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Isolation and Culture of Protoplasts from Kunugi (*Quercus acutissima* Carruth.) Callus Cultures

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Isolation and culture of protoplasts from Kunugi (*Quercus acutissima* Carruth.) callus cultures were examined with various culture conditions. The peeling treatment of twigs largely excluded contamination during the induction and culture of callus, leading to successful formation of callus. Callus was induced most effectively from the twigs using half strength MS solid medium containing 10 μM 2, 4-dichlorophenoxyacetic acid (2, 4-D). The callus growth in the medium was largely promoted without causing a browning of the callus in the subculture. In preparation of protoplasts, the addition of dithiothreitol (DTT) to the enzyme solution gave a high yield of protoplasts and enhanced the viability of the protoplasts in the subsequent culture. Cell division into 2 cells was observed after 24 hr of culture, then micro colonies formed after 7 days, followed by colony formation within 1 month. Callus was not induced from the colony, although the colony formation continued over 3 months of culture.

Introduction

Kunugi (*Quercus acutissima* Carruth.) is an important species as bed logs for cultivating Shiitake mushrooms (*Lentinus edodes* (Berk.) Sing.). The selection of the elite trees for bed logs and a stable supply of their clonally propagated seedlings are required. Generally, Kunugi trees are sexually propagated by seed. However, there are several problems concerning the production of seedlings. The seed production varies considerably from year to year. Seed storage is also difficult. Furthermore, propagation by cuttings is also difficult because of the poor rooting[1]. Hence, the propagation of seedlings of the elite trees by using tissue culture techniques is desired.

The clonal seedling propagation in the *Quercus* species has been investigated so far using mainly apical and axillary buds[2] or seed embryos[3-7]. Several investigators succeeded in the formation of multiple shoots from the axillary or hidden buds of cuttings[8-10]. In the *Quercus* species, however, little is known about the culture of the callus and protoplast, which is expected to be a promising technique, for the genetic transformation or induction of mutants. In some woody plants such as *Fagus* and *Zelkova* containing large amounts of polyphenolics, the compounds become an obstacle to progress in the tissues culture of these trees[11]. The polyphenolics also make it difficult to induce callus from the cuttings of the *Quercus* species.

Brison and Lamant[12] have reported callus formation from root protoplasts of *Q. rubra*, in which the protoplast-derived callus developed into green and compact callus, however, they gradually became brown and then died. On the other hand, although Koyama *et al.*[13] have isolated protoplasts

from the callus induced from the midribs of leaf disks in *Q. serrata*, the isolated protoplasts formed no colonies. Ide *et al.*[14] have reported that the isolated protoplasts from *Q. acutissima* shoot cultures showed viability only during three weeks of culture. Further research on the culture of *Quercus*-species protoplasts is needed. Material-tissue source, the components of enzyme solution and the culture conditions of protoplasts should be investigated in order to enhance the viability of protoplasts during the culture, and to establish a regeneration system into plantlets through protoplast culture.

In this study we induced callus tissues from the current twigs of *Q. acutissima*, isolated protoplasts from the subcultured callus, and then cultured the protoplasts under various culture conditions. Protoplasts were also isolated from mesophyll cells of the fresh leaves to compare with those from the callus.

Materials and Methods

Induction and culture of callus

Current twigs were collected from 15-year-old trees of *Q. acutissima*. After washing with running tap water, the twigs were surface-sterilized with 70% ethanol for 30 sec, further sterilized with 1% sodium hypochlorite aqueous solution containing a few drops of Tween 80 for 5 min, and then rinsed three times in sterilized distilled water.

The bark was peeled from the twigs leaving the cambium. The peeled twigs were inoculated in half strength Murashige and Skoog medium (1/2 MS)[15] solidified by 0.8% agar, whose pH was adjusted to 5.8, and cultured for callus induction at 25°C under fluorescence illumination of about 2,000 lux (16 hr/day) throughout the culture period. Four kinds of auxins, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-naphthaleneacetic acid (NAA) and 2, 4-D, were added to the culture medium in various concentrations (IAA, IBA: 0, 2.5, 5, 7.5, 10 μM; NAA, 2, 4-D: 0, 0.1, 1, 10, 100 μM). Ten specimens were used for callus induction in their respective conditions. The induced callus was subcultured in the dark at one-month intervals on the same solid medium as that for callus induction.

