Bull. Tokyo Univ. For., 85, 43-47 (1991)

Isolation and Culture of Protoplasts from *Quercus acutissima* Shoot Cultures*

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I. Introduction

There are many tree species which are important for timber products in the genus *Quercus* and some species including 'kunugi' (*Q. acutissima* CARR.) have been used as bed logs for Shiitake mushroom (*Lentinus edodes* (BERK.) SING.) culture in Japan.

Micro propagation methods in *Quercus* species have been progressed remarkably by recent studies, but little is known about their protoplast culture. Although callus formation from root protoplasts of *Quercus rubra* L. was reported (BRISON and LAMANT, 1990), there is no report on isolation and culture of protoplasts from *Q. acutissima.*

In this study, we could isolate sound protoplasts from shoot cultures of *Q. acutissima* and could observe cell buddings.

II. Materials and Methods

Preparation of shoot cultures

Shoots derived from aseptically cultured nodal segments of seedlings were used for protoplast isolation.

Acorns of *Quercus acutissima* were sown on vermiculite in a plastic container in January, 1989. The nodal segments about 1.5 cm long with axillary buds were isolated from young seedlings which grew about 15 cm tall, at mid-June, 1989. After removal of leaves, they were surface sterilized by stirring in 70% ethanol solution for one minute and in sodium hypochlorite solution, containing about one percent effective chloride, for 10 min. They were rinsed with sterilized distilled water three times. The basal ends of the explants damaged by the surface sterilization were cut back slightly by sterilized scissors.

They were inoculated in modified ANDERSON'S medium (ANDERSON, 1986, IDE and YAMAMOTO, 1988) with the concentrations of 5 mg/*l* of $AgNO_3$ and 0.8 mg/*l* of BAP(6-benzylaminopurine) (Table 1). The cultures were kept at 25℃ under fluorescent light at 5,000 lux for 16 hr photoperiod.

Isolation and culture of protoplasts

Seventy days after the inoculation, shoot derived from axillary buds on explants which grew about 2 cm were harvested. Whole shoots including leaves and stems were cut into small pieces by sterilized scissors and were used for protoplast isolation.

Enzyme solution, sucrose solution and wash solution were prepared for isolation and purification of protoplast (Table 2). Modified liquid MS medium (MURASHIGE and SKOOG, 1962) with a concentration of 3 mg/*l* of 2,4-D(2,4-dichlorophenoxyacetic acid) and of 5.5 g/*l*

* A part of this study was supported by a Grant-in-Aid for Scientific Research (No. 02660154) from the Ministry of Education, Science and Culture of Japan.
本研究の一部は文部省科学研究費（一般C：02660154）によった
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Table 1. Media for establishing shoot culture and for culturing protoplasts of *Quercus acutissima* (mg/*l*)

Components	Medium for shoot culture*	Medium for protoplast culture**
NH_4NO_3	400	1650
KNO_3	480	1900
$CaCl_2 \cdot 2H_2O$	440	440
$NaH_2PO_4 \cdot 2H_2O$	380	
KH_2PO_4		170
$MgSO_4 \cdot 7H_2O$	370	370
$MnSO_4 \cdot 4H_2O$	16.9	22.3
$ZnSO_4 \cdot 7H_2O$	8.6	8.6
$CuSO_4 \cdot 5H_2O$	0.025	0.025
H_3BO_3	6.2	6.2
KI	0.3	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25
$CoCl_2 \cdot 6H_2O$	0.025	0.025
FeEDTA***	5.6	28
Nicotinic acid		0.5
Thiamine HCl	0.4	0.1
Pyridoxine HCl		0.5
myo-Inositol	100	100
Glutamine		370
2,4-D****		3
BAP*****	0.8	
$AgNO_3$	5	
Sucrose	20000	
Glucose		5500
Mannitol		5500
Agar	8000	

* Modified ANDERSON's medium (ANDERSON, 1986)
pH was adjusted at 5.8 by KOH and HCl
** Modified MURASHIGE and SKOOG's medium (MURASHIGE and SKOOG, 1962)
pH was adjusted at 5.6 by NaOH and HCl
*** Ferric monosodium ethylenediaminetetraacetic acid
**** 2,4-dichlorophenoxyacetic acid
***** 6-benzylaminopurine

of glucose were prepared for culture of protoplasts (Table 1). These solutions and a medium had been used for isolation and culture of mesophyll protoplasts from 'Japanese whitebirch' (*Betula. platyphylla* SUKATCHEV var. *japonica* (MIQ.) HARA) (IDE *et al.*, 1991). They were filter sterilized by passing through a 0.22 μm membrane filter.

