HOKKAIDO UNIVERSITY

Title	Interspecific Heterokaryon Formation between Auricularia auricula-judae and Auricularia polytricha by Electrical Protoplast Fusion
Author(s)	SUNAGAWA, Masahide
Citation	北海道大學農學部 演習林研究報告, 49(2), 219-259
Issue Date	1992-08
Doc URL	http://hdl.handle.net/2115/21359
Type	bulletin (article)
File Information	49(2)_P219-259.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Interspecific Heterokaryon Formation between *Auricularia auricula-judae* and *Auricularia polytricha* by Electrical Protoplast Fusion*

By

Masahide SUNAGAWA**

キクラゲとアラゲキクラゲの電気的プロトプラスト融合によるヘテロカリオンの形成*

砂 川 政 英**

Abstract

Five fusants (Fu 9, 52, 58, 63 and 100) obtained by electrical protoplast fusion after treatment with two different metabolic inhibitors were investigated for mycelial-growth characteristics under various temperatures and on sawdust-medium, in paired cultures, by isozyme analysis and for biochemical test to clarify their characteristics. In addition, restriction endonuclease patterns of mitochondrial DNA of a fusant (Fu 9) which formed small fruiting-bodies, were compared to those of parental strains (*Auricularia auricula-judae* and *A. polytricha*) and momokaryotic strains (Mo A 11 and Mo P 16).

The optimal temperature of 5 fusants was 30℃ in mycelial growth on a PDA medium. Concerning mycelial-growth rate, 5 fusants were similar to the parental and monokaryotic strain, Mo P 16. In regard to mycelial density, all of the 5 fusants were thicker compared to parental and monokaryotic strains.

Five fusants exhibited superior mycelial growth on shirakamba (*Betula platyphylla* var. *japonica Hara*) sawdust-medium as compared to that on other sawdust-media (buna (*Fagus crenata Blume*) and mizunara (*Quercus mongolica* var. *grosseserrata Rehd et Wils*)).

In paired cultures between monokaryotic strains (Mo A 11 and Mo P 16) and 5 fusants, all the fusants formed antagonistic lines against both Mo A 11 and Mo P 16.

All of the 5 fusants were found to possess both specific bands which were present in the monokaryotic strains by analyzing electrophoretic isozyme.

In five fusants enzymic activity of α-galactosidase was observed. On the other hand, phenoloxidase activities were not exhibited by Fu 9, 52, 58, and 63.

Received March 31, 1992 1992年3月31日受理

* This paper is a part of the Doctoral thesis in Agriculture of Hokkaido University.

** Laboratory of Chemical Technology of Forest Products, Department of Forest Products, Faculty of Agriculture, Hokkaido University, Sapporo 060.
北海道大学農学部林産学科林産製造学講座

By using restriction endonuclease analysis, the molecular size of mitochondrial DNA of Fu 9, which had formed small fruiting-bodies, was confirmed to be 90.0 kbp.

From these evidences, it is concluded that all of the 5 fusants are heterokaryon between *A. auricula-judae* and *A. polytricha.*

Key words: *Auricularia auricula-judae, A. polytricha,* protoplast fusion, protoplast, mitochondrial DNA.

Contents

Introduction 221
1. Nature of *A. auricula-judae* and *A. polytricha* 223
 1.1 Materials and Methods 223
  1.1.1 Organisms 223
  1.1.2 Mycelial growth in *A. auricula-judae* and *A. polytricha* under various temperature conditions 223
  1.1.3 Preparation of sawdust-medium 223
  1.1.4 Cultivation of *A. auricula-judae* and *A. polytricha* 223
 1.2 Results and Discussion 224
  1.2.1 Morphological characteristics on *A. auricula-judae* and *A. polytricha* 224
  1.2.2 Mycelial growth under various temperature conditions 224
  1.2.3 Mycelial growth on sawdust-media 227
  1.2.4 Fruiting-bodies formation of *A. auricula-judae* and *A. polytricha* 228
2. Isolation and reversion of protoplasts from mycelia of *A. auricula-judae* and *A. polytricha* 229
 2.1 Materials and Methods 229
  2.1.1 Isolation of protoplasts from mycelia 229
  2.1.2 Regeneration and reversion of protoplasts 229
 2.2 Results and Discussion 230
  2.2.1 Effects of mycolytic enzyme on isolation of protoplasts 230
  2.2.2 Effects of culture period on isolation of protoplasts 230
  2.2.3 Effects of culture period on reversion of protoplasts 232
  2.2.4 Effects of regeneration media on reversion of protoplasts 233
3. Protoplast fusion between *A. auricula-judae* and *A. polytricha* 234
 3.1 Materials and Methods 234
  3.1.1 Treatment of metabolic inhibitors 234
  3.1.2 Protoplast fusion 234
 3.2 Results and Discussion 234
  3.2.1 Effects of protoplasts treated with metabolic inhibitors 235
  3.2.2 Effects of the pulse length and the field strength of DC pulse on electrical protoplast fusion 238
  3.2.3 Interspecific heterokaryon obtained by electrical protoplast fusion 239
4. Nature of fusants 240
 4.1 Materials and Methods 240
  4.1.1 Organisms 240
  4.1.2 Mycelial growth undedr various terpemature conditions 240

4.1.3 Preparation of sawdust-medium 240
4.1.4 Paired culture 240
4.1.5 Isozyme analysis 241
4.1.6 Biochemical tests 241
4.1.7 Formation of fruiting-bodies 241
4.1.8 Preparation of mitochondria and mitochondrial DNA 241
4.2 Results and Discussion 243
4.2.1 Comparison of mycelial growth under various temperature conditions 243
4.2.2 Mycelial growth on sawdust-media 245
4.2.3 Antagonism in paired culture between fusants and monokaryotic strains 246
4.2.4 Isozyme analysis of monokaryotic strains and fusants 246
4.2.5 Biochemical characterization 248
4.2.6 Fruiting-bodies formation 248
4.2.7 Restriction endonuclease analysis of mitochondrial DNA 249
Conclusion 252
Acknowledgement 253
References 253

Abbreviation: GMY: 1% Malt extract, 0.4% Yeast extract, LMY: 1% Lignin, 1% Mait extract, 0.4% Yeast extract, PCMY: 1% Polypeptone, 0.2% Casamino acid, 1% Malt extract, 0.4% Yeast extract, PDA: 20% Potato decoction, 1% Dextrose, 1.5% agar, PDMA: 20% Potato decoction, 1% Dextrose, 1% Malt extract, 1.5% Agar, PMY: 1% Polypeptone, 1% Malt extract, 0.4% Yeast extract, SMY: 1% Sucrose, 1% Malt extract, 0.4% Yeast extract, StMY: 1% Sucrose, 1% Starch, 1% Malt extract, 0.4% Yeast extract.

Introduction

Edible fungi, that is, mushrooms which are essential to the Jappanese diet in particular, are an important food source. Many types of mushrooms have also been noted as natural and dietetic foods. In recent years, concerning the medicinal effects of mushrooms, many studies have been carried out utilizing several mushroom types for their various preventive and therapeutic effects on major illness such as cancer viral infections and for the purpose of decreasing cholesterol in the blood. Characteristic effects have been clearly confirmed by studies on the oral administration of *Agaricus bisporas* and *Lentinus edodes* to mice (NANBA and others, 1988; KURASHIGE, 1988). Additionally, the mushrooms of *Auricularia* species, *A. auricula-judae* and *A. polytricha,* contain many nutrients, which have been used as herbal remedies since ancient times.

However, recently several problems have hindered research cultivation: lack of log-bed and sawdust of hardwood, such as kunugi and konara used for cultivation of *Lentinus edodes* and injury by pathogenic micro-organism, such as *Trichoderma* species. On the other hand, the cultivation of mycorrhizal mushrooms, *Tricholoma matsutake* and *Lyophyllum aggregatume,* yield good results, but those reports are not yet forthcoming.

Under the circumstances, application of biotechnology, such as cell fusion, genetic transfer and genetic recombination was expected to solve several of the problems as described above. For example, *L. edodes* will be able to cultivate by using softwoods,

such as sugi and hinoki instead of hardwood. Moreover, fungal genetic improvement and more successful and prolific breeding are possible by establishing those techniques.

Protoplasts are becoming increasingly important to achieve cell fusion in fungal genetics and breeding. Therefore, many investigators have investigated isolation, reversion and fusion of fungal protoplasts in relation to fungal genetics (ANNE and PEBERDY, 1975, 1976; DALES and CROFT, 1977; FOURNIER and others, 1977; HAMLYN and BALL, 1979; PEBERDY, 1979; HOPWOOD, 1981; YOKONO and others, 1988; TAMAI and others, 1988; MIURA, 1989; SUNAGAWA and others, 1989). PEBERDY and others (1976) revealed that the condition for protoplast isolation was affected by the nature and the molarity of osmotic stabilizers being used the pH of the mycolytic enzyme, and the period of lytic digestion mixture. Additionally, cell wall regeneration of fungal protoplasts has been investigated in various groups of fungi, such as *Saccharomyces cerevisiae* (NECAS, 1966; SOVODA and NECAS, 1966), *Geotrichum candidum* (DOOIJEWSSRD-kloosteriel and others, 1973), *Candida utilis* (ASES-Ledied and GARCIA-Mendoza, 1970), *Schizophyllum commune* (de VRIES and WESSELS, 1975), *Fusarium culmorus* (GARCIA and others, 1966), *Aspergillus nidulans* (PEBERDY and BUCKLEY, 1973), *Penicillium chrysogenum* (ANNA and others, 1974) and *Trichoderma viride* (BENITEZ and others, 1975). PEBERDY and GIBSON (1971) reported morphological changes of the hypha which occur during protoplast reversion in *Aspergillus nidulans,* and pointed out that the reversion frequency of the protoplasts was associated with the protoplast releasing sites of the parent hypha.

In order to obtain heterokaryons by protoplast fusion, it is necessary to eliminate the cells of each parental strains which have no fused and of homokaryons. However, most of the investigations were carried out using mutagenesis of cells lines which requires specific nutrients (FERENCZY and others, 1976; KEVEI and PEBERDY, 1977; GENTHNER and BORGIA, 1978; GOLD and others, 1983; HASHIBA and YAMADA, 1984; TOYOMASU and MORI, 1987; OHBA and others, 1988; YOKONO and others, 1988; SUNAGAWA and others, 1991). However, these techniques have several drawbacks. It is often difficult and time-consuming to introduce a specific marker into a given cell line, or to obtain lines that do not revert to the wild phenotype. On the other hand, iodoacetic acid sodium salt (IAS) and diethyl pyrocarbonate (DP), which have been used in these experiments, are metabolic inhibitors irreversibly affecting cell metabolism and are expected to allow selection of heterokaryons when performing protoplast fusion without the necessity of introducing heritable markers. From a result, fused heterothallic protoplast can survive by complementation of protoplasts which were treated with metabolic inhibitors.

In the present study, the author describes interspecific electrical protoplast fusion between *A. auricula-judae* and *A. polytricha* using two different metabolic inhibitors. The interspecific heterokaryons produced were investigated to clarify their characteristics.

1. Nature of *A. auricula-judae* and *A. polytricha*

1. 1 Materials and Methods

1. 1. 1 Organisms

The two parental strains, *A. auricula-judae* (Fr.) Quél. and *A. polytricha* (Mont.) Sacc., used in the present experiments were obtained respectively from Hokkaido Forest Products Research Institute, and Tottori Mycological Institute. Both strains were maintained on a PDA medium at 26℃.

1. 1. 2 Mycelial growth in *A. auriulaa-judae* and *A. polytricha* uderr various temperature conditions

The parental strains, *A. auricula-judae* and *A. polytricha,* were precultured on the PDA medium in Petri dishes (8.5 cm in diameter) for 7-14 days at 26℃. The mycelial tips of each strain were punched out with a cork borer 5mm in diameter, and the mycelial disks were then put in the center of the PDA medium in the Petri dishes. These strains were incubated under temperature conditions of 5, 10, 15, 20, 25, 30, 35, and 40℃ for 30 days. The diameter of the mycelia grown was measured in two directions perpendicular to each other at intervals of 2 days. I calculated the average value of five-replicate plates of each strain, and then compared the rate of increase of this average value between strains.

1. 1. 3 Preparation of sawdust-medium

I used sawdust from mizunara (*Quercus mongolica* var. *grosseserrata Rehd et Wils*), shirakamba (*Betula platyphylla* var. *japonica Hara*) and buna (*Fagus crenata Blume*) as the sawdust-medium to observe mycelial growth in *A. auricula-judae* and *A. polytricha.* The sawdust was obtained from a lumbermill in Esashi Town. It was passed through a sieve with a pore size of 10 meshes before use. Sieved sawdust was adjusted with subterranean water to a hydrous rate of about 70%. Portions of the sawdust-medium of 25 g were put in a Petri dish (8.5 cm in diameter), and then autoclaved at 121℃ under a 1.2 atmospheric pressure for 30 min. Parts of the mycelial tips of *A. auricula-judae* and *A. polytricha,* which had been subcultured on a PDA medium for 7-14 days at 26℃, were punched out with a corkborer (5 mm in diameter) and were then inoculated in the center of the sawdust-media in Petri dishes.

To examine effects of rice bran and bran as an additive, I inoculated *A. auricula-judae* and *A. polytricha* into the sawdust-media with a ratio of 4 : 1 (v/v) for sawdust and additive, according to the methods described above.

The diameter of the mycelia grown on the sawdust-medium was measured in two directions perpendicular to each other at intervals of 2 days. I calculated the average values of five-replicate plates of both strains, and compared the rate of increase of these average values among the strains grown on each of the sawdust-media.

1. 1. 4 Cultivation of *A. auricula-judae* and *A. polytricha*

Sawdust from shirakamba (*Betula platyphylla* var. *japonica Hara*) was used for the cultivation of *A. auricula-judae* and *A. polytricha.* The sawdust was passed through a sieve (pore size of 10 meshes) before a lumb of wheat bran was added as an additive. The ratio of sawdust to bran in the cultivation-medium was 4 : 1 (v/v). The hydrous

rate of the medium was adjusted to about 70%. The medium was autoclaved at 121℃ under a 1.2 atmosphere for 90 min. After autoclaving, liquid inoculum of 5 m*l* was inoculated in a hole in the medium. The liquid inoculum was obtained as follows: both strains were cultured in 40 m*l* of SMY liquid medium, shaken in 30 pieces of 5 mm glass beads in diameter at 2 day intervals for 10-20 days at 26℃. The cultivation -medium was then incubated for 40-50 days at 26℃ in R. H. of 70% in the dark. After incubation, the medium spread with mycelium in the culture bag was transferred to the fruiting room for forming fruiting-bodies. Room conditions of the room were controlled at temperatures of 15℃ (temperature of fruiting for *A. auricula-judae*) and 25℃ (temperature of fruiting for *A. polytricha*) in R. H. of 90% and lights on (200 lux).

1. 2 Results and Discussion

1. 2. 1 Morphological characteristics on *A.auricula-judae* and *A. polytricha*

Auricularia auricula-judae and *A. polytricha* are the two most popular edible fungi of the *Auricularia* genus. Figure 1 shows the *A. auricula-judae* and *A. polytricha* used in all the experiments. *Auricularia* is one of the most important mushrooms found in Japan. The fruiting-bodies of the mushroom contain many nutrients, which have been used as a herb medicine for hundreds of years. Among the *Auricularia* mushrooms, *A. auricula-judae* is a mushroom with a northern origin (temperature of fruiting; 10-15℃) and its commercial value is relatively high, while *A. polytricha* with a southern origin (temperature of fruiting; 25-30℃) is widely distributed in the subtropical zone. Both strains are commonly cultivated in bed logs and the sawdust of various trees, but *A. polytricha* can develop fruiting-bodies in a short time compared to *A. auricula-judae.*

The morphological characteristics of *A. auricula-judae* and *A. polytricha* are as follows:

Auricularia auricula-judae

Basidiocarp tough-gelatinous; single, gregarious, or caespitose; erect and foliaceous, slightly ear-or shell-shaped; yellow-brown to reddish-brown when fresh, yellow-brown to olive-brown when dry; sessile to sub-stipitate; up to 12 cm broad, 0.8 -1.2 mm thick; cyphae 2-3 μm in diameter; spores 10−15×5−6 μm, allantoid.

