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Plant Regeneration from Cell Suspension Cultures of *Betula platyphylla* var. *japonica*

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Cell suspension cultures were induced from a seed of *Betula platyphylla* var. *japonica* in MS medium supplemented with 1 μM 4-pu and 1 μM NAA. The cell suspension cultures developed into callus through the culture of them on the 1/2 MS solid medium. In the media with zeatin, 4-pu and TDZ, almost all of calluses developed into green calluses. Particularly, the phenylurea-type cytokinins, 4-pu and TDZ, were more effective for formation of green callus than the other cytokinins. Furthermore, the 1/2 MS solid medium containing 1 μM 4-pu successfully differentiated shoots with a combination of 1 μM GA_3. When the shoots obtained were transferred to cytokinin-free MS rooting medium, they rooted and developed into plantlets after one month of culture. From these results, 4-pu is expected to contribute to the successful tissue culture in other woody plants.

Introduction

Betula platyphylla var. *japonica* is one of the biomass resources in terms of the fast-growing nature in Japan. Breeding and improving this species, therefore, are required for forestry. In *B. platyphylla* var. *japonica*, the formation of adventitious buds from petioles has also been reported by several investigators[1,2]. The plant regeneration from the peeling twigs-derived callus has been reported by Saito and Ide[3]. Furthermore, differentiation of adventitious buds in the callus induced from stem segments was reported by Fu *et al.*[4]. However, no reports on plant regeneration from cell suspension cultures of *B. platyphylla* var. *japonica* have been provided to date.

In the present paper, the cell suspension cultures were induced from a seed of *B. platyphylla* var. *japonica* and cultured. By surveying the various hormonal combinations, successful plant regeneration was obtained from the cell suspension cultures in *B. platyphylla* var. *japonica*.

Materials and Methods

1. *Induction of Cell Suspension Cultures*

The seeds of *Betula platyphylla* var. *japonica* were obtained from the seed bank of the Forestry and Forest Products Research Institute, Tsukuba, Japan. They were sterilized with 0.5% NaClO solution for 15 min. Being washed several times with sterile water, they were transferred into 0.5 m*l* of liquid medium in a flat-bottomed 10 m*l* tube and cultured at 28°C in the dark with shaking (100 rpm). For induction of cell suspension cultures from a seed, three basal media; Murashige and Skoog's (MS)[5], half strength MS (1/2 MS) and modified MS eliminating ammonium nitrate

(MMS), all of which contained 0.09 M sucrose, were examined with various combinations of plant growth regulators as follows; 1-naphthaleneacetic acid (NAA; 0, 0.1, 1, 10 μM) or 2, 4-dichlorophenoxyacetic acid (2, 4-D; 0, 0.1, 1, 10 μM) as an auxin, and 6-benzylaminopurine (BAP; 0, 0.1, 1, 10 μM) or N-(2-chloro-4-pyridyl)-N'-phenylurea (4-pu; 0, 0.1, 1, 10 μM) as a cytokinin.

Small cell suspension cultures obtained from the seed were transferred into fresh medium of the same composition as the induction of cell suspension cultures in a flat-bottomed 50 ml tube, and cultured in the dark on a rotatory shaker at 100 rpm. After 5 months of culture, they were washed with the fresh medium on a 40 μm nylon mesh and subcultured every 2 weeks in 20 ml fresh medium in a 100 ml Erlenmeyer flask. Culture was performed at 28°C under the illumination from cool white fluorescence tubes of 2,500 lux for 16 hr per day with shaking (100 rpm).