About 0.5 g of shoot tissue was stirred in 100 m*l* of Erlenmeyer flask containing 40 m*l* of enzyme solution. The flask was shaken reciprocally for 19 hr with 80 turns/min at 30℃ in a water bath incubator.

The enzyme solution was filtered through 100 μm nylon mesh and centrifuged at 100 $\times g$ for 5 min. After removal of the supernatant, the pellet consisted of protoplasts and debris was resuspended in wash solution. The suspension was centrifuged at 100$\times g$ for 5 min. The pellet was resuspended in 8 m*l* of sucrose solution and 2 m*l* of wash solution was laid as an upper layer. It was centrifuged at 300$\times g$ for 10 min. Protoplasts floating in the border between sucrose solution and wash solution were pipetted and resuspended in

Table 2. Solutions for protoplast isolation*

Components	Enzyme solution	Wash solution	Sucrose solution
Isotonic solution[1]			
Mannitol	0.6 M	0.6 M	
Sucrose			0.6 M
H_3BO_3	2 mg		
$CaCl_2$		80 mg	80 mg
Pottasium citrate	162 mg		
Enzyme and others[2]			
Pectolyase Y-23[3]	0.1%		
Cellase 'Onozuka' R-10[4]	2%		
Driselase[5]	1%		
Potassium dextran sulfate[6]	0.1%		
Dextran T-40[7]			8%
Antibiotics[2]			
Ampicillin	40 mg		
Carbenicillin (Disodium salt)	3 mg		
Gentamicin sulfate	3 mg		

* After IDE *et al.* (in prep.)
pH was 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] Yakulut Pharmaceutical Co., Japan
[5] Kyowa Hakko Kogyo Co., Japan
[6] Meito Sangyo Co., Japan
[7] Pharmacia Co., Sweden

culture medium. Then number of protoplasts yielded was counted on a hemocytometer. The protoplasts suspension was centrifuged at $100 \times g$ for 5 min. The protoplasts pellet was resuspended in culture medium to the density of 1×10^5/m*l* and cultured in 35 mm × 10 mm plastic petri dish at 25℃ in the dark.

III. Results and Discussions

While the shoot tissues of *Quercus* acutissima apparently kept their original form even after 19 hr treatment in enzyme solution, small numbers of protoplasts were released. About 1×10^5 protoplasts of which diameters ranged from 20 μm to 40 μm were yielded from 0.5 g of shoot tissues after purification (Fig. 1). But it is not certain whether the origin of these protoplasts were derived from mesophyll cells or not, because we used whole of shoot.

The callus of *Quercus serrata* was digested perfectly within 4 hr treatment in the enzyme solution (KOYAMA *et al.*, 1988). And 6×10^6 protoplasts was released from 1 g of leaf tissue of *Betula platyphylla* var. *japonica* by 2 hr treatment in the same enzyme solution used in this experiment (IDE *et al.*, 1991). Moreover the yielded number of protoplasts from *Betula papyrifera* March. (TREMBLAY, 1988) and *Populus alba* L. (SASAMOTO *et al.*, 1989) which had been succeeded in callus formation from protoplasts, were more than 1×10^6.

From these facts, it was concluded that high protoplast yield from shoot tissue of *Q. acutissima* cannot be expected by the enzyme solutions used in this experiment. More research is needed to define appropriate components of enzyme solution for higher yield of protoplasts.

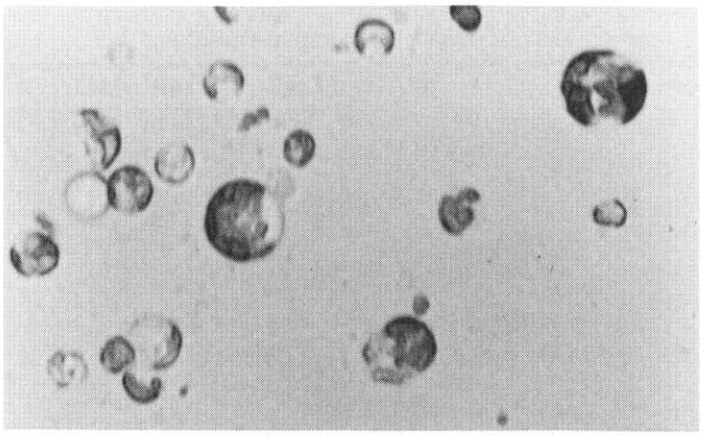

Fig. 1. Protoplasts isolated from shoot tissue of *Quercus acutissima* by enzyme solution.