Isolation of protoplasts

Protoplasts were prepared from the fully expanded young leaves and callus tissues induced from the twigs. After the midribs were removed from the leaves with forceps, the leaves were cut into small pieces, about 2 mm wide, with a surgical knife. About 0.2 g (fresh weight) of leaf pieces or about 50 mg (fresh weight) of callus were incubated in centrifuge tubes with 5 m*l* of enzyme solution for 1 to 6 hr at 30°C with shaking (90 reciprocation/min). For protoplast isolation, enyme solution of Koyama *et al.*[13] was modified and used in this experiment (**Table 1**). Cellulase "Onozuka" RS was used instead of Cellulase "Onozuka" R-10 which had been used by Koyama *et al.* Furthermore, 1% Meiselase Pl and 1% Hemicellulase were added originally to the solution. Two mM DTT was added to the enzyme solution to prevent oxidation of polyphenolics[11]. Five mM NH_4NO_3 was also added to the solution in order to protect protoplasts against burst[11]. The isolated protoplasts were washed three times with 0.6 M D-mannitol solution by centrifugation at $100 \times g$ for 5 min. The number of protoplasts was counted on a hemacytometer (Thoma counting chamber). Yields of protoplasts were determined by averaging triplicates in each treatment.

Culture and viability of protoplasts isolated from callus

Protoplasts were cultured with 1/2 MS liquid medium containing 0.1 μM of BAP, 0.1 μM of 2, 4-D and 0.6 M D-mannitol without adding saccharose in micro plates (Corning Cell Wells) at 25°C in the dark. The density of protoplasts was finally adjusted to 1.0×10^5 ml^{-1}. Protoplasts were

Table 1. Enzyme solution for isolating protoplasts

Pectolyase Y-23	0.1%
Cellulase "Onozuka" RS	2.0%
Meiselase P1	1.0%
Driselase	0.5%
Hemicellulase	1.0%
D-Mannitol	0.6 M
KH_2PO_4	1.0 mM
NH_4NO_3	5.0 mM
Dithiothreitol (DTT)	2.0 mM
Sodium citrate	5.0 mM
pH	5.8

This enzyme solution is modified from that of Koyama *et al*[13].

Table 2. Effects of IAA, IBA, NAA and 2, 4-D on callus formation in *Quercus acutissima* twigs

	Concentration (μM)				
	0	2.5	5.0	7.5	10.0
IAA	1	4	1	1	2
IBA	1	1	1	3	3
	Concentration (μM)				
	0	0.1	1.0	10.0	100.0
NAA	3	6	7	3	4
2, 4-D	3	4	2	7	7

Ten explants were used for callus formation in each treatment.

observed at 6 hr intervals using an inverted microscope (Olympus CK 2) during the culture.

The viability of the protoplasts was examined with fluorescein diacetate (FDA) staining. One drop of the protoplast suspension was mixed with one drop of acetone solution of FDA (5 mg/m*l*)[16]. The viability of protoplasts were observed with a fluorescence microscope (Olympus BH2-RFK) using excitation with blue light. The excitation filter was an IF490, and an O 530 served the barrier filter.

Results

Induction and culture of callus

In preliminary experiments to induce callus from twigs, more than 50% of the explants were contaminated without peeling treatment. In contrast, callus was induced successfully with low contamination (below 10%) by the peeling treatment of twigs. The media containing NAA or 2, 4-D effectively induced callus as shown in **Fig. 1**, while the media containing IAA or IBA were less effective for callus induction. In particular, the better results were obtained with the medium containing 1 μM of NAA, and 10 or 100 μM of 2, 4-D, as shown in **Table 2**. The results also showed that 2, 4-D induced callus earlier (7 days) than did NAA (15 days). However, all of the media caused browning of callus in the subculture process, except for the medium containing 10 μM of 2,

