus formation from protoplasts in *B. platyphylla* is needed.

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《和文要約》

シラカンバにおける細胞分裂促進のためのプロトプラストの培養条件

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シラカンバ葉肉プロトプラストからのカルス誘導・培養条件を確立するために，培養細胞密度，植物成長調節物質の種類と濃度，培地中の窒素源，リン酸塩濃度および培地pHについて検討した．プロトプラストの単離は，1% Cellulase Onozuka R-10と1% Driselaseを含む酵素溶液中に直接葉を切らずに浮かべ，20時間室温で静置培養した．酵素処理後，0.6 Mマンニトール溶液中で，ピンセットを用いて葉の表皮を破り，葉の内部でプロトプラスト化していた細胞を遊離させた．その結果，葉1 g当り5×10^7個のプロトプラストが得られ，生存率は80%以上の高い値を示した．プロトプラストの培養においては，培地pHは4.5-5.8，細胞密度は5×10^4/mlが最適であった．リン酸塩の添加濃度は，0.31-0.625 mMが最も適しており，この濃度範囲以外では，細胞分裂を抑制した．基本培地としては1/2 MS培地が最適であり，NAA 1 μMおよびBAP 10 μMを添加した培地において細胞分裂が最も促進された．