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Callus Proliferation from Protoplasts Isolated from Cell Suspension Cultures of *Alnus firma* Sieb.

Yohichi WAKITA*, Hamako SASAMOTO**, Shinso YOKOTA*
and Nobuo YOSHIZAWA*

* *Forestry and Forest Products, Department of Forest Science, Faculty of Agriculture, Utsunomiya University*, Utsunomiya 321, Tochigi, Japan
** *Forestry and Forest Products Research Institute, Ministry of Agriculture, Forestry and Fisheries*, Tsukuba 305, Ibaraki, Japan

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Protoplasts were isolated from cell suspension cultures induced from a seed of *Alnus firma* Sieb. in 1/2 MS liquid medium containing 1 μM 2, 4-D and 0.1 μM BAP. The optimal enzyme combination for protoplast isolation was 1% Cellulase Onozuka RS and 0.5% Pectolyase Y-23. Protoplast yield was of 1×10^7/g fresh weight, and the protoplasts showed high viability of more than 90%. Colony formation from the protoplasts was induced efficiently in 1/2 MS liquid medium containing 0.6 M mannitol, 0.09 M sucrose and factorial combinations of 0.1-1 μM 2, 4-D and 0.1-10 μM BAP or 4-PU at the plating density of 5×10^4/m*l*. Continuous callus proliferation was achieved by transferring colonies to fresh liquid medium containing 1 μM 2, 4-D and 0.1 μM BAP without mannitol.

Introduction

Alnus species have the ability to fixatmospheric nitrogen through the symbiosis with *Frankia* spp.[1,2]. Somatic hybrids between the *Alnus* and other species will be expected to assist in natural fertilization of soil[3]. Tremblay *et al.*[4] succeeded in callus formation from protoplast-derived cell suspensions of *A. incana*. We have also succeeded in the callus proliferation from leaf protoplasts of *A. firma* Sieb[5]. In *Alnus* species, however, successful plant regeneration from the protoplasts of *Alnus* species has not yet been reported.

Recently we have succeeded in plant regeneration from leaf protoplasts of *Betula platyphylla* var. *japonica*[6-8] which has a fast-growing nature. Production of somatic hybrids between *Alnus* and *Betula* by protoplast fusion is expected, and it is expected that such hybrids will be also utilized as soil fertilizer and tree planting as well as biomass resources, if the plant regeneration system from protoplasts is established in the *Alnus* species.

Cell suspension cultures have the advantage of providing cell populations which are relatively homogeneous and undifferentiated and easily accessible to enzyme action. In addition, the cell suspension cultures are useful as a plant material for the isolation and culture of protoplasts, because they can be obtained in large quantities with high stability. As the first step for producing somatic hybrids between *Alnus* and *Betula*, we attempted the isolation and culture of protoplasts from cell suspension cultures of *Alnus firma* Sieb. In the present paper, we report on the sustained callus proliferation from the protoplasts of *A. firma* Sieb.

Materials and Methods

1. *Induction of cell suspension cultures*

The seeds of *Alnus firma* Sieb. 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 reciprocation/min.). For induction of cell suspension cultures from seed, three basal media, Murashige and Skoog's (MS)[9], 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.

After two months of culture, small cell clusters obtained were transferred into the same fresh medium which successfully induced suspension cells among the media tested here, in a flat-bottomed 50 m*l* tube, and cultured in the dark on a rotary shaker at 100 rpm. Furthermore, after five months of culture, they were subcultured every two weeks to 20 m*l* fresh medium with the same composition in a 100 m*l* Erlenmeyer flask after washing them with the fresh medium on a 40 μm nylon mesh. Culture was performed at 28°C in the dark with shaking (100 rpm).

2. *Isolation of protoplasts*

Cell suspension cultures induced from the seed were used as materials for the protoplast isolation. Twenty-four enzyme combinations were examined to select the best one for protoplast isolation. The enzymes used were as follows: Cellulase Onozuka RS, Cellulase Onozuka R-10, Driselase, Pectolyase Y-23, Macerozyme R-10 and Hemicellulase.

Isolation of protoplasts was performed after 2 weeks from subculture of cell suspension cultures. The cell suspension cultures of 0.3 g fresh weight were washed with 0.6 M mannitol solution on a 40 μm nylon mesh, and put into the 20 m*l* of enzyme solution. After 6 hr incubation at room temperature, protoplasts were passed through a 40 μm nylon mesh, collected by centrifugation at 100×g for 3 min., then washed three times with 0.6 M mannitol solution.