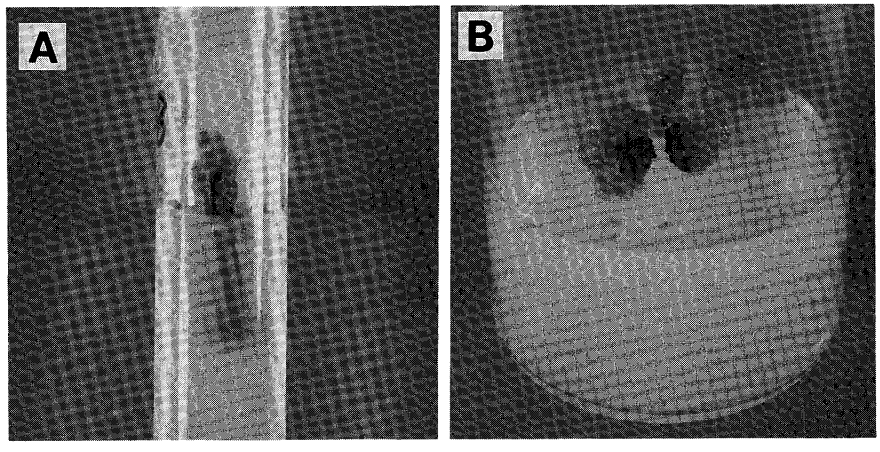

Fig. 1 Callus formation from a peeled twig of *Q. acutissima*. A: Callus formation. B: Subcultured callus.

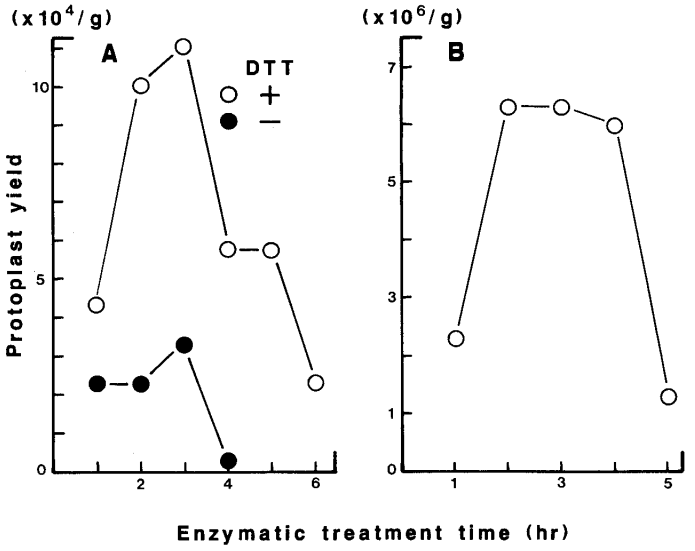

Fig. 2 Effect of DTT addition to the enzyme solution on protoplast yields. A: Leaf. B: Callus.

4-D, indicating that 1/2 MS solid medium containing 10 μM of 2, 4-D is suitable for the culture of callus in *Q. acutissima*. Callus growth in the medium was fairly active and the callus continued to grow over 3 months without browning. However, any indications of differentiation from callus to shoot or root were not observed in the present study.

Isolation of protoplasts

In the pre-experiment, about 3.0×10^4 g^{-1} protoplasts were isolated on average from 0.2 g of leaf tissues as shown in **Fig. 2**. The diameter of protoplasts induced ranged from 20 μm to 40 μm. Since tissues of *Q. acutissima* contain much polyphenolics, the protoplast yield seems to be considerably low compared to that of other species[11]. To obtain a large number of protoplasts, effects of the addition of DTT to the enzyme solutions were examined. The results are shown in **Fig. 2.** In leaf tissues, the protoplast yield was considerably increased by the addition of DTT, particularly in enzymatic treatment time of 2-3 hr. By the addition of DTT to the enzyme solution, protoplasts were isolated more easily from callus tissues than from leaf tissues (**Fig. 2**). The yields of protoplasts from callus tissues were also increased considerably by adding DTT, and were more than 6.0×10^6 g^{-1} (**Fig. 2**) in the enzymatic treatment time of 2-4 hr.