2. *Differentiation*

The cell suspension cultures induced from a seed were cultured on 12 ml of solid 1/2 MS medium (0.8% agar) in a 50 ml flat-bottomed tube containing 0.09 M sucrose and various combinations of NAA (0, 0.1, 1, 10 μM) and the following cytokinins; BAP, zeatin, 4-pu, kinetin, thidiazuron (TDZ) and N^6-(2-isopentenyl) adenine (2ip) (0, 0.1, 1, 10 μM). Furthermore, differentiation of shoots or roots from the callus were tried using the 1/2 MS solid media containing NAA (0, 0.1, 1 μM), 4-pu (0, 0.1, 1, 10 μM) and combinations of abscisic acid (ABA; 0, 0.1, 1, 10 μM) or gibberellic acid (GA_3; 0, 0.1, 1, 10 μM). The cell suspension cultures were transferred onto the differentiation media described above when it grew up to about 5 mm in diameter, and then incubated at 28°C under the illumination from cool white fluorescence tubes of 3,500 lux for 16 hr per day. The data were obtained from triplicate cultures of callus.

Results and Discussion

1. *Induction of Cell Suspension Cultures*

Many cell protrusions, probably resulting from active cell division, occurred on the surface of the seed cultured for 2 weeks in the two liquid media; MS and 1/2 MS liquid media containing 1 μM 4-pu and 1 μM NAA. Thereafter, they actively grew and developed into calluses. After 2 months of cuture, suspension cells consisting of 2 to 100 cells were released from the calluses (**Fig. 1-A**).

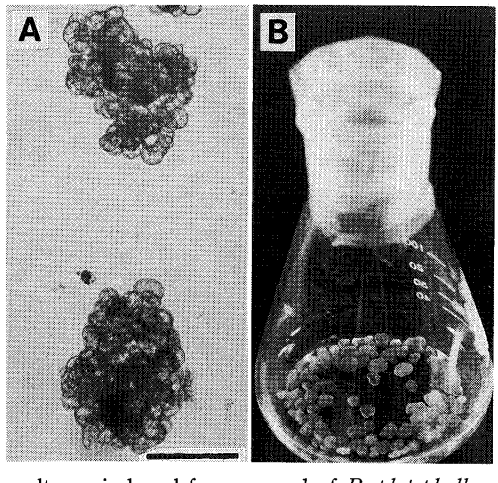

Fig. 1 Cell suspension cultures induced from a seed of *B. platyphylla* var. *japonica* in the MS medium with 1 μM 4-pu and 1 μM NAA.
A: Cell suspension cultures after 2 months of culture.
B: Cell suspension cultures subcultured at intervals of 2 weeks.
Bar=100 μm.

After 3 months of culture, the suspension cells developed into green-colored calluses of about 5 mm in diameter (**Fig. 1-B**). Thereafter, they grew well for more than one year by subculturing them in the same medium every 2 weeks. However, the cell suspension cultures subcultured in the 1/2 MS medium containing 1 μM 4-pu and 1 μM NAA caused browning during 6 months of culture. Eventually, active growth of the cell suspension cultures was observed only in the MS medium. From these results, it is considered that the MS medium containing 1 μM 4-pu and 1 μM NAA is effective on the culture of cell suspension cultures induced from a seed of *B. platyphylla* var. *japonica*.

2. *Differentiation*

Active proliferation of callus derived from suspension cultures occurred on the solid 1/2 MS media containing zeatin, 4-pu and TDZ (**Table 1**), irrespecctive of the presence or absence of NAA. In contrast, the media containing BAP, kinetin and 2 ip gave almost no proliferation of callus (**Table 2**). These results were in line with those of leaf protoplast-derived callus of *B. platyphylla* var. *japonica*[6].

In the media with zeatin, 4-pu and TDZ, many calluses obtained from the cell suspension cultures grew actively during 2 months of culture and almost all of them gradually developed into green calluses. Particularly, the phenylurea-type cytokinins, 4-pu and TDZ, were more effective for formation of green callus than the other cytokinins. Furthermore, NAA in combination with a low concentration of 4-pu and kinetin exhibited an effect on formation of green callus. After 3 months of culture, root differentiation from the callus occurred on the two media containing 4-pu; rooting from the green callus cultured on the 1/2 MS solid medium containing 1 μM NAA and 0.1 or 1 μM 4-pu (**Fig. 2-A**). This was in contrast with the case of leaf protoplast-derived callus of *B. platyphylla*, in which root differentiation was obtained from white calluses[6]. The adding of zeatin and 4-pu promoted the differentiation of roots in the leaf protoplast-derived callus of *B. platyphylla*, while only 4-pu showed an effect for root differentiation in the cell suspension cultures. Unfortu-

