# クヌギ不定胚から単離したプロトプラストからのカルス形成


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短　報

# Callus Formation from Protoplasts Isolated from Somatic Embryos of *Quercus acutissima*[*1]

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## I. Introduction

The national program for the genetic improvement of *Quercus acutissima* CARR. through plus-tree selections has been in effect since 1978 in Japan.

Somatic hybridization between different genotypes of *Q. acutissima* or with other species of *Quercus* seems to be promising for further improvement of its wood quality and/or expansion of its site adaptability in the course of breeding.

The technique of plant regeneration from protoplasts is essential for somatic hybridization, but little is known about the protoplast culture of *Q. acutissima*. While we reported the isolation and culture of protoplasts from whole shoots of shoot cultures (*5*), we did not succeed in colony or callus formation.

Recently, callus formations from the root protoplasts of *Quercus rubra* L. (*1*) and from the protoplasts of embryogenic cells of *Quercus serrata* MURRAY (*9*) were reported. They indicate that there is a great possibility for callus formation of *Q. acutissima* through protoplast culture.

In this paper, we report successful callus formation from protoplasts isolated from the somatic embryos of *Q. acutissima*.

## II. Materials and Methods

Somatic embryos were derived from a cotyledon of *Q. acutissima* in liquid modified MS (MURASHIGE-SKOOG) medium (*7*) with nitrogen at half strength (SC medium) (*2*). They were maintained by seven successive subculturing in a liquid modified SC medium (MSC medium) with an interval of 1.5～2.0 month. These subcultured somatic embryos were used for the protoplasts isolation.

The concentration of inorganic nitrogen in SC medium (*2*) was reduced by half of that in MS medium. MSC medium was modified from SC medium by the elimination of giberrellic acid, and by the addition of 100 mg/*l* of acid-hydrolyzed casein, and by the enrichment of glutamine to 100 mg/*l*. MSC medium contained 0.3 mg/*l* of PBA ($\gamma$-phenylbutylic acid), 0.25 mg/*l* of NAA ($\alpha$-naphtylacetic acid), and 30 g/*l* of sucrose (*3*).

Secondary somatic embryos emerged frequently in the culture, and they were in various developmental stages. They were classified into three stages by their developmental feature before being used for protoplast isolation. In the juvenile stage, somatic embryos were undeveloped, clear white, and 2～3 mm in diameter (Stage 1). In the middle stage, they were in a cotyledonary form, yellowish, and 3～7 mm in diameter (Stage 2). In the last stage, they were in cotyledonary form with well-developed cotyledons, had an opaque yellowish-white color, and than 7 mm in diameters (Stage 3).

Somatic embryos in Stage 2 which could provide a sufficient number of vital protoplasts for culture (*4*) were collected after three weeks of subculture. One gram of somatic embryo was cut into 2～3 mm fragments with a surgical knife and digested in 20 ml of the enzyme solution for 5 h at 30°C on a reciprocal water-bath shaker (70 rpm).

The enzyme solution contained 1.0% of Cellulase "Onozuka" RS (Yakult Pharmaceutical Co., Japan) and 0.25% of Pectolyase Y-23 (Seishin Pharmaceutical Co., Japan) in a 0.6 M mannitol solution. The solution

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also contained 0.1% of potassium dextran sulfate, 1.62 g/*l* of potassium citrate (monobasic), and 20 mg/*l* of boric acid to prevent isolated protoplasts from breaking (*8*). The enzyme solution was filtrated through a 0.22 $\mu$m membrane filter before using.

After 5 h of digestion of the somatic embryo tissue in the enzyme solution, large debris was removed by filtration through a 100 $\mu$m nylon mesh. The filtrate was centrifuged at 100×*g* for 5 min. The pellet consisted of protoplasts and debris was washed twice in a 0.6 M mannitol solution containing 0.8g/*l* of $CaCl_2 \cdot 2H_2O$ by centrifuging at 100×*g* for 5 min.

The debris was not removed completely by this washing procedure, but the pellet was suspended in 8 ml of 0.6 M sucrose solution which contained 0.8 g/*l* of $CaCl_2 \cdot 2H_2O$ and 8% Dextran T-40 (Pharmacia Co., Sweden) with 2 ml of mannitol solution laid as an upper layer. After centrifugation at 300×*g* for 10 min, protoplasts floating between the sucrose solution and the mannitol solution were pipetted and suspended in the culture medium. Then they were centrifuged at 100×*g* for 5 min and suspended in the culture medium again.