Auricularia polytricha

Basidiocarp leathery gelatinous; cup-or ear-shapes with a strongly convex dorsal surface; one or several lobes, up to 10 cm wide, 1.0-1.5 mm thick; red-brown when fresh, light gray or tan when dry; sessile or slightly stipitate; hyphae 2-3 μm in diameter; spores 12−17×5−6 μm, allantoid.

This species grows on dead wood of various kinds, such as *Acacia confusa* Murr., *Ficus retusa* L., *F. carica* L., *Citirus unshu* Mare., *C. tankan* Hay., and *Albizzia* sp..

1. 2. 2 Mycelial growth under various temperature conditions

The mycelial growth of *A. auricula-judae* and *A. polytricha* was compared under various temperature conditions on the PDA medium. Both strains showed similar patterns under each temperature condition, that is, *A. auricula-judae* and *A. polytricha* had an optimal temperature of 25-30℃ for mycelial growth on 12-day-old culture, as shown

Fig. 1 *Auriculraria auricula-judae* (A) and *A. polytricha* (B) used in the present study.

in Fig. 2. A Similar result was obtained by HIROE (1982). He have already reported that an optimal temperature of mycelial growth for *A. auricula-judae* and *A. polytricha* was 20-34℃. KINJO and KONDO (1979) have reported mycelial growth of *A. polytricha* under several temperature conditions on a OSA (Onion-Sucrose-Agar). They found that the mycelium of *A. auricula-judae* was able to grow under temperature within the

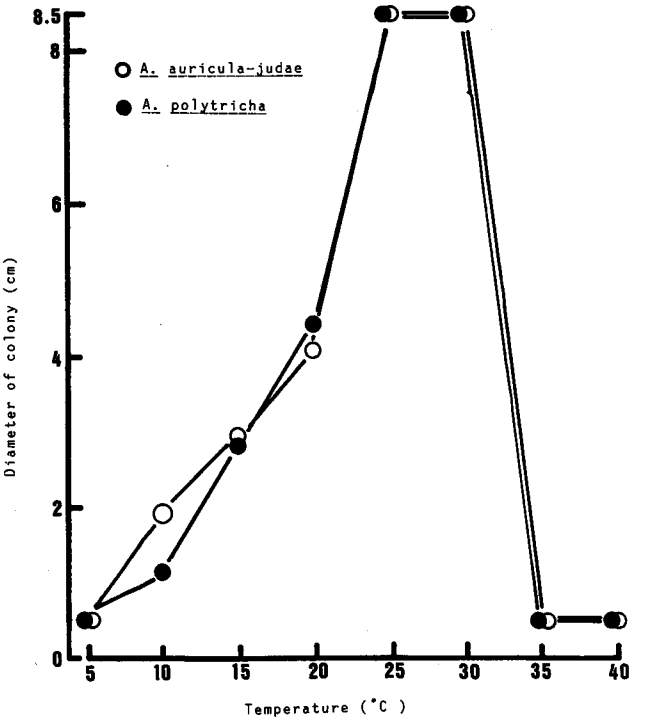

Fig. 2 Mycelial growth of *A. auricula-judae* and *A. polytricha* in each temperature on 12-old-day culture on a PDA medium.

range of 10℃ to 35℃ and that the mycelial growth was good at 30℃. This indicates that the results obtained here are similar to those reported in their study. Neither *A. auricula-judae* nor *A. polytricha* showed any growth at temperatures of 5, 35, and 40℃, as Hiroe also reported (1982).

Both strains also showed similar patterns of mycelial-growth rate on a 12-day-old culture.

Table 1 Mycelial growth of *A. auricula-judae* and *A. polytricha* in each sawdust-medium on 28-day-old culture

Sawdust-medium	Diameter of colony (cm)	
	A. auricula-judae	*A. polytricha*
Buna	0.6	7.5
Shirakamba	7.0	8.5
Mizunara	0.0	0.0

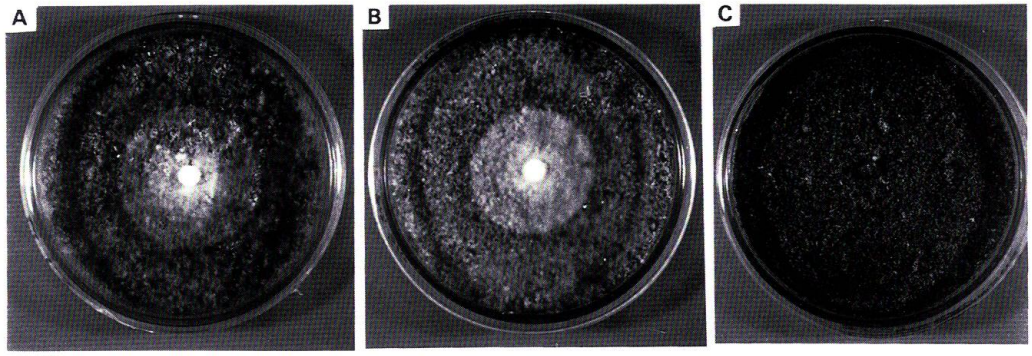

Fig. 3 Mycelial growth of *A. auricula-judae* in each sawdust-medium on 20-day-old culture.
Legend: A: Sawdust-medium used with buna sawdust.
B: Sawdust-medium used with shirakamba sawdust.
C: Sawdust-medium used with mizunara sawdust.

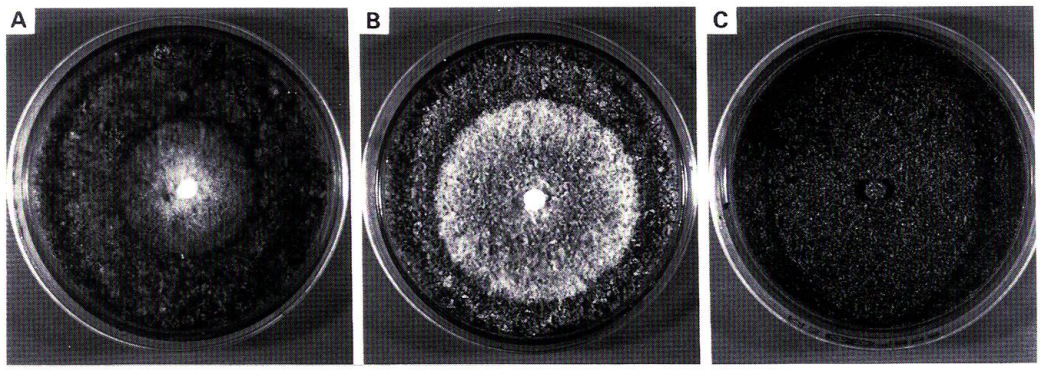

Fig. 4 Mycelial growth of *A. polytricha* in each sawdust-medium on 20-day-old culture.
Legend: A: Sawdust-medium used with buna sawdust.
B: Sawdust-medium used with shirakamba sawdust.
C: Sawdust-medium used with mizunara sawdust.

1. 2. 3 Mycelial growth on the sawdust-media

I compared the mycelial growth of *A. auricula-judae* and *A. polytricha* in the various sawdust-media collected from buna, shirakamba and mizunara. The results are shown in Table 1. Shirakamba-sawdust medium gave superior mycelial growth for both *A. auricula-judae* and *A. polytricha* when compared with the other sawdust-media. The sawdust-medium from buna also gave relatively superior mycelial growth for *A.*

Table 2 Mycelial growth of *A. auricula-judae* and *A. polytricha* on shirakamba sawdust-medium with additives on 12-day-old culture

Additive	Diameter of colony (cm)	
	A. auricula-judae	*A. polytricha*
Rice bran	4.1	6.4
Bran	6.4	8.5

polttricha. With the sawdust-medium from mizunara, however, no mycelial growth for either strain was observed, even for 28-day-old culture. In both strains, the mycelial colony grown on shirakamba sawdust-medium was thicker than that grown on buna, as shown in Figures 3 and 4. This fact may be useful for the cultivation of both *A. auricula-judae* and *A. polytricha.*

In order to investigate effects of additives, I used the shirakamba sawdust-medium, which gave the greatest mycelial growth described above. Table 2 shows the mycelial growth of *A. auricula-judae* and *A. polytricha* on shirakamba sawdust-media with rice bran and bran as additives. The shirakamba sawdust-medium with bran added induced greater mycelial growth of *A. auricula-judae* and *A. polytricha* than the medium with rice bran added. The addition of additive to the sawdust-medium promoted mycelial growth of both *A. auricula-judae* and *A. polytricha.* In general, the effects of additives, such as rice bran and bran, have been reported by many investigators (IWADE, 1958; KINJO and KONDO, 1979; NAKAMURA, 1982; KINJO and others, 1987).

1. 2. 4 Fruiting-bodies formation of *A. aurdcula-judae* and *A. polytricha*

I used shirakamba sawdust-medium with bran as a cultivation medium, which gave superior mycelial growth of *A. auricula-judae* and *A. polytricha* for fruiting-bodies formation of both strains. *Auricularia auricula-judae* required an incubation period of 50

Table 3 Fromation of fruiting-bodies using shirakamba sawdust-medium with bran for *A. auricula-judae* and *A. polytricha*

Strains	Incubation period of mycelium (days)	Days required for fruiting-bodies formation (days)	Yield of fruiting-bodies fresh weight/bag (g)
A. auricula-judae	50	30	196
A. polytricha	40	24	246

days until covered with mycelium in the culture bag, as shown in Table 3. On the other hand, *A. polytricha* required 40 days to achieve the mycelium spread. This fact indicates that the mycelial-growth rate of *A. polytricha* was relatively faster than that of *A. auricula-judae* (Tables 1 and 2). After mycelium had spread in the culture bag, *A. auricula-judae* required 30 days to form fruiting-bodies, while *A. polytricha* required 24 days; this suggests that the fruiting-bodies of *A. polytricha* can form easily compared to those of *A. auricula-judae. Auricularia auricula-judae* take 80 days until fruiting-bodies formation after inoculation of its liquid inoculum, whereas *A. polytricha* takes 60 days. These facts suggest that *A. polytricha* can be cultivated relative easily compared with *A. auricula-judae.*

Auricularia polytricha (246 g) had a higher yield of fruiting-bodies than *A. auricula-judae* (196 g). The fruiting-bodies of both *A. auricula-judae* and *A. polytricha* can gave yield of 5 times in a cultivation-medium. KINJO and YAGA (1988) have reported that the yield of fruiting-bodies in *A. polytricha* was 251.6 g, using cultivation -medium of baggasse with bran. The result in this experiment was similar to theirs.

2. Isolation and reversion of protoplasts from mycelia of *Auricularia auricula-judae* and *Auricularia polytricha*

2. 1 Materials and Methods

2. 1. 1 Isolation of protoplasts from mycelia

The mycelia of *A. auricula-judae* and *A. polytricha* grown on the PDA slant medium were preincubated into 40 m*l* of SMY liquid medium in a 100 m*l* Erlenmyer flask containing about 30 pieces of 5 mm glass beads in diameter for fragmenting the mycelia. Cultures were incubated with shaking at intervals of 2 days for 7-10 days at 26℃. Five m*l* of the cultures containing the fragmented mycelia were re-inoculated into 40 m*l* of SMY liquid medium, then incubated for 2-10 days at 26℃. Mycelia were collected by filtration with a miracloth (Calbiochem. Co., Ltd.) and washed several times with a buffer solution of 0.05 M MES (pH 5.3) containing $MgSO_4$ as osmotic stabilizers. The mycelia were then incubated in the same buffer containing mycolytic enzymes, with reciprocal shaking (60 reciprocation/min) at 29℃. After incubation for 2 hrs, the remaining mycelia were removed by filtration through a nylon cloth (mesh opening 10 μm, Nippon Rikagaku Kikai).

The yield of the protoplasts was determined by counting the number of protoplasts using a haemocytometer (Thoma counting chamber) after removal of the mycelial debris by filtration and centrifugation. The counting was done three times.

2. 1. 2 Regeneration and reversion of protoplasts

Protoplasts aseptically isolated for 2 hrs digestion with mycolytic enzyme solution were passed through a nylon cloth (mesh opening 10 μm). To purify protoplasts, the filltrates were washed with MES buffer three times by centrifugation (700×g) for 5 min. Suspension of protoplasts was diluted to the concentration of 2×10^5/m*l* with a buffer (0.5 M $MgSO_4$ and 0.05 M MES, pH 5.3). The diluted protoplast suspension (0.1 m*l*) was plated on 1.5% agar media containing various nutritional supplements and incubated at 26℃. The number of colonies, which developed on plates after 10-20 days incubation, was counted on three plates.

The reversion frequency was estimated with the following equation:

Table 4 Effects of enzyme combinations on yields of isolated protoplasts

Combination of enzymes	The number of protoplasts (×10^7/m*l*)	
	A. auricula-judae	*A. polytricha*
N	2.5	3.8
N+Driselase 2%	2.7	3.9
N+Chitinase 0.1%	5.3	7.2
C+Driselase 2%	0.03	0.04
C+Chitinase 0.1%	0.03	0.08
C+Zymolyase 20T 0.2%	0.01	0.01
C+β-Glucronidase 0.05m*l*/m*l*	0.02	0.05
C+Driselase 2%+Zymolyase 20T 0.2%	0.01	0.03

Notes: N : Novozym 234 1%
C : Cellulase "Onozuka" RS 2%

Reversion frequency (%) = (number of colonies formed on a plate/number of protoplasts spread on a plate) × 100.

2. 2 Results and Discussion

2. 2. 1 Effects of mycolytic enzyme on isolation of protoplast

It is well known that combination of mycolytic enzyme affects on the yield of protoplast isolation (MOORE, 1975; TOYODA and others, 1984; YANAGI and TAKEBE, 1984; YANAGI and others, 1985; KITAMOTO and others, 1987). Since the growth of aerial hypha became significant after 7-day-old culture in both *A. auricula-judae* and *A. polytricha,* mycelia of 4- and 6-day-old culture were, respectively, used as the source of protoplasts. This is because isolation of protoplasts from aerial hypha is difficult. The effects of the culture period on the protoplast isolation will be described in the next section. Various enzymes were used singly and in combinations for protoplast isolation from mycelia. The results are shown in Table 4. Verious combination of cellulase "Onozuka" RS (cellulase) and another enzymes were reported to be effective for the isolation of protoplasts from mycelium of various fungi (ARIMA and TAKANO, 1979; ISHIZAKI and others, 1983; OHMASA and others, 1983; GUNGE, 1984; AKAHANE and others, 1986; TOYOMASU and others, 1986). In both *A. auricula-judae* and *A. polytricha,* however, all combinations of cellulase gave poor yields of protoplasts. On the other hand, the Novozym 234 systems gave considerably larger yields of protoplasts compared to the cellulase systems. A combination of Novozym 234 and Driselase gave almost the same yield of protoplasts as Novozym 234 alone. The maximal yield of protoplasts was obtained using a combination of Novozym 234 and chitinase, suggesting that Novozym 234 system is relatively suitable for the protoplast isolation from *Auricularia* mycelium. HAMLYN and others (1981) also reported similar effects of Novozym 234 for *Penicillium chrysogenum.* As shown in Table 4, addition of chitinase to Novozym 234 was found to be effective for yield of protoplasts compared to the single application of Novozym 234. However, a mixture of cellulase and chitinase merely gave poor yield. Enzyme system containing cellulase could not easily digest the cell wall components of both strains. Cellulase is considered to be ineffective for digestion of the cell wall, when used for the protoplast isolation from the mycelia of *Auricularia* fungi. This fact is also true for *Lentinus edodes,* as reported by USHIYAMA (1983).

Different effects of enzyme systems on yields of protoplasts were found among species in the experiments. For example, the Novozym 234 systems gave smaller yields in *A. auricula-judae* than in *A. polytricha.*

Some investigators have reported that the yield of protoplasts is affected not only by the enzyme system but also by the concentration and treatment time of enzyme used (de VRIES and WESSELS, 1972 and 1973; ANNE and others, 1974; BOS and SIAKHORST, 1980; AKAMATSU and others, 1983; TOYODA and others, 1984; YANAGI and TAKEBE, 1984).