Table 1. Differentiation from cell suspension cultures of *B. plantyphylla*.

		zeatin (μM)			4-pu (μM)			TDZ (μM)		
		0.1	1	10	0.1	1	10	0.1	1	10
NAA (μM)	0	—	G	G	—	G	G	G	G	G
	0.1	—	G	G	G	G	G	G	G	G
	1	—	G	G	G R(3)	G R(3)	G	G	G	G

Three calluses induced from cell suspension cultures were cultured for differentiation.
Figures in parenthesis indicate the number of callus from which roots differentiated.
G: Formation of green callus. R: Formation of adventitious roots from callus.
—: No formation of callus.

Table 2. Differentiation from cell suspension cultures of *B. platyphylla*.

		BAP (μM)			2ip (μM)			kinetin (μM)		
		0.1	1	10	0.1	1	10	0.1	1	10
NAA (μM)	0	—	—	—	—	—	—	—	—	—
	0.1	—	—	—	—	W	W	—	—	—
	1	W	W	—	—	W	W	G	W	W

Three calluses induced from cell suspension cultures were cultured for differentiation.
G: Formation of green callus. W: Formation of white callus.—: No formation of callus.

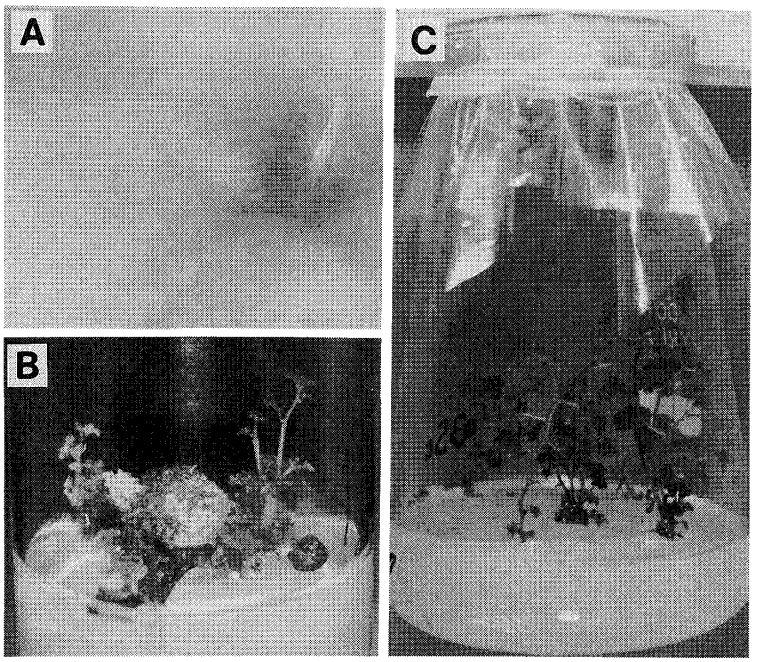

Fig. 2 Organogenesis from callus obtained from cell suspension cultures of *B. platyphylla* var. *japonica*.

A: Root differentiation from the callus on the medium containing 1 μM 4-pu and 1 μM NAA.

B: Shoot differentiation from the callus on the medium containing 1 μM 4-pu and 1 μM GA_3.

C: Plantlet regeneration from the callus on the medium containing 2.5 μM IBA and 0.1 μM NAA.

nately, no proliferation of shoots has been observed on all of the calluses induced throughout the experiments.

Therefore, in order to regenerate shoots from the callus, supplemental experiments were attempted using ABA and GA_3. In the experiments, 1/2 MS media containing NAA and 4-pu, which showed certain effects for root differentiation, were used in combination with ABA and GA_3. As a result, shoots successfully differentiated from the green callus cultured with one NAA-free medium containing 1 μM 4-pu and 1 μM GA_3 after 3 months of culture(**Table 3**). The shoots

Table 3. Differentiation from cell suspension cultures of *B. platyphylla*.