Protoplast yield was measured using a hemocytometer under an inverted microscope. Protoplasts were stained with 0.02% (w/v) fluorescein diacetate (FDA) in 0.6 M mannitol solution to examine their viabilities[10].

3. *Culture of protoplasts*

The protoplasts were cultured in MS, 1/2 MS and MMS liquid media. The medium contained 0.09 M sucrose, 0.6 M mannitol and various combinations of the following auxins and cytokinins: 2, 4-D or NAA (0, 0.1, 1, 10, 30 μM), and BAP or 4-PU (0, 0.1, 1, 10, 30 μM). Cell densities were adjusted to 1-10×10^4/m*l* by adding 2-5 μ*l* of the concentrated protoplast suspension to 50 μ*l* of the medium in a well of a 96-well plastic culture plate. About 10 m*l* of sterilized ultrapure water was dispensed between the wells to maintain the humidity, and the plate was tightly sealed with Parafilm. The protoplasts were cultured in the dark at 28°C using an incubator. After 2 months of culture, the number of colonies in each well was counted twice under an inverted microscope.

4. *Callus proliferation*

Colonies obtained from the culture of protoplasts were cultured with 18 kinds of liquid medium. The medium was supplemented with plant growth regulators combined as follows: 1 μM 2, 4-D and BAP (0.1, 1, 10 μM), 0.1 μM 2, 4-D and 4-PU (0.1, 1 μM), 1 μM 2, 4-D and 0.1 μM 4-PU. The

liquid medium contained mannitol (0, 0.3, 0.6 M). After culturing protoplasts for 2 months, the colonies were transferred into 3 m*l* liquid 1/2 MS medium in a 3 cm in diameter plastic Petri dish, and cultured in the dark or under the illumination from cool white fluorescence tubes of 3, 500 lux for 16 hr per day at 28°C.

Results and Discussion

1. *Induction of cell suspension cultures*

Many cell protrusions probably resulting from cell divisions occurred on the surface of the seed cultured for 2 weeks in the three liquid media: MS and 1/2 MS containing 1 μM 2, 4-D and 0.1 μM BAP, and MS containing 1 μM 2, 4-D and 0.1 μM 4-PU. However, MMS media failed to induce the cell suspension cultures. Thereafter, they actively grew and developed into calli. After 2 months of culture, many cells were released from the calli and actively divided, resulting in developing into cell clusters (**Fig. 1-A**). After 3 months of culture, they developed into yellowish-white cultures about 1-5 mm in diameter (**Fig. 1-B**). The suspension cultures subcultured in the two MS media eventually caused browning during up to 6 months of culture. Only the cell suspension cultures in the 1/2 MS medium showed active growth without browning. Thereafter, they grew well for more than one year by subculturing in the same fresh medium every two weeks (**Fig. 1-C**). It is considered that the 1/2 MS medium containing 1 μM 2, 4-D and 0.1 μM BAP is

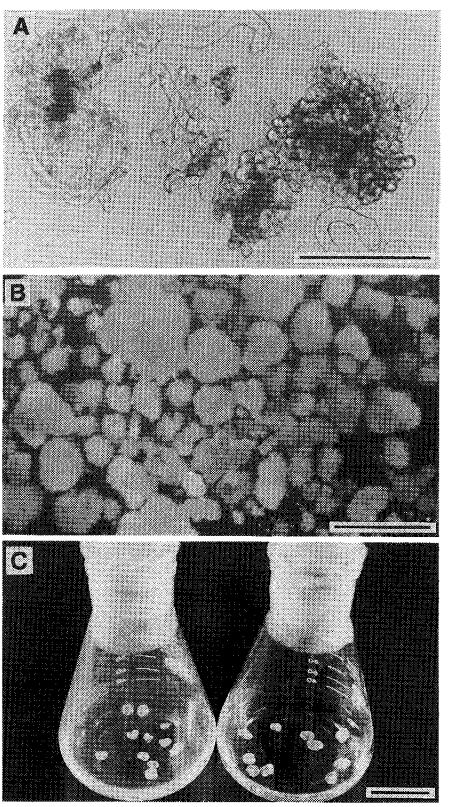

Fig. 1 Cell suspension cultures induced from a seed in the 1/2 MS medium with 1 μM 2, 4-D and 0.1 μM BAP.
A : Cell suspension cultures after 2 months of culture (bar=200 μm).
B : Cell suspension cultures after 3 months of culture (bar=5 mm).
C : Cell suspension cultures subcultured at intervals of 2 weeks (bar=20 mm).