2. 2. 2 Effect of culture period on isolation of protoplasts

Different culture periods of mycelia also influenced protoplast isolation. Figure 5

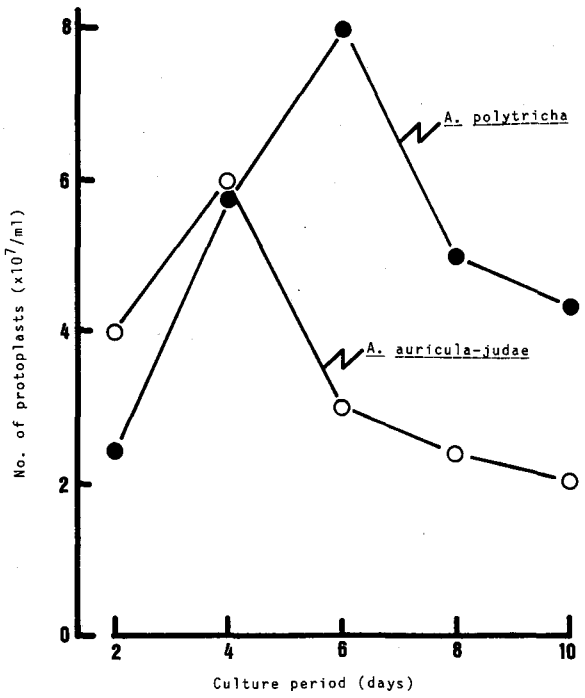

Fig. 5 Protoplast isolation from mycelia of *A. auricula-judae* and *A. polytricha* cultured for different periods.

indicates that the yields of protoplasts are affected strongly by the culture period. The number of protoplasts increased with the increase in the period of culture up to 4 days in *A. auricula-judae* and to 6 days in *A. polytricha*. After reaching the maximum, the yields of protoplasts decreased. The similar effect of culture period has been reported for other fungi (de VRIES and WESSELS, 1972; PEBERDY and others, 1976; YANAGI and others, 1985; OHMASA and others, 1987; KAWASUMI and others, 1987). De VRIES and WESSELS (1972) and PEBERDY and others (1976) reported that the largest yields of protoplasts were obtained from mycelia in fresh cultures (2-3 days). In this experiment, however, the culture period of mycelia for giving the greatest yield of protoplasts was slightly longer than those of other fungi. The effect of the culture period on the number of protoplasts may be different among fungi. The reason for the effects of culture period on the number of protoplasts is unknown. It may be associated with certain changes in the hyphal-wall components. In fact, the aerial hyphae appear to be relatively resistant to digestion by the mycolytic enzymes (de VRIES and WESSELS, 1972; YANAGI and others, 1985; OHMASA and others, 1987). ZONNEVELD (1972) suggested that the deposition of α-1, 3-glucan in an outer-wall layer in the old hyphae enhances resistance to the attack by mycolytic enzymes. In the strains used here, the aerial hyphae became predominant in the 7-day-old culture and the yield of protoplasts greatly decreased when the mycelia grew for more than 6 days, suggesting the increase

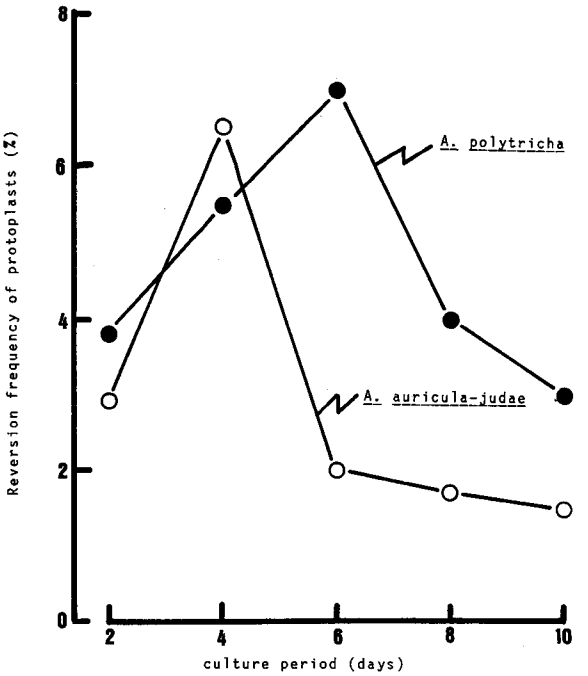

Fig. 6 Effects of culture period of *A. auricula-judae* and *A. polytricha* on protoplast reversion.

in resistance of the cell walls to the mycolytic attack. NISHIGUCHI and others (1987) have reported with *Pleurotus ostreatus* and *Pleurotus citrinopileatus* that the mycelia from 3- to 7-day-old culture gave the high yields of protoplast isolation. The high density of hyphae produced in 13-day-old culture caused the cell wall connection between adjacent hyphae (NISHIGUCHI and others, 1987), suggesting the decrease in releasing protoplasts from filamentous fungi.

2. 2. 3 Effects of culture period on reversion of protoplasts

Protoplasts isolated from different culture periods influenced their reversion on PCMY medium as regeneration media. Figure 6 indicates that the reversion frequency of protoplasts are affected strongly by culture period. In *A. polytricha,* the reversion frequency of protoplasts increased with the increase in the period of culture up to 6 days, whereas they were maximum at 4 days in *A. auricula-judae.* After reaching maximum, the frequencies decreased. KAWASUMI and others (1987) have been reported with *Lentinus edodes* that young mycelia (3-day-old culture) showed a higher regeneration frequency than old mycelia (5-day-old culture). They found that a number of reversion colonies from *L. edodes* on GMY agar medium containing 0.4% Sanpearl CP and 0.6 M sucrose were obtained up to 4 days, which clearly decreased after 5 days. The report of them is in accordance with the results for *A. auricula-judae.* The result with *A. polytricha,* however, the culture period of mycelia for giving the greatest reversion frequency of protoplasts was slightly longer than that with *A. auricula-judae.*

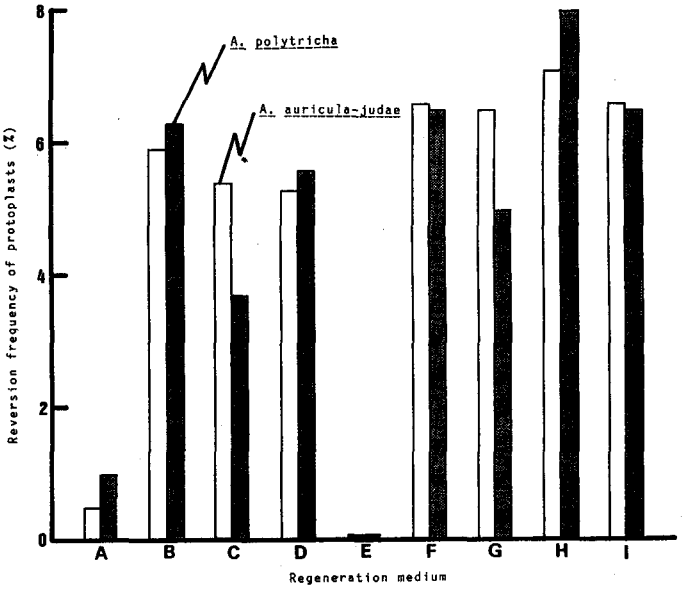

Fig. 7 Effects of regeneration media on the protoplast reversion.
Legend: A: PD B: MY C: PDMY D: GMY
E: LMY F: PMY G: SMY H: StMY I: PCMY

These facts indicate that culture period of mycelia is different among fungi and factors affecting the formation of the reversion colonies from the protoplasts.

2. 2. 4 Effects of regeneration media on reversion of protoplasts

Several regeneration media were examined for the development of the isolated protoplasts. Figure 7 shows the effect of media used for the formation of reversion colonies on reversion frequencies of protoplasts. In both *A. auricula-judae* and *A. polytricha,* a number of reversion colonies formed on regeneration media containing MY combination, expect for LMY medium. In particular, the maximal reversion frequency of protoplasts was obtained when starch was added to the MY for both strains. The smallest reversion frequency of protoplasts was obtained in case of using LMY medium for both strains. MOORE and PEBERDY (1976) have been reported that composition of regeneration media affects activity of cell-wall sythetase, and also frequency and rate of reversion. In fact, the StMY medium used in this experiments which gave maximal frequency of the reversion colonies is brought good result as regeneration medium for both *A. auricula-judae* and *A. polytricha.* It is reported that addition of *N*-acetylglucosamine to the regeneration medium stimulated the reversion of protoplasts isolated from *Coprinus macrorhizus* (YANAGI and others, 1985) and *Pleurotus ostreatus* (OHMASA and others, 1987). This fact suggests that *N*-acetylglucosamine has an effeet of promoting synthesis of the cell wall components of basidiomycetes. In this experiment, however, the regeneration of protoplasts could not be improved significantly by adding *N*-acetylglucosamine to the StMY medium in both *A. auricula-judae* and *A. polytricha.*

3. Protoplast fusion between *A. auricula-judae* and *A. polytricha*

3. 1 Materials and Methods

3. 1. 1 Treatment of metabolic inhibitors

In order to select hybrid cells by protoplast fusion, both monokaryotic strains, named Mo A 11 isolated from *A. auricula-judae* and Mo P 16 isolated from *A. polytricha,* were treated with two different metabolic inhibitors, iodoacetic acid sodium salt (IAS) and diethyl pyrocarbonate (DP), respectively. Protoplasts of both monokaryotic strains (Mo A 11 and Mo P 16) were isolated as described in the previous section 2. 2. 1 and counted with a haemocytometer. The protoplasts isolated from Mo A 11 and Mo P 16 were respectively suspended in IAS and DP solutions containing 0.05 M MES buffer (pH 5.3) and 0.5 M Mannitol. Treatments of metabolic inhibitors were performed in the final concentrations between 0.1 and 10%. After treatment for an hour at 4℃, each protoplast was washed three times by centrifugation with a solution of 0.5 M mannitol and 50 mM $CaCl_2$, and then suspended in 0.5 M mannitol, 0.5 mM $MgCl_2$, and 0.1 mM $CaCl_2$. Protoplasts of each suspension were counted using a haemocytometer to examine the broken frequency of protoplasts and spread on a StMY medium containing 1.5% agar to investigate the reversion frequency of the protoplasts. The suspensions of each protoplast were used for electrical protoplast fusion.

The broken frequency of protoplasts was estimated with the following equation:

Broken frequency(%)=(number of protoplasts after metabolic-inhibitor treatment/number of protoplasts before metabolic-inhibitor treatment)×100.

The reversion frequency of prtotoplasts was determined as described in the previous section 2. 1. 2.

3. 1. 2 Protoplast fusion

Both protoplasts of Mo A 11 and Mo P 16 which were treated with metabolic inhibitors as described above were used for electrical protoplast fusion. The protoplasts were suspended in 0.5 M mannitol, 0.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$ and each of protoplast suspensions were then mixed. Fusion apparatus used in the experiment were shown in Fig. 8. For protoplast fusion, 10 μl of the suspension was put in a fusion chamber. Protoplasts were aligned in chains by dielectrophoresis in an AC-field strength with 2 MHz and 500 V/cm. Protoplast fusion was initiated by the application of a DC pulse of 10-30 μsec, with a field strength of 3 and 4 kV/cm. After the application of the DC pulse, the AC field was gradually decreased to zero over a period of 30 sec. Then protoplast suspension in the fusion chamber was plated on a StMY medium. Reversion colonies were picked up from the StMY medium containing 0.4 M sucrose as osmotic stabilizer after protoplast fusion and maintained on a PDA medium at 26℃ until used next.

The fusion frequency was estimated with the following equation.

Fusion frequency (%)=(number of colonies on the StMY medium/number of protoplasts spread on StMY medium)×100.

3. 2 Results and Discussion

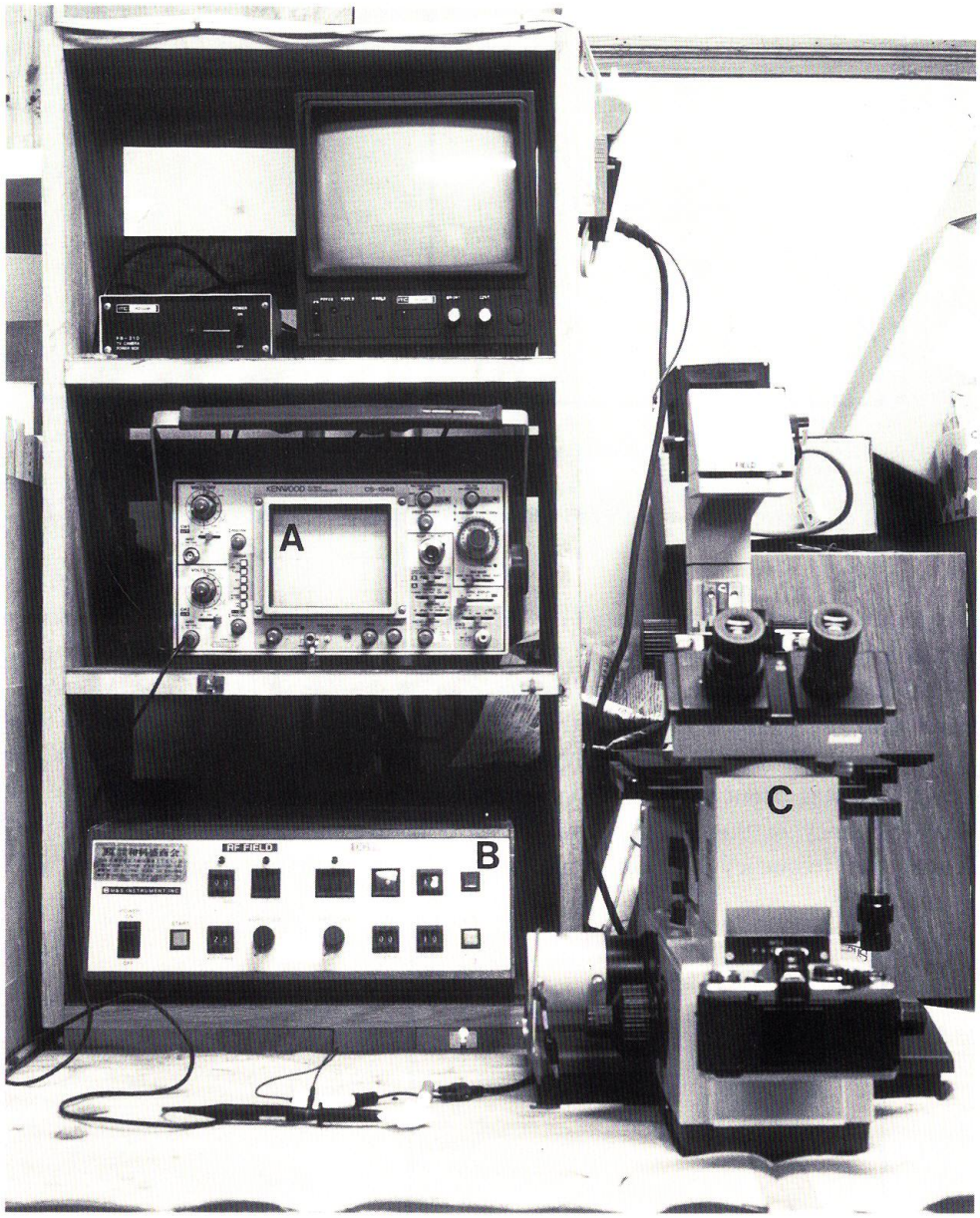

Fig. 8 System of the apparatus for electrically induced protoplast fusion.
Legend : A : Oscilloscope
B : Fusion controller
C : Inverted light microscope

3. 2. 1 Effects of protoplasts treated with metabolic inhibitors

Protoplasts isolated from monokaryotic strains (Mo A 11 and Mo P 16) were treated with a lethal dose of different inhibitors, IAS and DP, respectively, irreversibly affecting cell metabolism. Several concentrations were examined with broken and

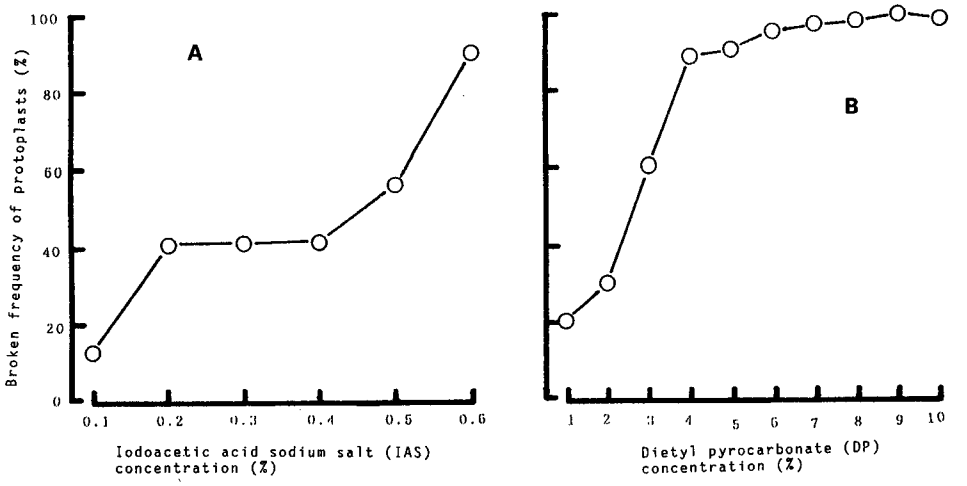

Fig. 9 Effects of concentrations of IAS and DP on broken frequency of protoplasts for Mo A 11 (A) and Mo P 16 (B).

reversion frequencies of protoplasts to select fusants from the StMY medium before the protoplast fusion was performed.

