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Characteristics of Fusion Products between *Pleurotus ostreatus* and *Pleurotus florida* Following Interspecific Protoplast Fusion

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느타리버섯과 사철느타리버섯의 종간 원형질체융합 균주의 특성

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Abstract: Interspecific heterokaryons were obtained between auxotrophic mutants of *Pleurotus ostreatus* and *Pleurotus florida* by polyethylene glycol-Ca^{++} induced fusion of somatic protoplasts. The fusion products produced colonies of dense growing mycelium after 10-14 days culture on hypertonic Mushroom Minimal Medium. When they were transferred to Mushroom Minimal Medium plates, the sectors showed normal vegetative morphology but the colonies of irregular shape varied in growth rate. All of the colonies produced fruit body of normal pilei. Some heterokaryon colonies bearing none or only a small amounts of basidiospores were isolated. Fusion products, generally, gave higher basidiocarp yields than the parents.

Keywords: Interspecific protoplast fusion, Sporeless heterokaryon, *Pleurotus ostrearus*, *Pleurotus florida*, Basidiomycetes.

Protoplast fusion holds great potential as a tool for improvement of industrially important microorganisms (Hamlyn and Ball, 1979; Peberdy 1980; Chang *et al.*, 1982; Peberdy and Bradshaw, 1982). Fusion of fungal protoplasts after treatment with polyethylene glycol (PEG) has been described as a new technique for interspecific heterokaryon formation or hybridization (Anne and Peberdy, 1975, 1976; Ferenczy *et al.*, 1975).

In relation to the formation of interspecific heterokaryon or hybridization many investigations have been studied on fungi including *Aspergillus nidulans* and *A. rugulosus* (Kevei and Peberdy 1977, 1979; Bradshaw, 1983), *Aspergillus nidulans* and *A. fumigatus* (Ferenczy, 1976; Ferenczy *et al.*, 1977), *Mucor pusilus* and *M. miehei* (Ohnuki *et al.* 1982), *Penicillium roquefortii* and *P. chrysogenum* (Anne *et al.*, 1976), *Penicillium chrysogenum* and *P. cyaneo-fulvum* (Peberdy *et al.*, 1977), *P. citrinum* and *P. cyaneo-fulvum* (Anne and Eyssen, 1978). In basidiomycetes, however, few works on interspecies protoplast fusion were reported.

The oyster mushroom, *Pleurotus* spp., has been esteemed as edible and good for many years. The production and consumption of this fungi have increased rapidly all over the world. One of the problems

for the oyster mushroom growers is allergic to *Pleurotus* spores (Hausen *et al.*, 1974; Schulz *et al*, 1974; Sakula, 1974; Zadrazil, 1974; Noster *et al.*, 1976). The fungi spores cause allergy of the farmers working on the place and the persons sensitive living in the vicinity (Eger, 1976). Therefore, *Pleurotus* should be improved to be sporeless as well as be high yielding and good quality. Sporeless mutant reported previously in *Coprinus macrorhizus* (Takemaru and kamada, 1971), *Coprinus congregatus* (Ross *et al.*, 1976; Ross, 1977). *Pleurotus ostreatus* (Eger *et al.*, 1976), *Pleurotus pulmonarius* (Ohira, 1979) and *Schizophyllum commune* (Bromberg and Schwalb, 1977). These heterokaryon may be very useful for study of the genetic control of sporulation.

This investigation was attempted to examine protoplast fusion as a potential tool in the mushroom breeding technique.

Materials and Methods

Strains and Growth Conditions

The genotypes and origins of the strains used in these experiments were listed in Table I. They were maintained on the Mushroom Complete Medium (MCM; Raper *et al.*, 1972), containing (g/l) $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 0.46, K_2HPO_4 1.0, Bacto-peptone (Difco) 2.0, Bacto-yeast extract (Difco) 2.0, glucose 20.0. Bacto-agar (Difco) 20.0. Heterokaryon selection after protoplast fusion was carried out on a osmotically stabilized Mushroom Minimal Medium (MMM; Raper *et al.*, 1972). It consists of (g/l) $MgSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 0.46, K_2HPO_4 1.0, Thiamin-HCl 120 μg; DL-asparagine 2.0, glucose 20.0, Bacto-agar 20.0, and is supplemented with 0.6 M KCl. Bottom agar was of 2.0% while overlaying soft agar was of 0.75% Bacto-agar (Difco).