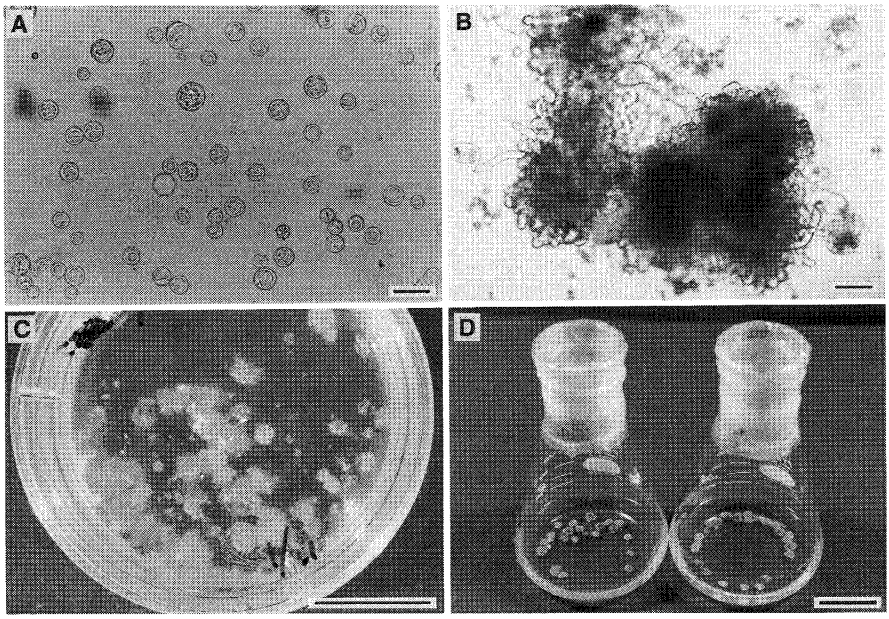

Fig. 2 Culture of protoplasts isolated from cell suspension cultures.
A : Protoplasts immediately after isolation (bar=50 μm).
B : Colony formation after 2 months of culture (bar=100 μm).
C : Starting materials for callus induction (bar=10 mm).
D : Callus formed after 5 months of culture (bar=20 mm).

effective in the culture of cell suspension cultures induced from the seed of *A. firma* Sieb.

2. *Isolation of protoplasts*

In preliminary experiments, effects of enzymes were examined on isolation of protoplasts from cell suspension cultures. Neither Macerozyme R-10 nor Hemicellulase was effective in combination with Cellulase Onozuka R-10 or RS. Driselase plus Cellulases gave much debris, particularly in combination with Cellulase Onozuka RS, which frequently gave brown and inactive protoplasts without showing any FDA fluorescence. Of 24 enzymatic combinations examined, an enzyme mixture of 1% Cellulase Onozuka RS and 0.5% Pectolyase Y-23 was suitable for protoplast isolation. The protoplasts obtained were colorless, and ranged from 20 to 30 μm in diameter (**Fig. 2-A**). The protoplast yield was 1×10^7 protoplasts/g fresh weight, and they showed high viability of more than 90%, this viability almost being the same as that of leaf protoplasts[5].

3. *Culture of protoplasts*

After 2-week-culture of protoplasts in the 1/2 MS medium, non-spherical cell enlargement indicating that cell wall formation had commenced was observed, whereas the non-spherical cell growth did not occur in the MS and MMS media. Thereafter, the cell division occurred in the 1/2 MS medium containing 2, 4-D during one month of culture. The colony formation from the protoplasts was observed with liquid 1/2 MS medium containing 0.6 M mannitol and hormonal combinations of 2, 4-D (0.1, 1 μM) and BAP (0.1, 1, 10 μM) or 4-PU (0.1, 1 μM) (**Fig. 3**). The colonies actively proliferated for 2 months of culture, and grew up to 1 mm in diameter (**Fig. 2-B**). These results indicate that in *A. firma* protoplasts obtained from cell suspension cultures, a small quantity of ammonium ion is necessary for their growth. This fact was also true for the induction of cell suspension cultures. In contrast, in leaf protoplasts of *A. firma*, the MMS medium eliminating ammonium nitrate from MS gave relatively better results for both cell division and colony

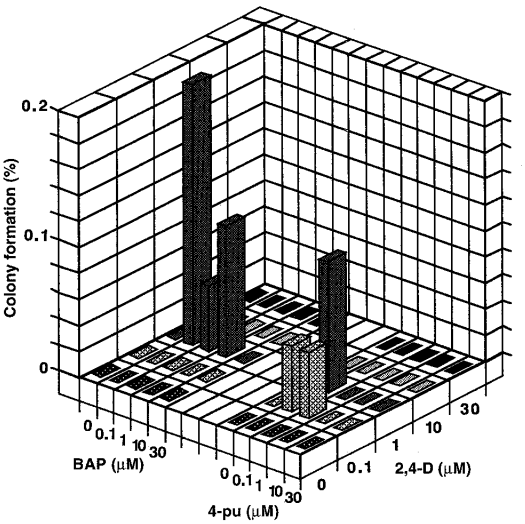

Fig. 3 Percentage of colony formation from protoplasts cultured in the 1/2 MS medium at the plating density of 5×10^4/m*l*.