The broken frequencies of both protoplasts of the Mo A 11 and the Mo P 16 increased with the increase of concentration of metabolic inhibitors, as shown in Fig. 9. In Mo P 16, the broken frequency increased up to 4% concentration of DP (Fig. 9B). The Mo P 16 was about 90% in DP concentrations between 4 and 10% (Fig. 9B). The broken frequency of the Mo A 11 was about 90% in concentration of IAS 0.6% (Fig. 9A), and the frequency did not appreciably change with the concentration of DP

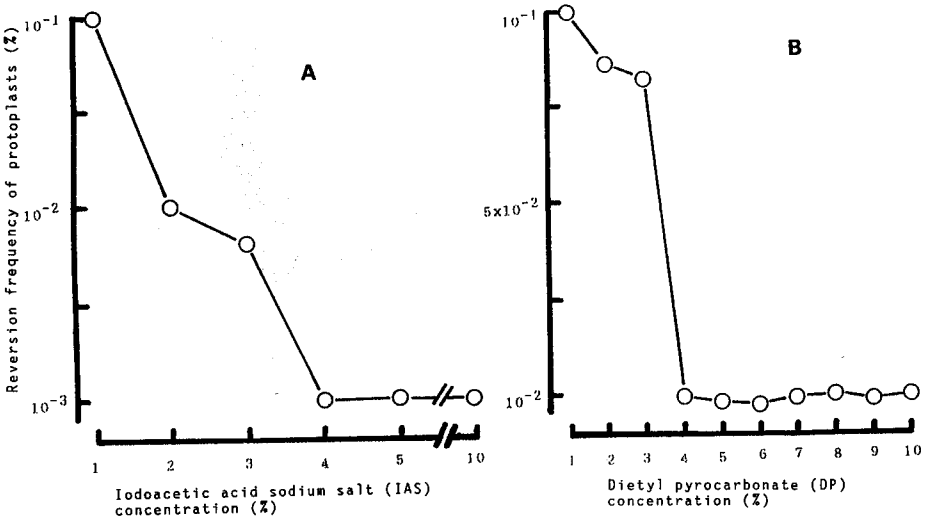

Fig. 10 Effects of concentrations of IAS and DP on reversion frequency of protoplasts for Mo A 11 (A) and Mo P 16 (B).

between 0.6 and 10%. Breakdown of both protoplasts was affected not only by concentration of metabolic inhibitors but also by centrifugation for washing protoplast. In fact, the number of protoplasts decreased to 20-30% after being washed three times as shown in Materials and Methods. However, the number of protoplasts for achieving protoplast fusion was present even at a broken frequency of 90% (10^6 protoplasts/ml). It is found that the addition of 100 mM $CaCl_2 \cdot 2H_2O$ to IAS and DP solution somewhat prevented the breakdown of protoplasts.

Reversion frequencies of the Mo A 11 and the Mo P 16 were approximately 7%, which was almost similar to those of parental strains (*A. auricula-judae* and *A. polytricha*). In the reversion frequencies of both prtotplasts of the Mo A 11 and the Mo P 16, they decreased with the increase of concentration of metabolic inhibitors up to 4% concentration (Fig. 10). Similar effects of metabolic inhibitors were also obtained using a rat myoblast cell line (WRIGHT, 1978). WRIGHT (1978) found that the lethal effects of each cell line increased with the increase of the concentrations of diethyl pyrocarbonate and idoacetamide. These facts suggest that metabolic inhibitors protected the enzyme system or other molecules in a cell which are necessary to keep the cell alive. In the concentrations of IAS and DP between 4 and 10%, the frequency of Mo A 11 was 10^{-3}%(Fig. 10A), while Mo P 16 was about 10^{-2}%(Fig. 10B). These facts suggest that some fusants after protoplast fusion can be selected from a regeneration medium by metabolic-inhibitor treatment. TAMAI and others (1990) have reported that concentrations of metabolic inhibitors, DP and IAS were respectively 0.1 and 0.5% for *Pleurotus ostreatus* and *P. cornucopiae*. This suggests that the concentrations of metabolic inhibitors were affected by differences of sensitivity to them among species.

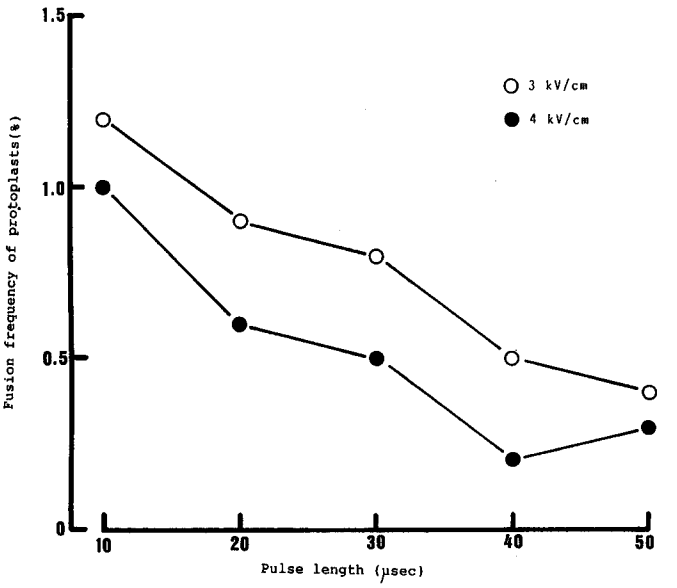

Fig. 11 Effects of the pulse length and the field strength of DC pulse on fusion frequency of protoplasts.

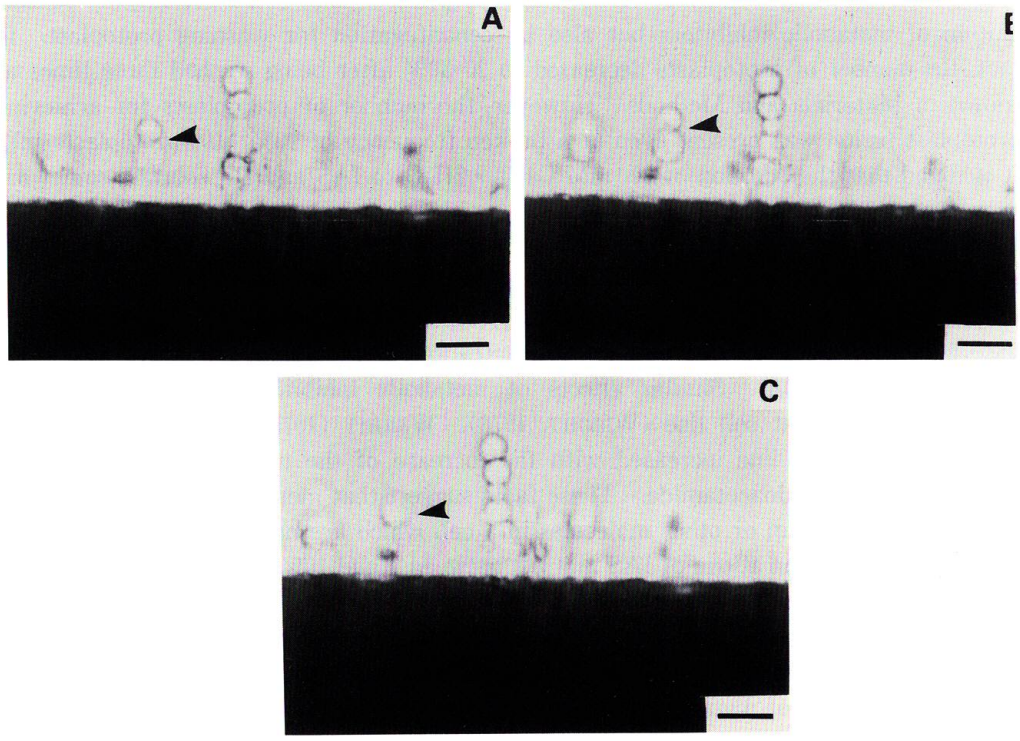

Fig. 12 Time course of protoplast fusion.
Note: Arrowheads show fusion sequence of protoplasts.
Legend: A: After the application of 2 MHz 500 V/cm AC
B: Immediately after the 3 kV/cm 10 μsec DC pulse
C: Twenty sec later taken the 3 kV/cm 10 μsec DC pulse (Scale bars: 10 μm)

From these results, concentrations of IAS and DP were determind to be 4% for Mo A 11 and Mo P 11.

3. 3. 2 Effects of the pulse length and the field strength of DC pulse on electrical protoplast fusion

Figure 11 shows the effects of the pulse length and the field strength of DC pulse on fusion frequency. At the pulse length of 10 μsec, fusion frequencies were good in field strength of both 3 kV/cm and 4 kV/cm. However, the fusion frequencies gradually decreased with the increasing pulse length. It is thought that the pulse length with above 10 μsec causes the breakdown of membranes. Compared to the fusion frequencies on the field strength of DC pulse, the fusion frequency of 3 kV/cm was greater than that of 4 kV/cm in all of the pulse lengths. In particular, the maximal fusion frequency (1.2%) was obtained when fusion was performed in conditions of DC pulse of 10 μsec with a field strength of 3 kV/cm. On the other hand, MIURA (1989) have reported that using 10 kV/cm 6 μsec DC pulse induced fusion products between *Lentinus edodes* and *Pleurotus ostreatus* by electrical protoplast fusion. These facts suggest that the difference of a field strength in DC pulse caused by the properties of protoplasts,

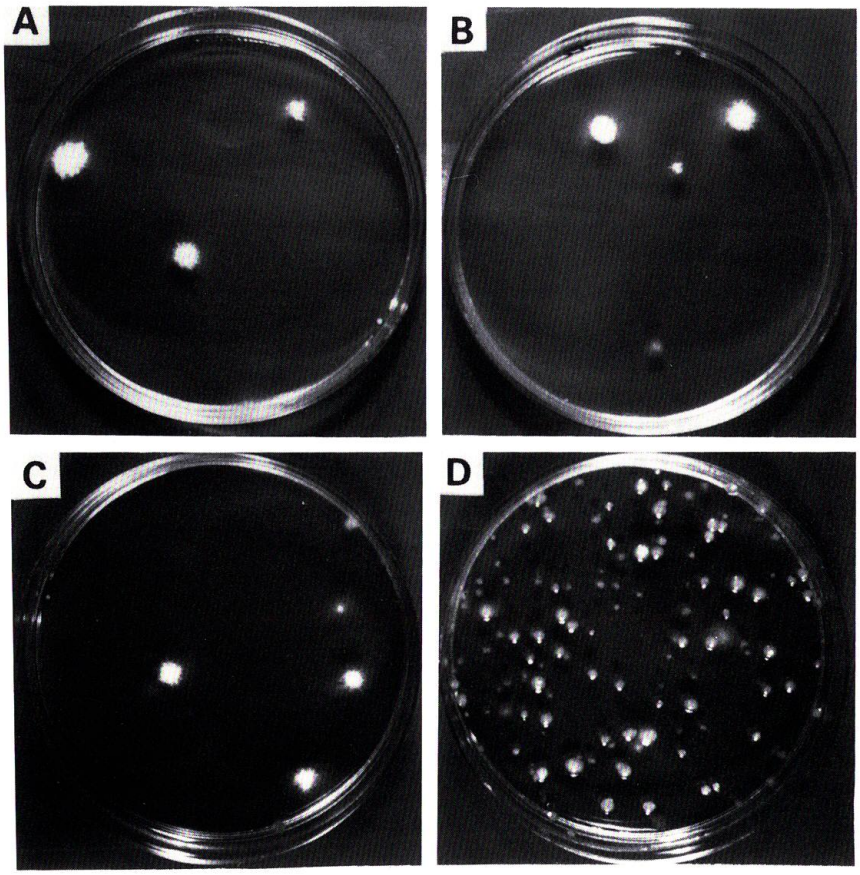

Fig. 13 Protoplast fusion of Mo A 11 and Mo P 16 after metabolic-inhibitor treatment. The plates A and B show Mo A 11 treated by IAS and Mo P 16 treated by DP without protoplast fusion, respectively. The plate C shows a mixture of Mo A 11 and Mo P 16 without protoplast fusion. Plate D shows protoplast fusion between Mo A 11 and Mo P 16.

such as size, condition of the membrane and origin (mycelium used when isolated protoplasts).

Figure 12 shows the time course of protoplast fusion when carried out under the condition of 10 μsec pulse length with a field strength of 3 kV/cm, having the maximal fusion frequency as shown in Fig. 11. The "Peal-chain" formation of protoplasts was caused by dielectrophoresis in an AC-field with a frequency of 2 MHz and field strength of 500 V/cm (Fig. 12A). Protoplast fusion was achieved by the application of DC pulse of 10 μsec, 2-5 times with intervals of approximately 1 sec, and a field strength 3 kV/cm (Figures 12B and C).

3. 2. 3 Interspecific heterokaryon obtained by electrical protoplast fusion

Both protoplasts of Mo A 11 and Mo P 16 which were treated with metabolic inhibitors (IAS and DP) were fused electrically with conditions as described in the previ-

ous section 3. 2. 2. The results are shown in Fig. 13. In the platings of A and B, protoplast suspensions obtained from each monokaryotic strain (Mo A 11 and Mo P 16) were spread on a StMY medium after treatment of metabolic inhibitors without protoplast fusion, as described in the previous section 3. 2. 1. In the plating C, two suspensions of both protoplasts (Mo A 11 and Mo P 16) which were treated with each of the metabolic inhibitors were mixed without fusion treatment. Several reversion colonies were only formed in three plates (platings A. B and C). These facts suggest that the formation of reversion colonies was inhibited by metabolic inhibitors as shown in Fig. 10. A large number of reversion colonies, however, were observed only in plating D, when protoplast suspensions of each monokaryotic strain (Mo A 11 and Mo P 16) were fused after the treatment with metabolic inhibitors. The appearance of reversion colonies is also in good accord with the results for heterospecific fusion between *Solanum* and *Petunia* reported by NEHLS (1978). NEHLS's results have revealed that protoplasts of *Solanum nigrum* and *Petunia hybrida* treated with IAS and DP formed cell walls and underwent mitosis after protoplast fusion by 30%(w/v) PEG 1500. It is considered that their colonies were formed by complementation of inhibited portions which were caused by metabolic-inhibitor treatment after protoplast fusion.

4. Nature of fusants

4. 1 Materials and Methods

4. 1. 1 Organisms

Five fusants (Fu 9, 52, 58, 63 and 100) were obtained by protoplast fusion as described in the previous section 3. 1. 2. All of the strains used in this experiments were maintained on a PDA medium at 26℃

4. 1. 2 Mycelial growth under various temperature conditions

Monokaryotic strains (Mo A 11 and Mo P 16) and fusants (Fu 9, 52, 58, 63 and 100) obtained by protoplast fusion were used in this section for observation of mycelial growth under various temperature conditions (5, 10, 15, 20, 25, 30, 35 and 40℃). Most experimental procedures were performed utilizing methods as mentioned in the previous section 1. 1. 2.