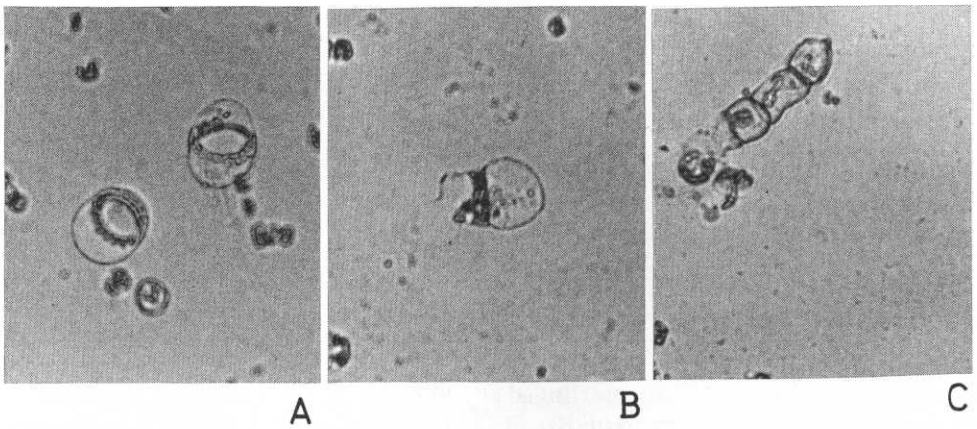

Fig. 2. Cells in different budding stages derived from 9 days culture of *Quercus acutissima* protoplasts.
A, First cell buddings after 24 hr culture of protoplasts; B and C, Continuous cell buddings after 2 weeks culture of protoplasts.

After 24 hr of protoplast culture, several two cell derivatives were observed (Fig. 2A). But it was not sure whether they were the products of cell divisions or the products of cell buddings. Because we did not confirm the cell wall regeneration.

The number of budded cells was increased and continuous cell buddings also were observed in the following three week (Fig. 2B and C). But cells stopped new buddings at the fourth week of culture, and died within a short period.

In this experiment, it was proved that a part of isolated protoplasts had viability for continuous cell buddings and maintained it for more than three weeks. We supposed that cell division in the culture of *Q. acutissima* protoplasts would be possible through studies on the status of material tissue, the components of enzyme solution and on the culture conditions of protoplasts.

Acknowledgments

We thank Ms. M. KOYAMA of Gunma Prefecture Forestry Experimental Station for her

kind consultation. And we also thank Professor K. SUZUKI of The Tokyo University for his critical reading of this manuscript.

Summary

Protoplasts were isolated from shoot tissues which were derived from culture of nodal segments of *Quercus acutissima* young seedlings by an enzyme solution. About 1×10^5 protoplasts were isolated from 0.5 g of shoot tissue. The protoplasts were cultured in the liquid modified MS medium containing 3 mg/*l* of 2,4-D(2,4-dichlorophenoxyacetic acid) at the density of 1×10^5/m*l*. First cell buddings were observed after 24 hr of protoplast culture, and cell buddings were continued three weeks.

Key words: *Quercus acutissima*, Shoot culture, Protoplast, Cell budding

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(Received, October 30, 1990)

クヌギの培養シュートからのプロトプラストの単離と培養

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要　　旨

クヌギの若い実生の節間部の小片の培養により，えき芽から伸長させたシュートから，酵素液を用いてプロトプラストを単離した。0.5 g のシュート組織から約 1×10^5 個のプロトプラストが得られた。このプロトプラストを，2,4-D(2,4-dichlorophenoxyacetic acid)3 mg/*l* を含んだ改変 MS (Murashige and Skoog, 1962) 培地に約 1×10^5/m*l* の密度で懸濁して培養した。24 時間後に最初のバディングが観察された。バディングはさらに 3 週間にわたり連続的に起こった。

キーワード: クヌギ，培養シュート，プロトプラスト，バディング