After purification the viability of the yielded protoplasts was determined by the FDA (fluorescein diacetate) staining method (*6*) and their numbers were counted on a hemocytometer.

MS medium from which $NH_4NO_3$ was eliminated was prepared for protoplast culture (MMS medium). In this medium 5.6 mg/*l* of Fe-EDTA (Ferric monosodium salt of etylenediaminetetraacetic acid) replaced $FeSO_4 \cdot 7H_2O$ and $Na_2$-EDTA (Disodium salt of etylenediaminetetraacetic acid).

The medium which contained 20 g/*l* of sucrose and 0.6 M of mannitol was supplemented with various concentrations of BAP (6-benzylaminopurine) (0, 0.2, and 0.4 mg/*l*) and/or 2, 4-D (2,4-dichlorophenoxiacetic acid) (0, 1.0, and 2.0 mg/*l*).

Protoplasts were suspended in the media and plated in a 24 hole multi-well plate (Falcon Co., U.S.A.) at a density of about $9\times10^4$/ml (500 $\mu$l for each hole). Forty days after plating, 100 $\mu$l of fresh medium with 0.5 M mannitol was added to each culture. After 66 days of culture, the culture medium was replaced by 500 $\mu$l of fresh MMS medium where mannitol had been reduced to 0.4 M for reducing the osmotic pressure. After an additional 11 days of culture, 500 $\mu$l of the same fresh medium were added again.

The plates were kept under continuous dark conditions at 25°C.

Liquid MSC medium was used for the culture of colonies. Three milliliters of medium was poured into 18×180 mm test tubes. The colonies were cultured in the test tubes by shaking slowly at 1 cycles/min on a gyrated drum. They were maintained at 25°C under 1,000~2,000 lx fluorescent illumination for a 16 h photoperiod.

MSC medium solidified by 0.7% agar was used for calluses formations from proliferated colonies. Cultures were kept under same conditions as those for colony proliferation.

All culture media used in this experiment had their pHs adjusted to 5.6 with NaOH and HCl and were autoclaved at 121°C for 20 min.

## III. Results and Discussion

The yield of protoplasts after purification was approximately $4.5\times10^5$ from one gram of somatic embryos. This yield appears to be of the same order as that from shoot cultures of *Q. acutissima* ($2\times10^5$/g) (*5*) and that from the roots of *Q. rubra* ($4\times10^5$) (*1*).

Although the number of protoplasts were not counted before purification in this experiment, approximately $1\times10^7$ protoplasts were obtained before purification in our previous isolation under the same conditions (*4*). The number of protoplasts were reduced considerably by the purification, but it is impossible to remove cell debris from protoplast isolations by filtration and by washing in a mannitol solution. A procedure of purification by a sucrose solution was necessary for the successive culture.

The diameter of protoplasts varied from 5 to 20 $\mu$m (Fig. 1 a). The viability of the protoplasts estimated by the FDA staining method was over 90%.

During the first ten days of protoplast culture only occasional cell-buddings were observed, but cell