Protoplast Formation

Disks of sterile cellophane membrane were placed on the surface of MCM in petridishes. The mycelial disks were ready for protoplast production when mycelia had grown over the disks. Mycelial disks of *P. ostreatus* from 4 days and *P. florida* from 5 days culture at 25°C were removed to clean sterile petri dishes and the lytic enzyme stabilizer solution was added immediately. The lytic mixture consisted of three kinds of enzyme in buffered osmotic stabilizer (Table II).

Protoplast Fusion

The procedure of protoplast fusion were based on those of Anne and Peberdy (1976), and Peberdy and Kevei (1979). At least 10^7 protoplasts of each parent were combined in a fusion tube and centrifused at 500×g for 10 min. The pellet of protoplasts was resuspended in 1 ml of a solution of 30% polyethylene glycol 4000 (PEG) containing 0.1 M $CaCl_2 \cdot 2H_2O$ and 0.05 M glycine, adjusted to pH 8.0 with 0.01M NaOH. After incubation for 5 min. at 30 C, the

Table I. List of strains used.

Species	Strain no.	Marker genotype*	Origin
P. ostreatus	ASI 2-1	Arg	Mutants induced (U.V.) in *P. ostreatus*
	ASI 2-2	Gly Ser.	ASI 106-6** (monokaryon)
P. florida	ASI 2-3	Ribo-1	Mutants induced (U.V.) in *P. florida*
	ASI 2-4	Ribo-2	ASI 124-30** (monokaryon)

* Mutant symbols: Arg (Arginine), Gly (Glycine), Ser (Serine), Ribo (Riboflavin).
** *Pleurotus ostreatus* 106-6 and *P. florida* 124-30 were obtained from spores of *P. ostreatus* 2018, *P. florida* 2016.

Table II. Enzymes and osmotic stabilizers systems used to protoplast release.

Enzymes	Osmotic stabilizer	Buffer system
β-D-Glucanase (BDH)+		
Novozym 234 (Novo)+	0.6M	0.05 M Maleate
Snail enzyme	$MgSO_4$	pH 5.8

suspension was diluted with 0.6 M KCl, washed once by centrifugation, and resuspended in 5 ml osmotic stabilizer. Serial dilutions of treated protoplasts were plated onto hypertonic MCM for viability and onto MMM to select for fusion products.

Results and Discussion

Isolation of Fusion Products

After PEG solution treatment of a mixture of complementing *P. ostreatus* and *P. florida* protoplasts, small prototrophic colonies developed on MMM. Fusion frequencies for the various interspecific crosses were shown in Table III. After 10-14 days culture on hypertonic MMM and MCM fusion products produced sectors of dense growing mycelium (Fig. 1). When transferred to MMM plates the sectors gave normal mycelium. (Peberdy *et al.*, 1977). The dense colony might be attributed to the presence of osmotic stabilizer in the medium.

Characteristics of the Fusion Products

Fusion products were different from the parental colony forms, and morphological variants obtained as fusion products from crossed *Pleurotus ostreatus* and *Pleurotus florida* (Table IV). Based on the growth rate and cultural characters of each individual fusion products grown on MMM they could be classified into threetypes; fast growing, moderate growing and slow growing colonies (Fig. 2). Distinct differences were detected between the cultural morphologies of some colonies. All of the colonies produced fruit body and clamp connection. The presence of clamp connection is generally indicative of the dikaryotic condition. There are exceptions, however, since homokaryotic

Table III. Percentage of fusion between protoplasts of *P. ostreatus* and *P. florida* complementing auxotrophs.