Culture and viability of protoplasts isolated from callus

Vigorous protoplasts exhibited fluorescence of yellow-green under a fluorescence microscope, whereas the damaged protoplasts did not (**Fig. 3**). **Figure 4** shows the influence of enzymatic treatment time on protoplast viability 6 hr after isolating protoplasts from callus. The viability decreased considerably with increasing enzymatic treatment time. After 12 hr from the protoplast isolation, the viability decreased below 50%. One hr treatment gave the highest viability of protoplasts (**Fig. 4**). In the pre-experiment, protoplasts were largely damaged in their preparation from callus, and they tended to lose their viability and died during the short period of culture. However, the addition of DTT to the enzyme solution apparently enhanced the viability of protoplasts, by which the protoplasts were grown actively throughout the culture. After protoplast culture for 24 hr, several masses composed of two cells showing first cell division were observed, and 4-cell-stage cells appeared after 2-days of culture (**Fig. 3**). Then, many micro colonies were observed after 7-days of culture. Furthermore, colony formation was observed within one month. However, callus

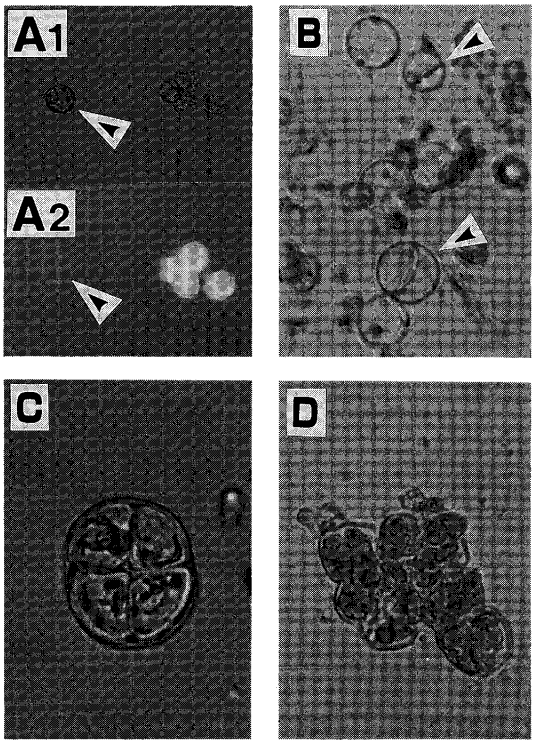

Fig. 3 Protoplast culture. A1: Fresh protoplasts isolated from callus. A2: Fluorescence microphotograph of protoplasts stained with FDA in the same field as A1. Vigorous protoplasts show yellow-green fluorescence. Arrowhead: Damaged protoplast. B: First cell division after 1-day culture (arrowheads). C: Four-cell-stage cell after 2-day culture. D: Micro colony formation after 7-day culture.

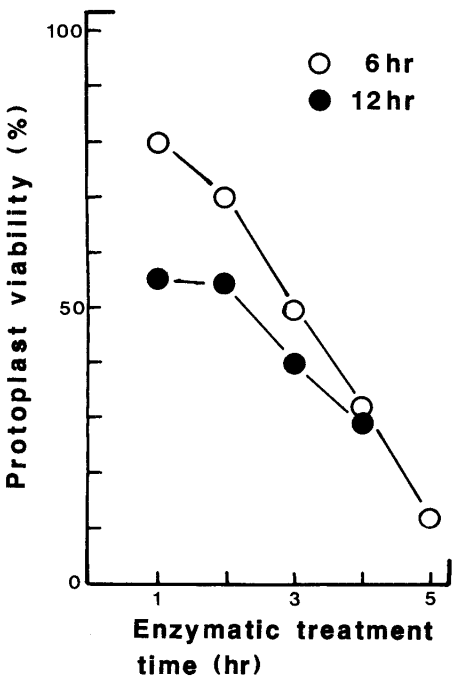

Fig. 4 Influence of enzymatic treatment on the viability of protoplasts isolated from callus.

formation from the colonies was not observed.