formation than the other two media tested here, MS and 1/2 MS[5]. On the other hand, Tremblay *et al.*[4] succeeded in colony formation of *A. incana* protoplasts isolated from cell suspensions of leaf-derived callus cultured in KM 8p medium[11] containing ammonium nitrate. It is considered, therefore, that the requirement of ammonium ion as a protoplast growth agent differs among species and the tissue sources even in the same species.

In general, the optimal plating density of protoplasts ranges from 10^3 to 10^5 protoplasts/m*l* in some forest tree species[12,13]. In the culture of *A. firma* protoplasts, cell densities lower than 2×10^4 protoplasts/m*l* were less effective in cell division, and those of more than 7×10^4 protoplasts/m*l* caused cell aggregation rather than cell division. A plating density of 5×10^4 protoplasts/m*l* gave the most active growth and cell division of protoplasts. This value was similar to that of leaf protoplasts in *A. firma* ($5\text{-}7\times10^4$ protoplasts/m*l*)[5], and in line with those ($10^3\text{-}10^5$ protoplasts/m*l*) reported for other forest species[6,12,13].

The most active colony formation was observed under the following condition: 1/2 MS medium containing 1 μM 2, 4-D and 0.1 μM BAP, plating density at 5×10^4 protoplasts/m*l* (**Fig. 3**). However, colony formation rate was only 0.2%.

4. *Proliferation and subculture of callus*

When calli obtained from the protoplasts were cultured in darkness in 1/2 MS liquid media containing no mannitol, with 1 μM 2, 4-D and BAP (1, 10 μM), and 2, 4-D (0.1, 1 μM) and 4-PU (0.1, 1 μM), they grew for 3 months. After 5 months, however, a portion of the callus became brown, and the whole callus ceased to grow. On the other hand, only the 1/2 MS liquid medium containing no mannitol and the combination of 1 μM 2, 4-D and 0.1 μM BAP gave active callus growth in the dark even after 5 months of culture (**Fig. 2-C**). Thereafter, the calli obtained were subcultured every 2 weeks to 20 m*l* fresh medium with the same composition in a 100 m*l* Erlenmeyer flask (**Fig. 2-D**). This suggests that low concentration of BAP is suitable for callus growth in the medium. However, active callus proliferation did not occur in the light condition in the same medium. It is concluded that 1/2 MS liquid medium containing no mannitol, 0.1 μM of BAP and 1 μM of 2, 4-D is suitable for the proliferation of callus derived from protoplasts of cell suspension cultures in *A. firma* Sieb. The successful callus proliferation system established in the present study was the first in *A. firma* Sieb. We are further investigating the culture conditions for plantlet regeneration from

protoplast-derived callus of *A. firma* Sieb.

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《和文要約》

ヤシャブシ液体培養細胞由来プロトプラストからのカルス形成

脇田陽一*・笹本浜子**・横田信三*・吉澤伸夫*

* 宇都宮大学農学部
** 農林水産省森林総合研究所

ヤシャブシの種子から，1 μM 2,4-D と 0.1 μM BAP を含む 1/2 MS 液体培地中，暗条件，100 rpm の振盪培養により，液体培養細胞を誘導した．得られた液体培養細胞を，1% セルラーゼオノズカ RS 及び 0.5% ペクトリアーゼ Y-23 を含む 0.6 M マントニール溶液中，6 時間，100 rpm で振盪し，酵素処理することにより，液体培養細胞 1 g 当り 1×10^7 個のプロトプラストが得られ，生存率も 90% 以上の高い値を示した．プロトプラストの培養については，培養密度を 5×10^4/m*l* に調節し，0.1-1 μM の 2, 4-D に 0.1-10 μM の BAP あるいは 0.1-1 μM の 4-PU を組み合わせて添加した 1/2 MS 液体培地中で培養した時，プロトプラストからコロニーが形成され，0.2% のコロニー形成率が得られた．さらに，得られたコロニーを 1 μM 2, 4-D と 0.1 μM BAP を添加した 1/2 MS 液体培地で培養することにより，効果的にカルスの増殖が得られた．