4. 1. 3 Mycelial growth on sawdust-medium

In order to observe mycelial growth of 5 fusants (Fu 9, 52, 58, 63 and 100), they were inoculated into several sawdust-media from mizunara, shirakamba and buna, and also inoculated into sawdust-media with a ratio of 4 : 1 (v/v) to sawdust and additive (rice bran and bran). Experiments in this section were performed by using the method described in the previous section 1. 1. 3.

4. 1. 4 Paired culture

Paired culture was carried out between fusants (Fu 9, 52, 58, 63 and 100) and monokaryotic strains (Mo A 11 and Mo P 16). These strains were precultured on a PDA medium for 7-10 days at 26℃. Each mycelial disk of the fusants and the monokaryotic strains was then cultured in pairs on a MA medium in a Petri dish. After incubation for 7-14 days, antagonistic line was confirmed by visual observation at the mycelial contact zone between the fusant and the monokaryotic strain.

4. 1. 5 Isozyme analysis

Monokaryotic strains (Mo A 11 and Mo P 16) and 5 fusants (Fu 9, 52, 58, 63 and 100) were used for isozyme analysis. Each strain was inoculated into 40 m*l* of SMY medium and incubated for 10-20 days at 26℃. The mycelia were collected by filtration, washed with deionized water, and then suspended in 0.6 M phosphate buffer (pH 7.0) containing 1 mM dithiothreitol and 0.1 M sodium ascorbate. The mixture was homogenized two times in an ice bath for 30 sec with a supersonic disintegrator (Tomy Seiko Co., LTD). The homogenate was centrifuged at 11750×*g* for 30 min at 4℃. A part of the supernatant was used immediately as enzyme sample for electrophoresis, and the remainder was stored in a deep freezer at −80℃ until the next analyzed. Electrophoresis was perpormed on polyacrylamide slab-gel at a constant current of 12 mA/cm² for approximately 2 hrs at 4℃. Following electrophoresis, the isozyme bands were stained by the methods of Shiroishi (1987a and b): for the detection of esterase bands, the gel was stained with a solution containing 1 mM of α-naphtylacetate, 0.1% of Fast Blue RR salt in 0.1 M phosphate buffer at 37℃

4. 1. 6 Biochemical tests

Five fusants (Fu 9, 52, 58, 63 and 100) obtained by protoplast fusion were precultured on a PDA medium for 7-14 days at 26℃. Tips of mycelia were punched out with a cork borer (5 mm in diameter), and then inoculated on various media as described below in order to examine their biochemical reactions. The media and the biochemical test were performed as follows:

p-nitrophenyl-α-D-galactoside medium: *p*-nitrophenyl-α-D-galactoside as substrate was prepared in 1 ml of acetone and added to 9 m*l* of a PDA prepared with citrate buffer (pH 5.4). After 24 hrs incubation on the medium prepared, a drop of 5% $Na_2CO_3 \cdot 10H_2O$ was added surrounding mycelial disks and a positive reaction was recorded when a definite yellow coloring from free *p*-nitrophenol was produced.

Caffeic acid medium: caffeic acid of 0.3% was used as substrate in a PDA. Enzymic oxidation of the substrate producing a distinct brown coloring surround the mycelial disks after incubation of 2 days was recorded as a positive reaction.

Tannic acid medium: tannic acid of 0.06% was used as substrate in a PDA. Phenoloxidase activity produced a brown coloration surrounding the mycelial disks was recorded as a positive reaction.

4. 1. 7 Formation of fruiting-bodies

Five fusants (Fu 9, 52, 58, 63 and 100) were cultivated by cultivation-medium utilizing sawdust obtained from shirakamba containing bran in order to form fruiting-bodies. Cultivation of fusants were performed by the methods described in the previous section 1. 1. 4.

4. 1. 8 Preparation of mitochondria and mitochondrial DNA

Isolation of mitochondria

Parental strains, monokaryotic strains (Mo A 11 and Mo P 16) and a fusant (Fu 9) were inoculated into 1 *l* of SMY liquid medium in a 3 *l* Erlenmyer flask, then incubated for 20-30 days at 26℃. All of the steps described below were done at 4℃. Wet mycelia of 80-190 g were harvested and collected by filtration with a miracloth and

washed with both deionized water and a buffer A (0.4M sucrose, 50 mM KH_2PO_4, 25 mM disodium EDTA and 10 mM 2-mercaptoethanol (pH 6.8)). The mycelia were suspended in a buffer A of about 0.8 volume to mycelial weight, and then fragmented with a homogenizer for 30 sec. Further, the homogenate was ground in the buffer A of about 2 volume to mycelial weight containing sea sand (10-15 meshes) with mortar and pestle. After grinding for 10 min, the homogenate was filtrated through 4 layers of sterile cheesechoth to remove mycelial fragments. The filtrate was centrifuged at 4000×g for 10 min to remove cell debris and a proportion of nuclei. Subsequently, the supernatant was centrifuged at 20000×g for 20 min. The precipitated mitochondria was used to purify a mitochondrial DNA.

Purification of mitochondrial DNA

The mitochondria were incubated on ice bath for 60 min with an appropriate volume of lysis buffer (2% N-lauroylsarcosine (sodium salt), 0.5 M NaCl and 0.3 M disodium EDTA (pH 9.0)), and the incubation was further continued at 65℃ for 60 min. Following cooling to room temperature, the lysate was centrifuged at 11000×g for 10 min. The supernatant was dialyzed against TEN buffer (10 mM Tris-HCl (pH 8.0), 1 mM disodium EDTA and 100 mM NaCl) at 4℃. The α-amylase (100 μg/ml) and RNAase (50 μg/ml) were then added to the supernatant dialyzed, and the mixture was incubated at 25℃ for 2 hrs. Following further incubation at 25℃ for an hour with protinase K (100 μg/ml), the reaction mixture was dialyzed against dialyze buffer (0.15 M NaCl, 20 mM NaH_2PO_4, 6 mM disodium EDTA (pH 7.2)). The mixture was subjected to density gradient centrifugation of Bisbenzimide H 33258-CsCl. To 30 ml of solution, 29.38 g of CsCl and 440 μl of Bisbenzimide H 33258 (10mg/ml) were added and the mixture was centrifuged twice at 105000×g for 40 hrs at 15℃. The DNA was separated into nuclear (bottom), mitochondria (lower) and plasmid-like elements devoid of protein (upper) fractions. The mitochondrial DNA bands removed from gradients were extracted 5 times with 5 M NaCl-saturated isopropanol, and then dialyzed against TEN buffer.

Extraction of mitochondrial DNA

The solution containing mitochondrial DNA which had been dialyzed with TEN buffer were mixed with 0.1 volume of 0.3 M sodium acetate and 2 volumes of cold ethanol, the suspension was chilled in a deep freezer at −70℃ to precipitate the DNA. After 2 hrs the DNA was collected by centrifugation at 13500×g, washed with 100% cold ethanol, and dried under a vacuum. The mitochondrial DNA was dissolved in TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM disodium EDTA) and stored at −15℃ until further use.

Endonuclease digestion analysis of mitochondrial DNA

For analysis of mitochondrial DNA, restriction endonuclease Hind III was purchased from Takara Shuzo, Kyoto, Japan and digests were carried out under conditions specified by the manufacturer. Mitochondrial DNA (approximately 1-2 μg) of 5 μl dissolved in TE buffer was incubated with reaction mixture of 15 μl at 37℃ for 2 hrs. Reactions were stopped by mixing with one-tenth volume of TAE buffer (40 mM Tris acetate, 2 mM EDTA, pH 8.0) containing 0.1% bromphenol blue, 0.1% xylen cyanol and

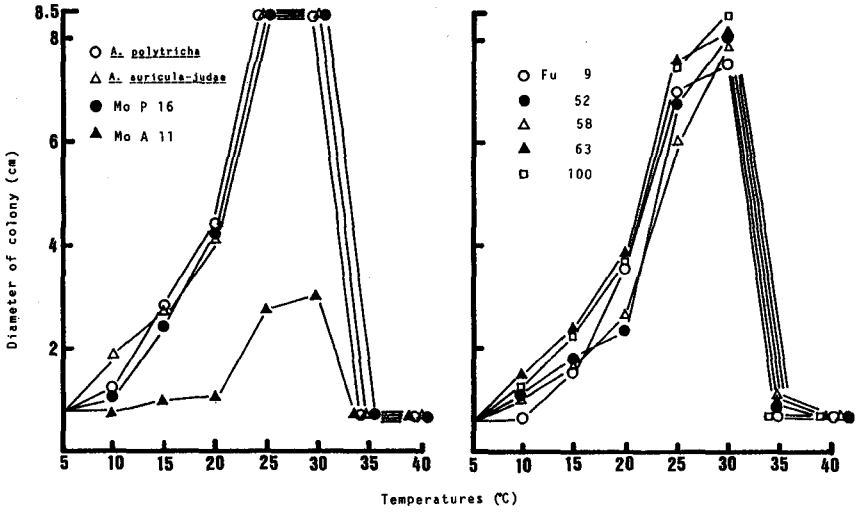

Fig. 14 Mycelial growth of the parental strains (*A. auricula-judae* and *A. polytricha*) and the monokaryotic strains (Mo A 11 and Mo P 16) (A), and fusants (Fu 9, 52, 58, 63, and 100) (B) in each temperature on 12-day-old culture.

50% glycerol. Electrophoresis was performed at a constant voltage (60 V). The gels were stained in a solution 0.5 μg of ethidium bromide per m*l* . After incubation at room temperature for 30 min, the mitochondrial DNA was visually observed using short-wave-length ultraviolet light and then photographed.

4. 2 Results and Discussion

4. 2. 1 Comparison of mycelial growth under various temperature conditions

Mycelial growth of 5 fusants (Fu 9, 52, 58, 63 and 100) were compared to those of parental strains and monokaryotic strains (Mo A 11 and Mo P 16). The parental strains and the Mo P 16 showed optimal temperature within 25-30℃ in mycelial growth on 12-day-old culture, as shown in Fig. 14. Whereas, optimal temperature of Mo A 11 was 30℃ in mycelial growth. The temperature characteristics in mycelial growth of 5 fusants were similar to that of Mo A 11. It is considered that the fusants obtained by protoplast fusion inherited optimal-temperature characteristics in mycelial growth from Mo A 11. All of the fusants exhibited minimal growth in temperatures of 5, 35 and

Table 5 Mycelial growth of 5 fusants (Fu 9, 52, 58, 63 and 100) in each sawdust-medium on 28-day-old culture

Sawdust-medium	Diameter of colony (cm)				
	Fusants				
	9	52	58	63	100
Buna	5.0	2.8	2.5	2.8	3.0
Shirakamba	6.3	3.7	2.8	4.0	3.8
Mizunara	0.0	0.0	0.0	0.0	0.0

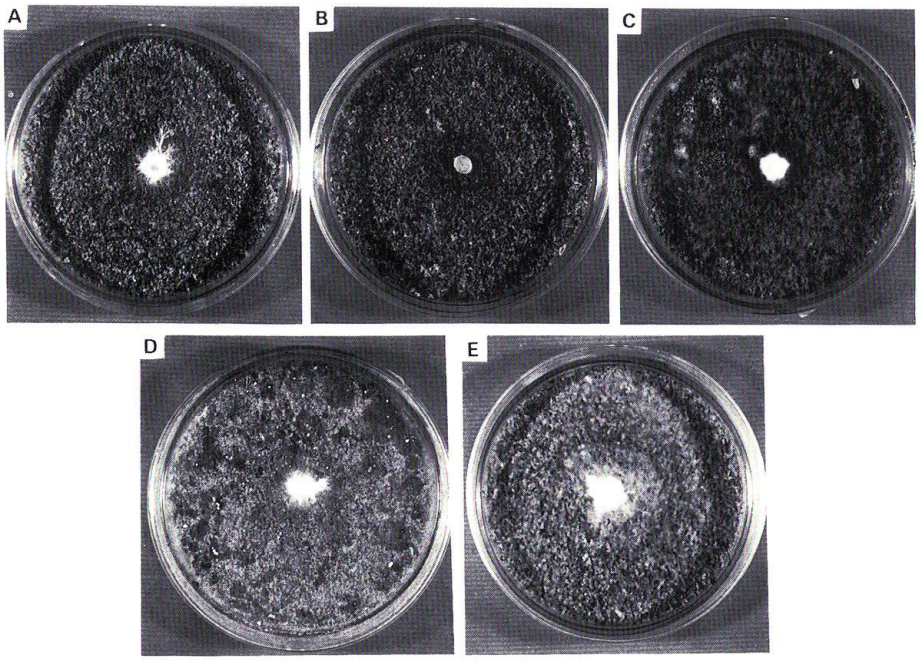

Fig. 15 Mycelial growth of fusants in buna sawdust-medium on 20-day-old culture.
Legend: A: Fu 9 B: Fu 52 C: Fu 58 D: Fu 63 E: Fu 100

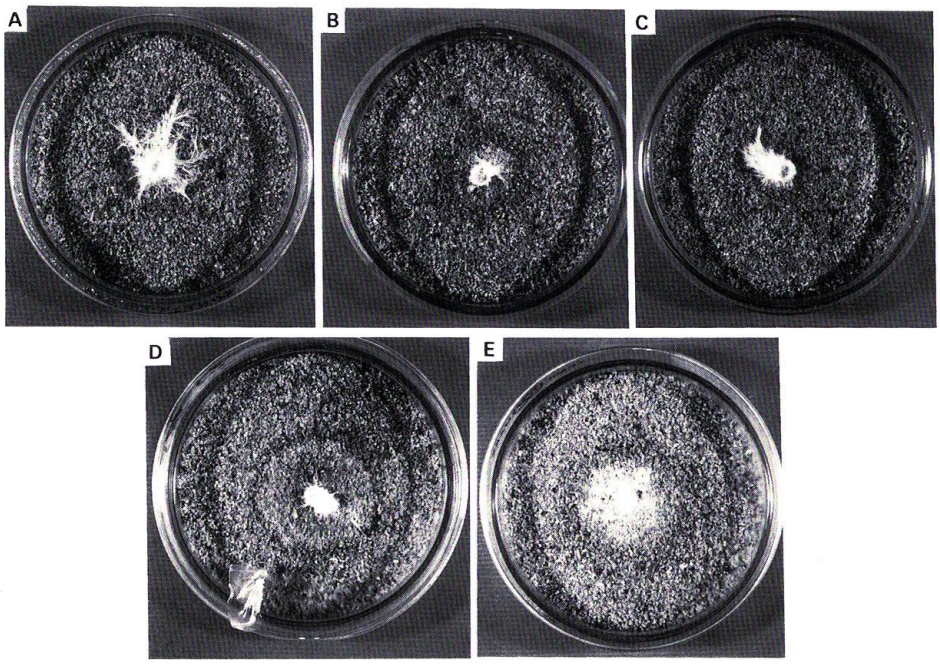

Fig. 16 Mycelial growth of fusants in shirakamba sawdust-medium on 20-day-old culture.
Legend: A: Fu 9 B: Fu 52 C: Fu 58 D: Fu 63 E: Fu 100

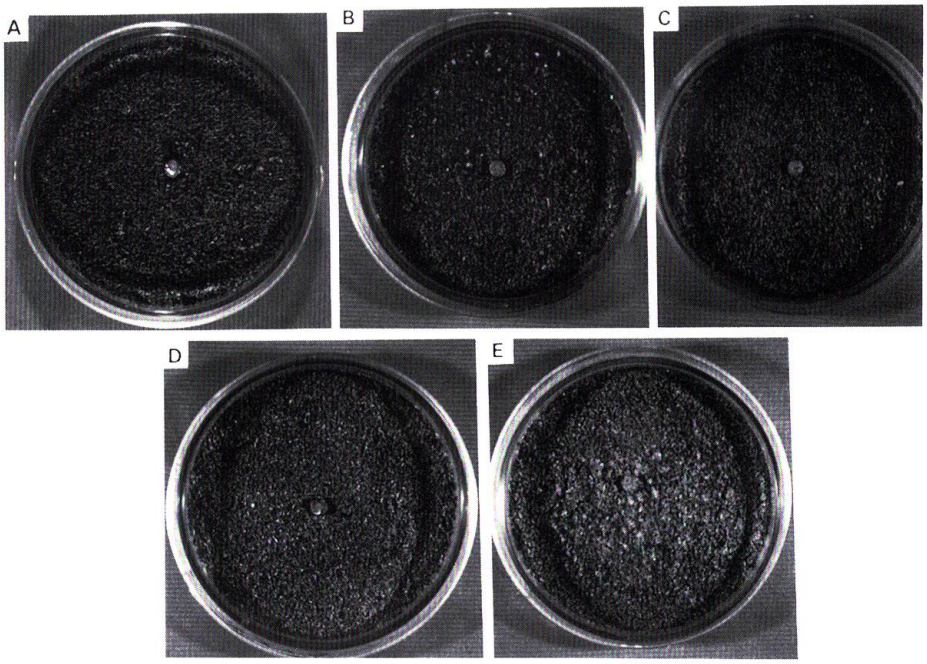

Fig. 17 Mycelial growth of fusants in mizunara sawdust-medium on 20-day-old culture.
Legend: A: Fu 9 B: Fu 52 C: Fu 58 D: Fu 63 E: Fu 100

Table 6 Mycelial growth of 5 fusants (Fu 9, 52, 58, 63 and 100) on shirakamba sawdust-medium with additives on 12-day-old culture

Additive	Diameter of colony (cm)				
	Fusants				
	9	52	58	63	100
Rice bran	5.6	3.7	4.0	5.5	3.2
Bran	8.5	4.0	4.6	5.6	4.1

40℃, suggesting that these fusants have similar characteristics of mycelial growth to parental and monokaryotic strains.