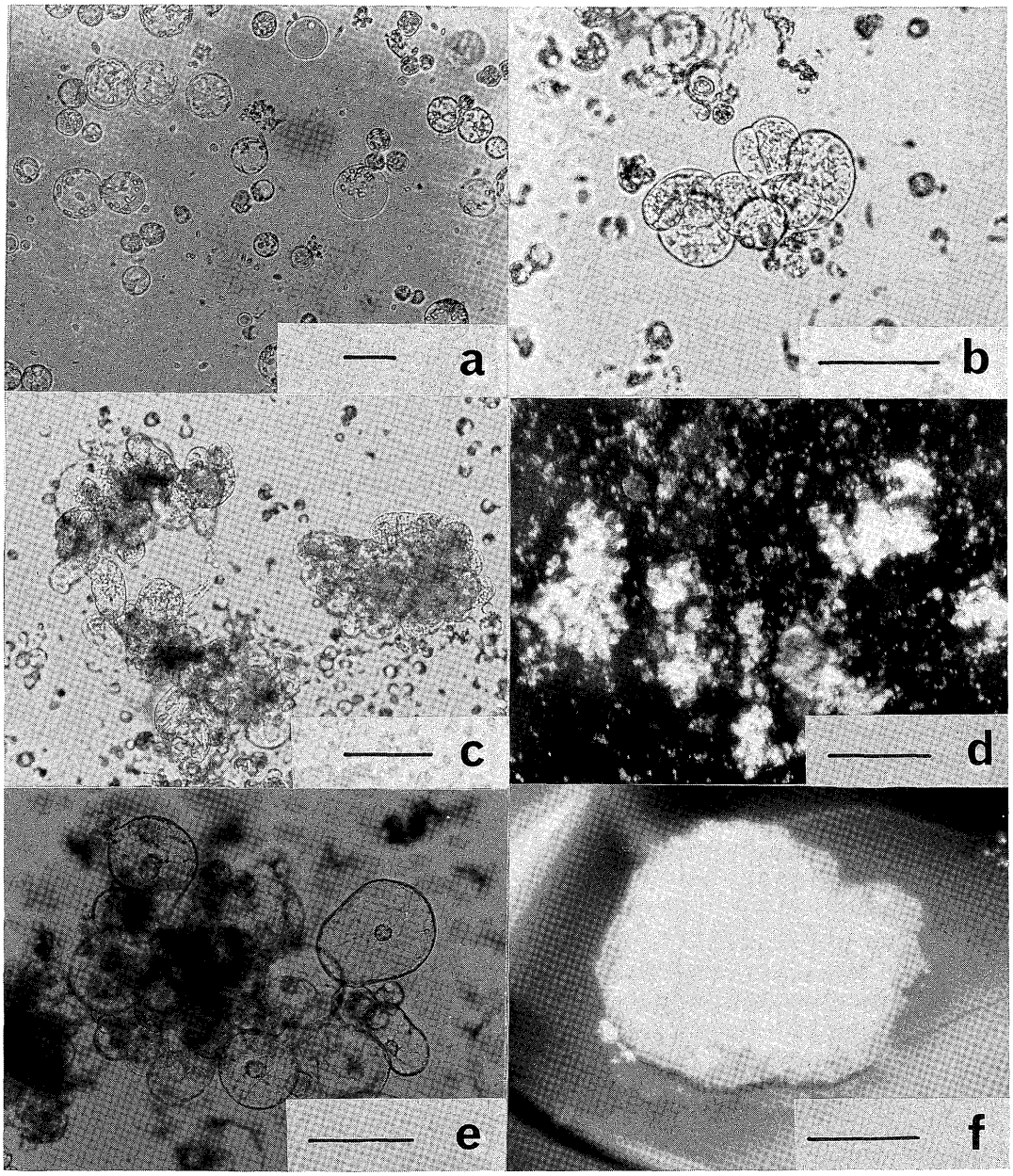


Fig. 1. Callus formation from somatic embryo protoplasts of *Quercus acutissima*
a: Protoplasts isolated from somatic embryos of *Quercus acutissima*.
Note: Bar=20 μm.
b: Cell clusters 22 days after protoplasts plating.
Notes: Medium: MMS, BAP 0.4 mg/*l*, 2,4-D 1.0 mg/*l*; Bar=30 μm.
c: Cell colonies 40 days after protoplasts plating.
Notes: Medium: MMS, BAP 0.2 mg/*l*, 2,4-D 1.0 mg/*l*; Bar=50 μm.
d: Enlarged colonies 65 days after protoplasts plating.
Notes: Medium: MMS, BAP 0.2 mg/*l*, 2.4-D 1.0 mg/*l*; Bar=0.5 mm.
e: Vacuolized cells among cell colonies 65 days after plating.
Notes: Medium: MMS, BAP 0.2 mg/*l*, 2,4-D 2.0 mg/*l*; Bar=100 μm.
f: Callus formed on solid MSC medium 60 days after transplanting to solid medium.
Note: Bar=2 mm.

Table 1. Effects of the concentrations of BAP and 2, 4-D with MMS medium on colony formation

(mg/$l$)

| BAP \ 2,4-D | 0 | 1.0 | 2.0 |
|---|---|---|---|
| 0 | −/0 | −/0 | −/0 |
| 0.2 | +/20 | +++/77 | ++/80 |
| 0.4 | +/21 | ++/60 | ++/56 |

Notes : Degree of colony formation is indicated an − for no colony formation, and +, ++, and +++, for varying degrees, increasing from rare to abundant, 40 days after protoplast plating. Numbers indicate the colony numbers over 0.5 mm in diameter formed from about $4.5\times10^4$ protoplasts 76 days after protoplast plating.

division was not observed. Thirteen days after plating some divided cells were found in every culture media.