		GA_3 (μM)								
		0.1			1			10		
		4-pu (μM)								
		0.1	1	10	0.1	1	10	0.1	1	10
NAA (μM)	0	—	G	G	G	G S(2)	G	—	G	G
	0.1	G	G	G	G	G	G	G	G	G
	1	G	G	G	G	G	G	G	G	G

Three calluses induced from cell suspension cultures were cultured for differentiation.
Figure in parenthesis indicates the number of callus from which shoots differentiated.
G: Formation of green callus. S: Formation of adventitious shoots from callus.
—: No formation of callus.

Table 4. Differentiation from cell suspension cultures of *B. platyphylla*.

		ABA(μM)								
		0.1			1			10		
		4-pu(μM)								
		0.1	1	10	0.1	1	10	0.1	1	10
NAA(μM)	0	—	G	G	W	G	G	W	W	G
	0.1	W	G	G	W	G	G	G	G	G
	1	G	G	G	G	G	G	G	G	G

Three calluses induced from cell suspension cultures were cultured for differentiation.
G: Formation of green callus. W: Formation of white callus.—: No formation of callus.

obtained were shown in **Fig. 2-B**. On the other hand, the supplementation of ABA frequently gave green calluses, but failed to regenerate both shoots and roots from these calluses (**Table 4**). These facts indicate that the supplement of GA_3 to the medium is effective for differentiation of shoots from the callus induced by the culture of cell suspension cultures of *B. platyphylla*. It is considered that GA_3 inhibited growth of callus probably by causing the decline of endogenous auxin activity, resulting in successful differentiation of shoots.

For rooting, the shoots obtained were transferred to the cytokinin-free MS solid medium supplemented with 2.5 μM indole-3-butyric acid (IBA) and 0.1 μM NAA. After one month of culture, they rooted and developed into plantlets (**Fig. 2-C**). The rooting rate was 100%.

In the present study, of interest is that 4-pu showed an effect on the differentiation of shoots with the aid of GA_3 in the culture of callus from cell suspension cultures. The effect of 4-pu was also true for the culture of leaf protoplast-derived callus of *B. platyphylla*[6]. This effect was confirmed with the culture of protoplasts in *B. grossa* as well[7]. It is interesting that 4-pu possesses a high activity as a cytokinin also in the *in vitro* culture of *Betula* protoplasts, as pointed out previously by Takahashi *et al.*[8] who succeeded in the proliferation of tobacco callus using 4-pu. These findings suggest that use of 4-pu in the tissue culture of *Betula* species is fairly useful. In *Alnus firma*, furthermore, callus formation from protoplasts of cell suspension cultures was also enhanced by using 4-pu[9]. From these findings, phenylurea-type cytokinin, 4-pu, which has a high activity for not only the growth of callus but also differentiation from callus, is expected to contribute to the successful tissue culture in other woody species.

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《和文要約》

シラカンバ液体培養細胞からの植物体再生

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シラカンバの種子を，1 μM 4-pu と 1 μM NAA を含む MS 液体培地中，暗条件下で 100 rpm で振盪培養することにより液体培養細胞が効果的に誘導された．継代培養している種子由来液体培養細胞を分化培地で培養した時，4-pu, zeatin, TDZ 添加培地において活発に増殖し，緑色カルスが数多く得られた．さらに，1 μM 4-pu を含む 1/2 MS 寒天培地に GA_3 1 μM を添加することによって，液体培養細胞からのシュートの分化に成功した．分化したシュートを 2.5 μM IBA と 0.1 μM NAA を含む MS 培地に移植することによって，シュートは発根し，幼植物体を再生した．液体培養細胞の誘導及びそれからの植物体再生において，4-pu はかなり有効であることが見い出された．これまで難しいとされてきた他の樹木の組織培養においても，このサイトカイニン 4-pu はかなり有効であると思われる．