In regards to mycelial-growth rate, all of fusants were similar to the parental strains and the Mo P 16 on 12-day-old culture. However, mycelial densities on a PDA medium of the 5 fusants were thicker than those of both parental and monokaryotic strains.

4. 2. 2 Mycelial growth on sawdust media

Mycelial growth of 5 fusants (Fu 9, 52, 58, 63 and 100) was compared with sawdust-media from buna, shirakamba and mizunara. The results are shown in Table 5 and Figures 15, 16 and 17. A Fu 9 showed the greatest mycelial growth of all the fusants using shirakamba sawdust-medium. A similar result was obtained from buna sawdust-medium. In mizunara sawdust-medium, all fusants exhibited no mycelial growth, as in the results described in the previous section 1. 2. 3 (Table 1). Shirakam-

Table 7 Paired culture between the fusants and the monokaryotic strains

Fusants	Monokaryotic strains	
	Mo A 11	Mo P 16
Fu 9	+	+
52	+	+
58	+	+
63	+	+
100	+	+

Note: +: Formation of the antagonistic line

ba sawdust-medium gave relatively good mycelial growth results compared to the other sawdust-media.

In order to investigate the effects of additives, rice bran and bran were added to shirakamba sawdust-medium which had exhibited the greatest mycelial growth potential with all the fusants. The effects of both rice bran and bran were found with all of fusants, as shown in Table 6. Mycelial growth of fusants was considerably superior as compared to that on sawdust-media without additives. The effect of bran was relatively superior over that of rice bran for all of fusants.

4. 2. 3 Antagonisms in paired culture between fusants and monokaryotic strains

In omder to examine whether or not fusants (Fu 9, 52, 58, 63, and 100) obtained by protoplast fusion have different properties from monokarytic strains, Mo A 11 and Mo P 16, the 5 fusants were cultured in pairs with the monokarytic strains on a MA medium.The results are shown in Table 7. All of the fusants formed antagonistic line against both Mo A 11 and Mo P 16 at the contact zones between fusants and monokarytic strains. Toyomasu and Mori (1987) have reported that a barrage-like phenomenon was observed at the contact zones between fusants and monokaryotic strains, when the fusants were cultured on GMY agar plates with the monokaryotic strains for *Pleurotus* species. The antagonistic line obtained in this experiment is in good accord with the barrage-like prenomenon reported by them. This fact suggests that these fusants have different properties from both monokaryotic strains. Similar results were also obtained with paired cultures between fusants and parental strains. In addition, all the 5 fusants apparently had clamp connections in their hypha, suggesting that the fusants was heterokaryon.

From the results, it is suggested that the 5 fusants are heterokaryon between *A. auricula-judae* and *A. polytricha.*

4. 2. 4 Isozyme analysis of monokaryotic strains and fusants

Figure 18 shows the electrophoretic isozyme pattern of monokaryotic strains (Mo A 11 and Mo P 16) and 5 fusants (Fu 9, 52, 58, 63, and 100) in esterase isozyme. The isozyme patterns are different between the Mo A 11 and the Mo P 16, suggesting that differences of isozyme patterns are caused by a difference among strains. These differences might result in a difference in the physiology, such as the number of nuclear and organella composition in the cytoplasm, of monokaryotic mycelia obtained by protoplasting the parental mycelia. OHMASA and FURUKAWA (1986) have reported that

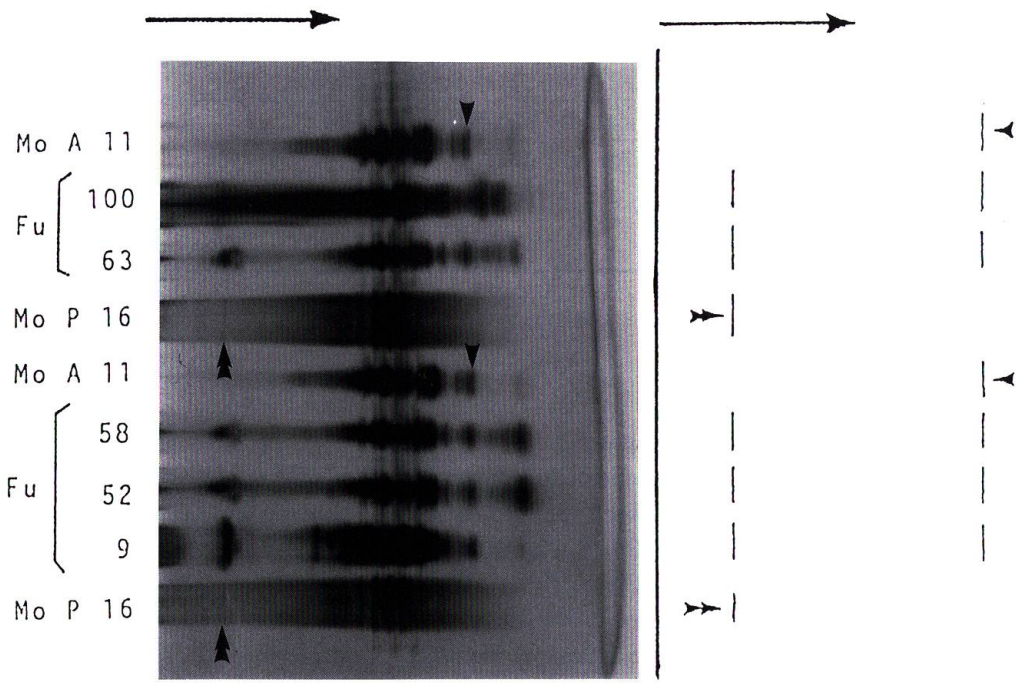

Fig. 18 Electrophoretic patterns of esterase for the monokaryotic strains (Mo A 11 and Mo P 16) and fusants (Fu 9, 52, 58, 63 and 100).
Note: Single and double arrowheads show specific isozyme bands to Mo A 11 and Mo P 16, respectively.

some factors, such as culture age of mycelium and medium composition, affect the stability of isozyme bands of *L. edodes*. They found that the substantial isozyme pattern did not change, although the staining intensity of bands varied with the difference of culture periods and medium composition. The results are nearly parallel with these.

Specific isozyme bands in each of the monokaryotic strains are shown in diagrammatic representation in Fig. 18. The Mo A 11 is indicated by single arrowheads, and the Mo P 16 is indicated by double arrowheads. The specific bands were used to prove hybrid cells obtained from the interspecific protoplast fusion. Figure 18 apparently indicates that all of the 5 fusants possess both specific bands of the respective monokaryotic strains. This result is nearly the same as that obtained by intraspecific protoplast fusion of *P. ostreatus* (OHMASA, 1986). OHMASA (1986) found that esterase-isozyme patterns of mycelia from fusants obtained by protoplast fusion possessed both of the isozyme bands of each parental strain. Similarly, TOYOMASU and MORI (1987) also reported that the fusants had specific esterase-isozyme bands relating to both parental strains of *Pleurotus* species, suggesting that they were hybrid cells. TAMAI and others (1990) have also reported similar results for *Pleurotus* species. These evidences suggest that these fusants have different properties from the monokaryotic strains.

From these evidences, it is concluded that the 5 fusants obtained from these experiments are heterokaryon characteristically located between *A. auricula-judae* and *A.*

Talbe 8 Biochemical tests in the parental and monokaryotic strains (Mo A 11 and Mo P 16) and the fusants (Fu 9, 52, 58, 63 and 100)

Character	Strains								
	Parental strains		Monokaryotic strains		Fusants				
	Kiku	Ara	Mo A 11	MO P 16	9	52	58	63	100
α-galactosidase	+	+	+	+	+	+	+	+	+
Caffeic acid	+	+	+	+	−	−	−	−	+
Tannic acid	+	+	+	+	−	−	−	−	+

Legend: Kiku: *A. auricula-judae* Ara: *A. polytricha*
+: Positive reaction −: Negative reaction

polytricha.

4. 2. 5 Biochemical characterization

In order to examine the biochemical characterization of fusants (Fu 9, 52, 58, 63 and 100), 3 biochemical tests were performed using *p*-nitropheny-α-D-galactoside, caffeic acid, and tannic acid. Table 8 shows an enzyme activity of α-galactosidase in *p*-nitrophenyl-α-D-galactoside as substrate. All strains on media used, colored to a definite yellow around the mycelial disks, suggesting that α-galactosidase activity was found. It is considered that the 5 fusants obtained by protoplast fusion inherited a characteristic of the enzymic activity of α-galactosidase from parental and monokaryotic strains.

For biochemical test of enzymic oxidation using caffeic acid as a substrate, 4 (Fu 9, 52, 58, and 63) out of 5 fusants did not show an enzymic activity (Table 8). This fact suggests that the 4 fusants possess a property, in respect to phenoloxidase activity, differing from the parental and monokaryotic strains. A Fu 100, however, exhibited enzymic activity, indicating that Fu 100 have a similar property to the parental and monokaryotic strains. Similar results were obtained from biochemical tests in tannic acid as a substrate concerning enzymic activity of phenoloxidase brown coloring around mycelial disks (Table 8).

Kiuchi (1988) have reported for 7 mushroom types that the biochemical reactions concerning various biological character, such as, catecholeoxidase, alkaline phosphatase and gelatinase were nearly all stable in the cases of both PDA plus 1% malt extract (PDAM) and GMYS (1% malt extract, 0.4% yeast extract, 0.5% sucrose, and 1.2% agar) plus CP (0.4% Sanparl CP) media as a basal media. Our results obtained in these experiments exhibited similar tendencies as his results using PDMA, GMYS plus CP, and PDA media. Also, the biological reaction concerning *p*-nitrophenyl-α-D-galactoside, caffeic acid, and tannic acid were stable during three repeated experiments.

4. 2. 6 Fruiting-bodies formation

Five fusants (Fu 9, 52, 58, 63 and 100) were cultivated with shirakamba cultivation-medium with added bran in order to form fruiting-bodies. All of the fusants required incubation periods of 60 days until surface covering with mycelium in culture bags was complete. Small fruiting-bodies formation was observed from only

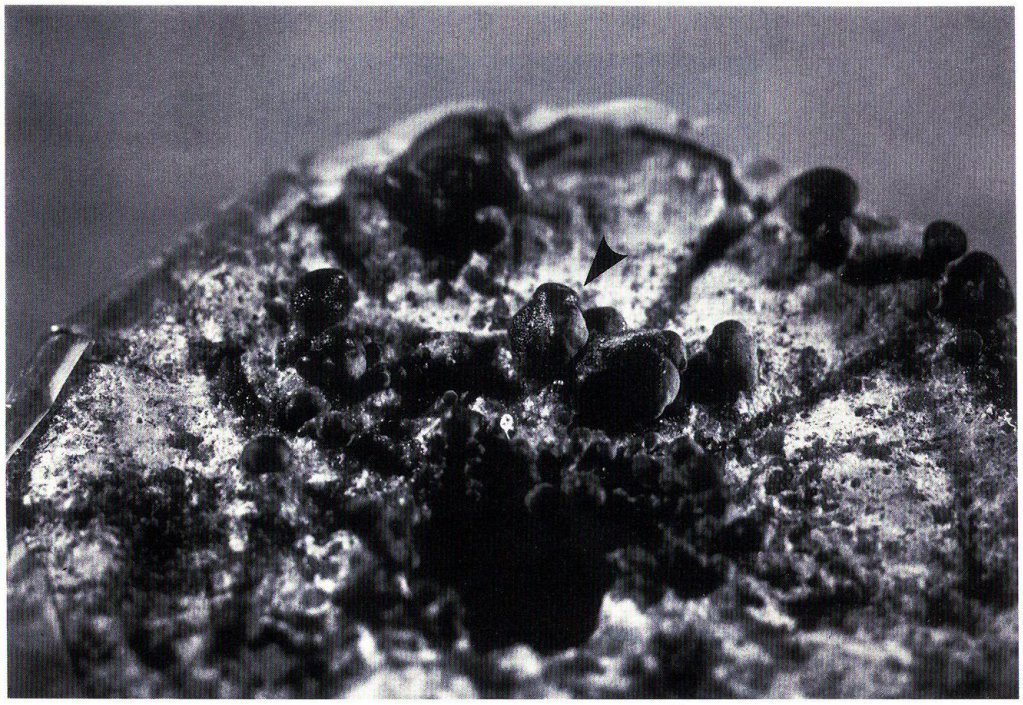

Fig. 19 Small fruiting-bodies formation of the Fu 9.
Note: Arrowhead shows small fruiting-bodies.

Fu 9, as shown in Fig. 19. After spread with mycelium in cultivation-medium. The Fu 9 required 20 days to form small fruiting-bodies formation. The remaining 4 fusants (Fu 52, 58, 63 and 100), however, did not form a primordium even after incubation of 100 days. The Fu 9 which formed fruiting-bodies in the shirakamba cultivation -medium with bran was used to analyze restriction endonuclease in the next section 4. 2. 7.

4. 2. 7 Restriction endonuclease analysis of mitochondrial DNA

Mitochondrial DNAs of Fu 9, parental strains and monokaryotic strains (Mo A 11 and Mo P 16) were obtained at between 350-500 μg per 100 g of wet mycelium. The molecular sizes of each strain were found to range in size from 70-90 kbp by agarose-gel electrophoretic analysis (Fig. 20), being in about the same ranges as mitochondrial DNA in *Neurospora crassa* (approximately 61 kbp; HINNEN and others, 1978). YUI and others (1988) have also reported that the molecular size of *Lentinus edodes* mitochondrial DNA is 69 kbp.