Twenty-one days after plating, cell clusters, consisted of about ten cells, were formed in the medium containing 0.4 mg/$l$ of BAP only. During another 20 days, cell clusters also were formed in every medium (Fig. 1 b), and the number of cell clusters increased and developed in to cell colonies which were composed of 20～30 cells after 40 days of culture (Fig. 1 c). The features of cell-cluster formation in each medium after 40 days of protoplast culture are shown in Table 1.

No colony formation was observed in media without BAP, but colonies were formed even in media without 2,4-D. It appeared that concentrations of 0.2 mg/$l$ or 0.4 mg/$l$ of BAP in MMS medium were essential for colony formation, but a concentration of 2,4-D is not always necessary. The most active colony formation was observed in the medium containing 0.2 mg/$l$ of BAP and 1 mg/$l$ of 2,4-D. It is obvious that special combinations of BAP and 2,4-D concentrations allow active colony formations.

Forty days after the plating of protoplasts, fresh medium was added to each culture. Colonies had cell divisions and formed larger colonies during the 25 days after the addition of fresh medium. The colonies were 0.5～1.0 mm in diameter and could be observed by the naked eye (Fig. 1 d).

Vacuolized cells with reticulated cytoplasm were observed frequently in each medium after 40～65 days of plating (Fig. 1 e). Vacuolization was serious especially in the medium containing 2.0 mg/l of 2,4-D. It was supposed that the greater 2,4-D concentration was inappropriate for continuous cell division.

Colonies continued their increases in number and their enlargements in size after the replacement and addition of fresh medium with osmotic pressure reduced to 0.4 M.

In *Q. rubra*, the intermittent addition of fresh media at intervals of ten days was effective for callus formation (*1*).

In our experiment fresh medium was added three times, and the osmotic pressure of culture medium was reduced from 0.6 to 0.4 M, but it is not certain whether they had effects on active colony formation or not.

Features of colony formation in each MMS medium after 76 days of protoplast culture are shown in Table 1. From these results, it was concluded that media containing 0.2mg/$l$ of BAP and 1.0 or 2.0mg/$l$ of 2,4-D were suitable for colony formation.

After 92 days of protoplast culture, 5～10 colonies were transferred to test tubes each containing 10 ml of liquid MSC medium and shaken on a gyrated drum for further proliferation and/or somatic embryo formation. The colonies proliferated in number (not counted precisely but about 30 colonies on average for each test tube) and enlarged to 1～3 mm diameters within a month after being transplanted to the MSC medium, but no somatic embryo were formed.

Two sets of enlarged colonies showing different growth rates were selected for callus formations. They originally were derived in the MMS media containing 0.2mg/$l$ of BAP and 1.0mg of 2,4-D, and 0.4mg/$l$ of BAP and 2.0 mg/$l$ of 2,4-D. They were transplanted to solid MSC medium after 153 days of protoplast culture (Colonies derived from another set of hormones were not tested in this experiment).

They grew slowly and formed soft white calluses which were more than 5 mm in diameter during the one month after transplanting to the solid MSC medium (Fig. 1 f). Larger calluses were obtained from the colonies originating in the MMS medium containing 0.2 mg/$l$ of BAP and 1.0 mg/$l$ of 2,4-D (mean diameter : 6.0 mm, range : 4.0～7.0 mm) than in the MMS medium containing 0.4 mg/$l$ of BAP and 2.0 mg/$l$ of 2,4-D (mean diameter : 2.7 mm, range : 2.0～5.0 mm).

Therefore, we concluded that the former combination of BAP and 2,4-D concentrations was suitable not

only for colony formation but also for the successive callus formation.

MS medium from which $NH_4NO_3$ was eliminated was effective for the protoplast culture of *Populus alba* L. (*10*). Then we choose this medium for the protoplast culture of *Q. acutissima*. As a result, it was effective for callus formation of this species.

Plating density of protoplasts seriously affects callus formation in *Q. rubra* (*1*) and *P. alba* (*10*). In *Q. rubra* $5\sim7\times10^3$ protoplasts/ml was the best result (*1*). Although other plating densities were not tested, the density of about $9\times10^4$/ml allowed the active colony formation in our experiment, but it is not certain whether this density is optimum or not.

We found the conditions allowing colony formation from protoplasts of *Q. acutissima* and could obtain calluses by successive culture in this experiment. The regenerated calluses now are being cultured for plant regeneration.

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