Discussion

In general, sterilization of materials obtained from field-grown woody plants is difficult. This difficulty limits callus formation from adult trees of many species. In this experiment, after surface-sterilization the bark was peeled from twigs under aseptic conditions and then the twigs were inoculated in the callus induction medium. Consequently, callus could be induced with almost no contamination from the peeling treatment of twigs. This treatment resulted in shortening the sterilization time to only *ca.* 5 min-sterilization time (70% ethanol for 30 sec and 1% sodium hypochlorite aqueous solution for 5 min). This sterilization time was satisfactory to prevent contamination. Callus was obtained successfully in the media containing 1 μM of NAA, and 10 or 100 μM of 2, 4-D. The growth rate of callus was greater in the media containing 2, 4-D than in those containing NAA. All the media containing NAA and the medium containing a high concentration of 2, 4-D (100 μM) caused browning in many calluses. These results suggest that the medium containing 10 μM of 2, 4-D is suitable for the induction and culture of *Q. acutissima* callus. In order to define the optimal condition, however, further research is needed to examine the effect of 2, 4-D concentration at about 10 μM.

Haraguchi[17] has reported plantlet regeneration from adventitious embryos induced by seed-cotyledon culture in *Q. acutissima*. In the present experiment, however, callus was induced from the current twigs of adult trees. Any adventitious embryos were not induced from the subcultured callus during the prolonged culture periods in this study. It is considered that the induction of adventitious embryos and buds from the callus derived from the twigs is very difficult. Further research is needed to establish the culture conditions for inducing adventitious embryos from callus.

In general, tissue culture of woody plants containing a large amount of polyphenolics appears to be difficult[11]. In this experiment, the polyphenolics also seemed to affect the yield and viability of protoplasts. Although about 3.0×10^4 g^{-1} protoplasts were isolated preliminarily from leaf tissues, high yields of protoplasts (1.0×10^5 g^{-1}) were obtained from leaf tissues by adding DTT to the enzyme solution (**Fig. 2**). Similarly, higher yields of protoplasts were easily obtained from the callus. The isolated protoplasts survived longer in the subsequent culture. *Quercus* species contain large amounts of polyphenolics which inhibit protoplast culture. Within a cell the polyphenolics are oxidized by phenol oxidases, resulting in the formation of quinones which denature proteins and then decrease cell activity[11]. Dithiothreitol used in this experiment had the effect of preventing oxidation of polyphenolics. However, the yield and viability of the protoplasts from leaf tissues were not satisfactory for subsequent protoplast culture. Other improvements are needed to prepare protoplasts from leaf tissues.

An examination of the viability of protoplasts by FDA staining showed that the viability decreased with increase of enzymatic treatment time (**Fig. 4**). This fact is in agreement with those of other woody plants[14]. After 12 hr of protoplast culture, the viabilities of the protoplasts obtained by the enzymatic treatments for 1 and 2 hr decreased below 60%. These results indicate that the enzymatic treatment time of 2 hr, which gave a high yield of protoplasts, is suitable for isolation of protoplasts in *Q. acutissima*. In the culture of protoplast derived from the callus, cell division first occurred after 24 hr and micro colonies were observed after 7 days. Then, colony formation continued over 3-month culture. Thereafter, however, callus induction from the protoplast culture was not obtained.

Protoplasting is an important technique for somatic hybridization by cell fusion and for genetic

improvement by gene transfer. In this study, determining the conditions of protoplast isolation and culture is the first step in transformation research of *Q. acutissima*, although further investigations are still needed to establish the appropriate culture conditions for plantlet regeneration from protoplast-derived callus.

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《和文要約》

クヌギ（*Quercus acutissima* Carruth.）カルスからのプロトプラストの単離と培養

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クヌギカルスからのプロトプラストの単離及び培養を行なった．枝条の剝皮処理によるカルス誘導及び培養を通じて，雑菌による汚染率を10％以下にすることができた．10 μM の 2，4-D を含む 1/2 濃度の MS 培地を用いて，剝皮枝条から多くのカルスを誘導することができた．継代培養の間，カルスは褐変することなく成長を続けた．プロトプラストの調製において，酵素溶液中に DTT を添加することにより，高いプロトプラスト収率が得られ，その後の培養での生存率も増加した．プロトプラストの培養24時間後に最初の細胞分裂が観察され，2日後には4分割した細胞が観察された．7日後には10～50細胞からなる多くの細胞塊が観察された．また，培養開始から1カ月以内にコロニーの形成が認められ3カ月以上にわたって成育を続けたが，カルス形成にまでは至らなかった．