Noteworthy, each of the Fu 9, the parental strains and monokaryotic strains (Mo A 11 and Mo P 16) was confirmed to have a mitochondrial plasmid-like DNA. The molecular sizes of plasmid-like DNA were estimated to be in the range of 9-10 kdp. NAKAYA and others (1991) have reported that using 32 strains of *P. ostreatus,* two different plasmid-like DNAs (molecular size of 9.5 and 10.1 kbp) were specifically distributed in the mitochondria of these strains. Similarly, KATAYOSE and others (1990)

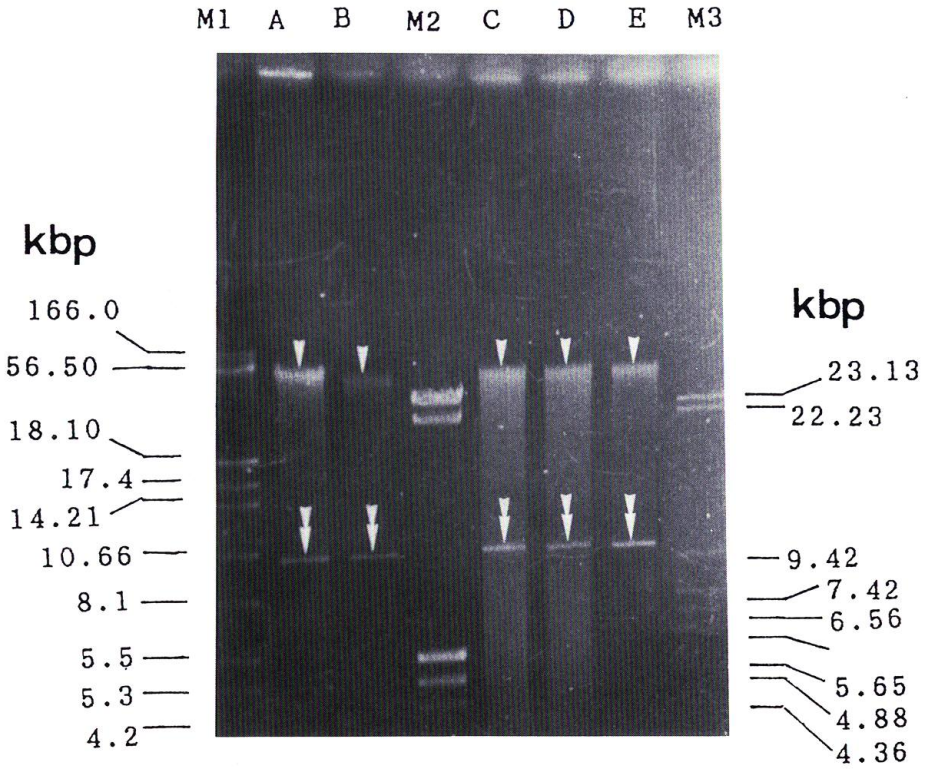

Fig. 20 Agarose (0.3%) gel electrophoresis of mitochondrial DNA without restriction endonuclease.
Lane M1, T4dc+T4dc digested with *Bgl*; Lane A, *A. auricula-judae*; Lane B, *A. polytricha*; Lane M2, lambda DNA digested with *Hind* III; Lane C, Mo A 11; Lane D, Mo P 16; Lane E, Fu 9; Lane M3, a mixture of lambda DNA digested with Hind III, and with Eco R I.
Notes: The numbers on the left and right sides indicate the molecular length (kbp) of size marker, M1 and M3 respectively. Single and double arrowheads show mitochondrial and plasmid-like DNAs, respectively.

have found a linear mitochondrial DNA plasmid with *L. edodes*. The liner DNA plasmids were observed in *Streptomyces rochei* (HAYAKAWA and others, 1979), the yeast *Kluyveromyces lactis* (GUNGE and others, 1981) and *Saccharomyces kluyveri* (KITADA and HISHINUMA, 1987), the ascomycete *Ascobolus immersus* (FRANCOU, 1981) and *Claviceps purpurea* (TUDZYNSKI and others, 1983), the basidiomycete *Agaricus bitorquis* (MOHAN and others, 1984), the plant pathogenic fugi *Rizoctonia solani* (HASHIDA and others, 1984) and *Fusarium oxysporum* (KISTLER and LEONGE, 1986) and plants *Zea mays* (PRING and others, 1983) and *Brassica campestris* (ERICKSON and others, 1985).

Mitochondrial DNA of the Fu 9 was compared to that of parental strains and monokaryotic strains (Mo A 11 and Mo P 16) using restriction endonuclease analysis. A report concerning comparison of mitochondrial DNAs between fusants and parental strains are not yet forthcoming. Agarose gel electrophoresis of mitochondrial DNAs of

Table 9 Sizes of Hind III restriction fragment with monokaryotic strains (Mo A 11 and Mo P 16) and Fu 9

Strains	Mo A 11	MO P 16	Fu 9
	17.4 kbp	17.4 kbp	17.4 kbp
	15.4	15.4	15.4
	8.5	8.5	8.5
	6.5	6.5	6.5
	5.8	5.8	5.8
	5.6	5.6	5.6
	4.5	4.5	4.5
	4.2	4.2	4.2
	4.0	4.0	4.0
	3.8	3.8	3.8
		3.5	3.5
	3.2		3.2
	3.0	3.0	3.0
			2.5
			2.1
Total	81.9	82.2	90.0

parental strains and monokaryotic strains showed similar patterns to each other by analysis of restriction endonuclease HindIII. Mitochondrial DNA of the Fu 9 was digested with HindIII restriction endonuclease in order to compare that of monokaryotic strains, Mo A 11 and Mo P 16. The result in shown in Table 9. Both Mo A 11 and Mo P 16 possessed 12 restriction fragments, while Fu 9 possessed 15 fragments. Specific restriction fragments in each monokaryotic strain were shown in Table 9. The molecular sizes of each fragment of Mo A 11 and Mo P 16 were, respectively, 3.2 and 3.5 kbp. The Fu 9 possessed both specific restriction fragments of respective monokaryotic strains. In addition, the Fu 9 possessed different two restriction fragments (2.1 and 2.5 kbp) from Mo A 11 and Mo P 16. The two different mitochondrial DNA fragments of Fu 9 obtained in this study may have occurred due to recombination of mitochondrial DNA between Mo A 11 and Mo P 16 after protoplast fusion. However, the Fu 9 had fragments common to each nonokaryotic strain. As shown in Table 9, the molecular size of Mo A 11 and of Mo P 16 and the Fu 9 were, respectively, 81.9, 82.2 and 90.0 kbp by analyzing HindIII restriction endonuclease. SPECHT and others (1983) have reported using *Shizophyllum commune* which mitochondrial DNA of that strain were revealed to be approximately 49.85 kbp with restriction analysis of 5 endonuclease. Similar molecular sizes of mitochondrial DNA were reported by CHYOUG-shwu and MEYER (1991) for the *Candida* species. For the analysis of two different fragments (2.1 and 2.5 kbp) of the Fu 9, which are not present in monokaryotic strains as described above, further detailed studies are necessary.

From these evidences, it is shown that the the Fu 9 is a heterokaryon between *A. auricula-judae* and *A. polytricha.*

Conclusion

In the present study, firstly, The nature of *A. auricula-judae* and *A. polytricha,* concerning cultivable characteristics were investigated. Secondly, various factors affecting the isolation and reversion of protoplasts were examined for *A. auricula-judae* and *A. polytricha.* Thirdly, protoplasts of monokaryotic strains which were treated with metabolic inhibitors were electrically fused. Fourthly, The nature of fusants obtained by electrical protoplast fusion were investigated.

1. Nature of *A. auricula-judae* and *A. polytricha*

Auricularia auricula-judae and *A. polytricha,* exhibited the greatest mycelial growth between 25℃ and 30℃ on a PDA medium. Regarding mycelial-growth rate, both strains exhibited similar patterns on a 12-day-old culture.

Superior mycelial growth was obtained from shirakamba sawdust-medium. Addition of additives (rice bran and rice) caused primitive effects concerning mycelial growth in both strains.

Auricularia polytricha formed fruiting-bodies at shorter incubation periods compared to *A. auricula-judae,* and yield a fruiting-body mass higher than *A. auricula-judae.*

2. Isolation and reversion of protoplasts from mycelia of *A. auricula-judae* and *A. polytricha*

Conditions for obtaining maximal yields of protoplast isolation from *A. auricula-judae* and *A. polytricha* were as follows:

Osmotic stabilizer: 0.5 M $MgSO_4$ in MES buffer (pH 5.3)
Mycolytic enzyme: Novozym 234 1%+chitinase 0.1%
Culture period of mycelia: 4 days (*A. auricula-judae*)
6 days (*A. polytricha*)

Several parameters afiecting the protoplast reversion, culture period of mycelia and regeneration medium were examined. High reversion frequency of protoplasts was obtained for protoplasts isolated from mycelia of 4-day-old culture for *A. auricula -judae* and of 6-day-old culture for *A. polytricha.* The StMY regeneration medium also provided the highest reversion frequency of protoplasts for both strains.

3. Protoplast fusion between *A. auricula-judae* and *A. polytricha*

Monokaryotic strains, Mo A 11 and Mo P 16 were, respectively, treated with iodoacetic acid sodium salt (IAS) and diethyl pyrocarbonate (DP) to select hybrid cells by protoplast fusion. The concentrations of IAS and DP were found to be 4% for both strains from the results of broken and reversion frequencies of protoplasts. High fusion frequency (1.2%) was obtained by the use of DC pulse of 10 μsec, 2-5 times with intervals of approximately 1 sec, and a field strength 3 kV/cm.

After electrical protoplast fusion, a number of reversion colonies were observed on StMY medium by complementation of protoplasts of each monokaryotic strain which were treated with metabolic inhibitors.

4. Nature of fusants

Five fusants (Fu 9, 52, 58, 63 and 100) obtained by protoplast fusion were investigated to clarify their characteristic. The 5 fusants had a optimal-temperature charac-

teristics of 30℃ in mycelial growth and were similar to the parental strains and the monokaryotic strain Mo P 16 on a PDA medium in mycelial-growth rate. Mycelial densities of the 5 fusants were thicker than those of the other strains (parental and monokaryotic strains).

Shirakamba sawdust-medium provided good mycelial growth for the 5 fusants.

All of the 5 fusants exhibited an antagonism to both monokaryotic strains (Mo A 11 Mo P 16) at the contact zone in paired culture. The 5 fusants had clamp connections in their hypha.

Five fusants had several bands in common with both monokaryotic strains in the analysis of esterase isozyme patterns.

In 5 fusants enzymic activity of α-galactosidase was observed, whereas, phenoloxidase activities were not exhibited by Fu 9, 52, 58 and 63.

By restriction endonuclease analysis, molecular size of mitochondrial DNA of Fu 9 which formed small fruiting-bodies was confirmed to be 90.0 kbp. All of the 5 fusants had a mitochondrial plasmid-like DNA. The 5 fusants had both specific restriction fragments of each monokaryotic strain. Additionally, Fu 9 had different restriction fragments (2.1 and 2.5 kbp) from Mo A 11 and Mo P 16.

From these evidences, it is concluded that all of the 5 fusants (Fu 9, 52, 58, 63 and 100) are heterokaryon.

Acknowledgment

The author wishes to express his sincere gratitude to Professor Minoru Terazawa, Department of Forest Products, Hokkaido University for his invaluable guidance and encouragement during the course of this study, and for the critical readings of this manuscript.

The author would like to express his appreciation to Associate Professor Kiyoshi Miura, Department of Forest Products, Hokkaido University for his helpful suggestions and discussions, and for critical readings of this manuscript.

The author is grateful to Professor Takashi Sasaya, and Professor Kazumi Fukazawa, Department of Forest Products, Hokkaido University, for their suggestions and critical readings of this manuscript.

Refferences

AKAHANE, K., M. KITOH and S. O. YANAGI. 1986. Preparation and regeneration of protoplasts from *Flammulina velutipes*. Proc. Nihon Kin Gakkai. 30: 69.

AKAMATSU, K., T. KAMADA and T. TAKEMARU. 1983. Release and regeneration of protoplasts from the oidia of *Coprinus cinereus*. Trans. mycol. Soc. Japan. 24: 173-184.

ANNE, J., H. EYSSEN and P. De SOMER. 1974. Formation and regeneration of *Penicillium chrysogenus* protoplasts. Arch. Microbiol. 93: 159-166.

ANNE, J. and J. F. PEBERDY. 1975. Condition for induced fusion of fungal protoplasts in polyethylene glycol solutions. Arch. Microbiol. 105: 201-205.

ANNE, J. and J. F. PEBERDY. 1976. Induced fusion of fungal protoplasts following treatment with polyethylene glycol. J. gen. Microbiol. 92: 413-417.

ARIMA, K. and I. TAKANO. 1979. Protoplast fusion in microorganisms. Hakko Kougaku, 57(5): 380-395.

ASES-Ledied, M. Nor and C. GARCIA-Menodoza. 1970. J. gen. Microbiol. 61: 335-342. (See Toyoda and others, 1984.)

BENITEZ, T., S. RAMOS and I. G. ACH. 1975. Arch. Microbiol. 103: 199-203. (See Toyoda and others, 1984.)

BOS, C. J. and S. M. SLAKHORST. 1980. Isolation of protoplasts from *Aspergillus nidulans* conidiospores. Can. J. Microbiol. 27: 400-407.

CHYONG-Shwu, S. and S. A. MEYER. 1991. Characterization of mitochondrial DNA in various *Candida* species: isolation, restriction endonuclease analysis, size, and base composition. Int. L. Syst. Bacleriol. 41: 6-14.

DALES, R. B. G. and J. H. CROFT. 1977. Protoplast fusion and the genetical analysis of vegetative incompatibility in *Aspergillus nidulans*. FEMS Microbiol. Lett. 1: 201-204. (See Peberdy, 1979.)

De VRIES, O. M. H. and J. G. H. WESSELS. 1972. Release of protoplasts from *Schizophyllum commune* by a lytic enzyme preparation from *Trichoderma viride*. J. gene. Microbiol. 73: 13-22.

De VRIES, O. M. H. and J. G. H. WESSELS. 1973. Release of protoplasts from *Schizophyllum commune* by combined action of purified α-1, 3-glucanase and chitinase derived from *Trichoderma vride*. J. gen. Microbiol. 76: 319-330.

De VRIES, O. M. H. and J. G. H. WESSELS. 1975. Chemical analysis of cell wall regeneration and reversion of protoplasts from *Schizophyllum commune*. Arch. Microbiol. 102: 209-218.

DOOIJEWAARD-Kloosteriel, A. .M P., J. H. SIETSMA and J. T. M. WOUTERS. 1973. J. gen. Microbiol. 74: 205-209. (See Toyoda et al., 1984.)

ERICKSON, L., W. D BEVERSDORF and K. P PAULS. 1985. Curr. Genet. 9: 679-682.

FERENCZY, L., F. KEVEI, M. SZZEGEDI, A. FRANKO and I. ROJIK. 1976. Factors affecting high-frequency fungal protoplast fusion. Experientia 32: 1156-1158.

FOURNIER, P., A. PRYVOST, C. BOURGUIGNON and H. HESLOT. 1977. Recombination after protoplast fusion in the yeast *Candida tropicalis*. Arch. Microbiol. 115: 143-149. (See Peberdy, 1979.)

FRANCOU F. 1981. Mol. Gen. Gent. 184: 440-444. (See Katayose, 1990)

GARCIA Acha, I., F. LOPEZ-Bemlonte and J. R. VILLANUEVA. 1966. Regeneration of mycelial protoplasts of *Fusarium culmorum*. J. gen. Microibiol. 45: 515-532.

GENTHNER, F. J. P. T BORGIA. 1978. Spheroplast fusion and heterokakyon on formation in Mucor racemosus. J. Bacteriol. 134: 348-352.

GOLD, M. H., T. M. CHENG, and M. ALIC. 1983. Formation, fusion, and regeneration of protoplasts from wild type and auxotrophic strains of the white rot basidiomycetes *Phamerochate chrysosporium*. Appl. Environ. Microbiol. 46: 260-263.

GUNGE, N. A. TAMURA, F. OZAWA and K. SAKAGUCHI. 1981. J. Bacteriol. 145: 382-390. (See Katayose, 1990)

GUNGE, N. 1984. Protoplast fusion of yeast and its utilization. Zyo Kyou. 79(4): 210-215.

HAMLYN, P. F. and C. BALL. 1979. Proc. 3rd Int. Symp. Genet. Ind. Microorg. Madison, Wisc. (See Peberdy, 1979.)

HAMLYN P. F., R. E. BRADSHAW, F. M. MELLON, C. M. SANTIAGO, J. M. WILSON and J. F. PEBERDY. 1981. Efficient protoplast isolation from fungi using commercial enzymes. Enzyme. Microb. Technol. 3: 321-325.

HASHIBA, T. and M. YAMADA. 1984. Intraspecific protoplast fusion between auxotrophic mutants of *Rhizoctonia solani*. Phytopathology 74: 398-401.

HASHIBA, T., Y. HOMMA, M. HYAKUMACHI and I. MATSUDA. 1984. J. Gen. Microbiol. 130: 2067-2070. (See Katayose, 1990)

HAYAKAWA, T., T. TANAKA, K. SAKAGUCHI, N. OTAKEA. poly and H. YONAHARA. 1979. J. Gen. Appl. Microbiol 25: 255-260. (See Katayose, 1990)

HINNEN, A., J. B. HICKS, and G. R. FINK. 1978. Proc. Natl. Acad. Sci. USA 75: 1929-1933. (See Yui, 1988)

HIROE, O. 1976. New application of fungology. Ed. by O. Hiroe. p. 1554-1561

HOOPWOOD, D. A. 1981. Genetic studies with bacterial protoplasts. Ann. Rev. Micribiol. 35: 237-272.

ISHIZAKI, H., A. YAJIMA, M. KOHNO and H. KUNOH. 1983. Effect of isoprothiolane on *Pyricularia ozyzae* GAVAGA(1) cell wall regeneration of protoplasts. Ann. Phytopath. Soc, Japan. 49: 471-480.

IWADE, I. 1958. Icubation methods for mushrooms. Ed. by I. Iwade. pp. 116-118.

KATAYOSE, Y., S. KAJIWARA, and K. SHISHIDO. 1990. The basidiomycete *Lentinus edodes* linear mitochondrial DNA plasmid contains a segment exhibiting a high autonomously replicating sequency activity in *Sacchromyces cerevisiae.* Nuc. Aci. Res. 18: 1395-1400.

KAWASUMI, T., N. KIGUCHI, Y. FUTATSUGI, K. OHBA and S. O. YANAGI. 1987. High yield preparation of *Lentinus edodes* ("Shiitake") protoplasts with regeneration capacity and mating type stability. Agric. Biol. Chem. 51(6): 1649-1656.

KEVEI, F. and J. F. PEBERDY. 1977. Interspecific hybridization between *Aspergillus nidulans* and *Aspergillus rugulosus* by fusion of somatic protoplasts. J. gen. Microbiol. 102: 255-262.

KINJO, K. and T. KONDO. 1979. Study on the cultivation culture media of basidiomycetes (III). With reference to cultural characteristics of *Auricularia polytricha* (Mont.) Sacc. Mokuza Gakkaishi. 25: 799-803.

KINJO, K., M. SUNAGAWA and S. YAGA. 1987. Study on the cultivation culture media of basidiomyceter medium aptitude of hard wood of low quality. The Bulletin of the College of Agriculture, University of the Ryukyus. 34: 89-94.

KINJO, K. and S. YAGA. 1988. Study on cultivation culture media of basidiomycete (V)-characteristics of Baggasse medium Mokuzai Gakkaishi. 34: 48-53.

KISTLER, H. C. and S. A. LEONG. 1986. J. Bacteriol. 167. 587-583. (See Katayose, 1990)

KITADA, K. and F. HISHINUMA. 1987. Mol. Gen. Genet. 206: 377-381. (See Katayose, 1990)

KITAMOTO, Y., I. KAGAWA, N. NAGAO, M. NAKAMATA and Y. ICHIKAWA. 1987. High-productivity protoplasting and reversion of protoplasts in *Coprinus cinereus* with a sigle preparation of lytic enzyme from *Trichoderma harizianum.* Trans. mycol. Soc. Japan. 28: 217-227.

KIUCHI, N. 1988. Biochemical characteristics of some edible mushrooms. Bull. For. Prod. Res. Ints. 15: 1-9.

KURASHIKI, S., Y. AKUZAWA, and K. MORI. 1987. Effect of *Lentinus edodes* administration on immunocompetent cells in mice treated with carcinogen. Proc. Nihon Kin Gakkai. 32: 120.

MIURA, K. 1989. Electrofusion of protoplasts from edible mushrooms. Res. Bull. Exp. For. Hokkaido univ. 46: 343-391.

MOHAN, M., R. J. MEYER, J. B. AMDERSON and P. A. HORGEN. 1984: Curr. Gent. r: 615-629. (See Katayose, 1990)

MOORE, D. 1975. Production of *Coprinus protoplasts* by use of chitinase or helicase. Trans. Br. mycol. Soc. 65: 134-136.

MOORE, P. M. and J. E. PEBERDY. 1976. Release and regeneration of protoplasts from the conidia of *Aspergillus flavus.* Trans. Br. mycol. Soc. 66(3): 421-425.

NAKAMURA, K. 1982. Dictionary of mushrooms. Ed. K. Nakamura. pp. 425-430.

NAKAYA, M., T. SAITO, F. TERAMI, J. OHINAKA, J. AOSHIMA, and H. NISHIKAWA. 1991. Distribution of mitochondrial plasmid-like DNA in *Pleurotus ostreatus.* Trans. Mycol. Soc. Jappan 32: 291-297.

NANBA, H., K. MORI and H. KURODA. 1987. Antitumor polysaccharide in fruit bodies of mushroom (*Agaricus bispora*). Proc. Nihon Kin Gakkai. 32: 119.

NECAS, O. 1965. Mechanisms of regeneration of yeast protoplasts (II). Formation of the cell wall de novo. Folia Biol. Praha. 11: 97. (See Garcia Acha, 1966.)

NECAS, O. 1966. Nature. 192. (See toyoda and others, 1984.)

NEHLS, R. 1978. The use of metabolic inhibitors for the selection of fusion products of higher plant protoplasts. Molec. Gene Genent. 166: 117-118.

NISHIGUCHI, Y., K. MIURA, S. FUJIKAWA and T. KAYAMA. 1987. Ultrastructure of mycelia and protoplasts of edible basidiomycetes. -Evolution of mycelia and protoplasts as the materials for cell fusion-Res. Bull. Exp. For. Hokkaido Univ. 44: 1435-1473.

OCBA, K., T. KAWASUMI and S. O. YANAGI. 1988. Protoplast fusion between common-B mating-type monokaryons of *Coprinus cinereus*. Trans. mycol. Soc. Japan 29: 271-280

OHMASA, M., Y. ABE, H. FURUKAWA,M. TANIGUCHI and H. NEDA. 1983. Preparation, culture and fusion of protoplasts of mushrooms of the gene *Pleurotus*. Proc. Nihon Kin Gakkai. 28: 59.

OHMASA, M. 1986. Intraspecific protoplasts fusion *Pleurotus ostratus* using auxotrophic mutants. Japan. J. Breed. 36: 429-433.

OHMASA M. and F. FURUKAWA. 1986. Analysis of esterase and malate dehydrogenase isozymes of *Lentinus edodes* by isoelectric focusing for the identification and discrimination of stocks. Trans. Mycol. Soc. Japan. 27: 79-90.

OHMASA, M., Y. ABE, H. FURUKAWA, M. TANIGUCHI and H. NEDA. 1987. Preparation and culture of protoplasts of some Japanese cultivated mushrooms. Bull. For. and For. Prod. Res. Inst. 343: 155-170.

PEBERDY, J. F. and R. K. GIBSON. 1971. Regeneration of *Aspergillus* nidulans protoplasts. J. gen. Microbiol. 69: 325-330.

PEBERDY, J. F. and C. E. BUCKLEY. 1973. J. gen. Microbiol. 74: 282-288. (See Toyoda and others, 1984.)

PEDERDY, J. E., C. E. BUCKLEY, D. C. DALTREY and P. M. MOORE. 1976. Factors affecting protoplast release in some filamentous fungi. Trans. Br. Mycol. Soc. 67(1): 23-26.

PEBERDY, J. F. 1979. Fungal protoplasts: istlation, reversion, and fusion. Ann. Rev. Microbiol. 33: 21-39.

PRING. D. R., C. S. LEVINGS, W. W. L. HU and D. H. TIMOTHY. 1983. Proc. Natl. Acad. Sci. USA. 74: 2904-2908. (See Katayose, 1990)

SHIROISHI, S. 1987a. Analysis of isozymes. Rinnboku no ikusyu. 142: 23-25.

SHIROISHI, S. 1987b. Analysis of isozymes. Rinnboku no ikusyu. 143: 34-37.

SVOBODA, A. and O. NECAS. 1966. Nature. 210. (See Toyoda and others, 1984.)

SPECHT, C. A., C. P. MOVOTNY, and R. C. ULLRICH. 1983. Isolation and characterization of mitochondrial DNA from the basidiomycete *Schizophyllum commune*. EXp. Myco. 7: 336-343.

SUNAGAWA, M., M. OHMASA, S. YOKOTA, N. YOSHIZAWA and T. IDEI. 1989. Isolation and reversion of protoplasts of *Auricularia auricula-judae* and *Auricularia polytricha*. Mokuzai Gakkaishi. 35: 1131-1138.

SUNAGAWA, M., M. OHMASA, S. YOKOTA, N. YOSHIZAWA and T. Idei. 1991. Interspecific protoplast fusion between auxotrophic mutants of *A. polytricha*. Mokuzai Gakkaishi. 37: 1069-1074.

TAMAI, Y., K. MIURA and T. KAYAMA. 1990. Electrical fusion of protoplast between two Pleurotus species. Mokuzai Gakkaishi 36: 487-490.

TAMAI, Y., K. MIURA and T. KAYAMA. 1988. Studies on breeding of edible basidiomycetes by electric-fusion-Characterization of protoplasts from various mycelia-Res. Bull. Exp. For. Hokkaido univ. 45: 833-854.

TOYODA, H., T. HIRAI, H. SUMIYA, Y. KAWAKAMI, M. SAKAMOTO and R. USHIYAMA. 1984. Preparation and cell wall regeneration of protoplasts of *Lentinus edodes*. Men. Fac. Agr. Kinki Univ. 17: 121-130.

TOYOMASU, T., T. MATSUMOTO and K. MORI. 1986. Interspecific protoplast fusion between *Pleurotus ostreatus* and *Pleurotus salmoneo-stramineus*. Agric. Biol. Chem. 50(1): 121-127.

TOYOMASU, T. and K. MORI. 1987. Fruit body formation of the fusion products obtained on interspecific protoplast fusion between *Pleurotus species*. Agric. Biol. Chem. 51(7): 2037-2040.

TUDZYNSKI, P., A. DUVELL, and K. ESSER. 1983. Cuur. Gent. 8: 651-619. (See Katayose, 1990)

USHIYAMA, R. 1983. Isolation and applicatiotive utilization of protoplasts from edible fungi. Proc. Nihon Kin Gakkai. 27: 10-11.

WRIGHT, W. E. 1978. The isolation of heterokaryons and hybrids by a selective system using irreversible biochemical inhibitors. Exp. Cell Res. 112: 395-407.

YANAGI, S. O. and I. TAKEBE. 1984. An efficient method for the isolation of mycelial protoplasts from *Coprinus macrorhizus* and other basidiomycetes. App. Microbiol. Biotechnol. 19: 58-60.

YANAGI, S. O., M. MONMA, T. KAWASUMI, A. HINO, M. KITO and I. TAKEBE. 1985. Conditions for isolation of and colony formation by mycelial protoplasts of *Coprinus Macrorhizus*. Agric. Biol. Chem. 49(1): 171-179.

YOKONO, T., Y. TAMAI, K. MIURA, T. KAYAMA and M. HYAKUMACHI. 1988. Electrofusion of the auxotrophic mutants of *Rizoctonia solani*. Mokuzai Gakkaishi. 34. 176-180.

YUI, Y., Y. KATAYOSE, and K. SHISIDO. 1988. Two linear plasmid-like DNA elements simultaneously maintained in *Pleurotus ostreatus*. Biochim. et Bioph. Acta. 951: 53-60.

ZONNEVELD, B. J. M. 1972. Morphogenesis in Aspergillus nidulans: the significance of α-1, 3-glucan of the cell wall and α-1, 3-glucanase for cleistothecium development. Biochemica et biophysica Acta. 1973: 174-187. (See Peberdy et al., 1976.)

摘　　要

本研究はキクラゲとアラゲキクラゲのプロトプラスト融合を行うにあたりプロトプロストの調製，培養条件を検討し，また雑種細胞を選抜するため代謝阻害処理を行い，プロトプロスト融合によって得られた融合株の性質を明らかにするため様々な解析を行った。内容は以下のように大要される。

1. キクラゲとアラゲキクラゲの性質

キクラゲとアラゲキクラゲの菌糸伸長に関する最適温度は共にPDA(20%ジャガイモ抽出液，1%デキストロース，1.5%寒天)培地上で25—30℃であった。また菌糸伸長速度に関しては，両菌株共に同程度であった。

ブナ，シラカンバ及びミズナラの木粉培地を用いて菌糸伸長の比較を行った結果，キクラゲ，アラゲキクラゲ共にシラカンバ培地で最も良い結果が得られた。

添加物の影響を検討するため，両菌株に対して菌糸伸長が良かったシラカンバ培地に米糠及びフスマが添加された。その結果，米糠，フスマ共に無添加培地よりもキクラゲ，アラゲキクラゲの菌糸伸長に対して促進効果が認められた。栽培特性において，アラゲキクラゲの方が，子実体を形成するまでの培養日数が短く，また子実体の収量も良い結果が得られた。

2. キクラゲとアラゲキクラゲのプロトプラストの調製・培養

キクラゲ及びアラゲキクラゲのプロトプラストの調製に影響を及ぼすと考えられる細胞壁溶解酵素，菌糸の培養日数等が検討された。その結果，菌糸の培養培地をSMY (1%ショ糖，1%麦芽エキス，0.4%酵母エキス）でキクラゲでは4日間，アラゲキクラゲでは6日間培養した菌体を用いて，酵素処理（ノボザイム234　1%とキチナーゼ0.1%）を行った場合にプロトプラストの高い収量（$5-7\times10^7$ 個/ml）が得られた。

作出されたプロトプラストを菌糸まで復帰させるため，菌糸の培養日数及び再生培地に関するプロトプラストの培養条件を検討した。その結果，キクラゲでは培養4日目，アラゲキクラゲでは6日目の菌体を用いて前述した条件でプロトプラストを単離し，それらをStMY（1%澱粉，1%麦芽エキス，0.4%酵母エキス）再生培地に塗布した場合に高い再生率（6.5—7.5%）が得られた。

3. キクラゲとアラゲキクラゲのプロトプラスト融合

キクラゲとアラゲキクラゲから誘導した一核菌糸（MoA 11，MoP 16）に対し融合前処理として代謝阻害処理を行った。処理後のプロトプラストの破壊率と再生率の結果により，MoA 11のプロトプラストに対しては4%のヨード酢酸ナトリウム，アラゲキクラゲのプロトプラストに対しては4%のジエチルピロカーボネートによる処理が適当であると判断された。それらのプロトプラストを用いて，電気的な融合条件を検討した。0.1 mMの塩化カルシウム

および0.5 mM塩化マグネシウムを含む0.5 Mマンニトール溶液に懸濁したプロトプラストを2 MHz 500 V/cmの高周波を10分間印加し，次いで10 μsec, 3 kV/cmの直流パルスを1秒間隔で2—5回印加して融合処理を行った。そして，それらのプロトプラストをStMY再生培地に塗布した場合に高い融合率（1.2%）が得られた。

4. 融合株の性質

5株の融合株の菌糸伸長に関する最適温度は，PDA培地上で30℃であり，これはMoA 11と類似していた。また，菌糸伸長速度は親株及びMoP 16と類似していた。

ブナ，シラカンバ及びミズナラの木粉培地を用いて菌糸伸長の比較を行った結果，5株の融合株のすべては親株と同様，シラカンバ培地で良い結果が得られた。また，木粉培地に添加物（米糠，フスマ）を加えた場合に無添加培地よりも菌糸の伸長が促進された。すべての融合株は親株，一核菌糸より菌糸密度が濃かった。

融合株を親株，一核菌糸のそれぞれと対峙培養した結果，5株の融合株すべては両方に対して拮抗線を形成した。また，その融合株はクランプコネクションを有していた。

エステラーゼのアイソザイム分析において，すべての融合株はそれぞれの一核菌糸に特異的なバンドを併せ持っていることが確認された。

α-ガラクトシダーゼ活性においてはすべての融合株に活性が認められたが，フェノールオキシダーゼ活性においては5株の融合株のうち1株においてそれが認められた。

融合株の子実体形成を試みたところ，1つの融合株において幼子実体の形成が認められ，その融合株のミトコンドリアDNAの解析を行った。その結果，ゲノムサイズが90 kbpであることが確認された。Hind IIIを用いた制限酵素断片パターンにおいては，その融合株はそれぞれの一核菌糸に特異的なバンド（3.2, 3.5 kbp）を併せ持っていた。また，その融合株に一核菌糸と異なる2つのバンド（2.1, 2.5 kbp）が存在することから，それらの断片は両菌株のミトコンドリアDNAがプロトプラストの融合によって相互に組み換えが起こった結果生じたものと考察された。

以上の結果により，5株の融合株はキクラゲとアラゲキクラゲのプロトプラスト融合によって得られたヘテロカリオンであることが結論された。