Fusion mixtures	Fusion frequencey(%)
ASI 2-1+ASI 2-3	0.50
ASI 2-1+ASI 2-4	3.80
ASI 2-2+ASI 2-3	0.18
ASI 2-2+ASI 2-4	2.00

Table IV. Characteristics of the fusion products.

Fusion mixture	No. of fusion products	1)Mycelial growth on MMM	2) spore	3) Yield of fruit body
ASI 2-1+ASI 2-3	1	M	+	++
	2	F	+	++
	3	S	+	++
	4	M	+	+
	5	F	+	+++
	6	F	+	+++
	7	F	+	+++
	8	F	+	++
	9	F	+	++
	10	M	+	++
ASI 2-2+ASI 2-4	11	M	+	++
	12	M	+	+
	13	M	−	+++
	14	M	−	++
	15	M	+	++
	16	S	−	++
	17	M	+	+
	18	M	+	+++
	19	F	−	+
	20	F	+	+++
ASI 2-1+ASI 2-4	21	F	−	+
	22	S	+	+++
	23	M	−	+
	24	M	•	+
	25	M	−	+
	26	F	+	++
	27	F	+	+++
	28	S	+	++
	29	M	+	++
	30	M	+	++
ASI 2-2+ASI 2-3	31	M	+	++
	32	M	+	++
	33	M	−	+
	34	S	+	++
	35	F	+	++
	36	M	+	+++
	37	M	+	++
	38	M	+	+++
	39	F	+	+++
	40	M	+	+++

1) F=Fast growing type, M=Moderate growing type, S=Slow growing type
2) +=Spore, −=Sporeless
3) Signs refer to estimates of yield of basidiocarp: Fusion products has a greater (+++), Similar (++) and lower yield (+) than parents.

mycelia of some species are known to possess clamps while dikaryotic mycelia of other species apparently lack them. (Raper, 1966).

Fusion products from different matings or growth characteristics of colonies were variable in fruiting behaviour. Morphological differences were recognized in appearance between the basidiocarps of the fusion products and the parents (Fig. 3). Parental *Pleurotus ostreatus* 2018 showed dark grey color and *P. florida* 2016 pale white color on pileus in the early stage, and then fusion products intermediate color, respectively.

Eight heterokaryon colonies bearing none or only a small amounts of basidiospores were isolated (Fig. 4). These strains also could not be distinguished by its growth or characteristics of mycelium from parent (Ohira, 1979) or other fusion products.

The yield of fruit body on fusion products were variable. Many heterokaryons gave higher yield than the parents which is probably due to heterosis (Burnett, 1975: Eger, 1978). Some colonies were lower than parents. These colonies were, generally, sporeless.

적 요

식용버섯의 재배와 소비가 증가해가는 중요성에 비추어 새로운 품종 육성을 위하여 국내의 주요 재배 버섯인 느타리버섯과 사철느타리버섯의 원형질체를 융합하여 종간 체세포 잡종을 선발하였으며, 그 특성을 조사한 결과를 요약하면 다음과 같다.

1. 느타리버섯과 사철느타리버섯의 종간 원형질체 융합율은 0.18-3.80%였다.
2. 융합된 균총의 생장은 빠른 것, 중간 그리고 아주 느린 것으로 나눌 수 있었으며 그 형태는 불규칙하였다.
3. 선발한 40개의 체세포 잡종 모두가 정상적인 자실체를 맺었으며, 자실체의 형태가 모본과는 달리 아주 다양하게 나타났다.
4. 체세포 잡종의 자실체색은 짙은 회색을 가진 느타리버섯과 연한 백색을 가진 사철느타리버섯의 중간색을 나타내었다.
5. 무포자 또는 극히 적은량의 포자를 지닌 균주를 선발하였으며 이들의 자실체 형태는 정상이었다.
6. 수량이 다소 낮은 무포자 균주를 제외하고는 대체적으로 체세포 잡종의 자실체 수량이 모본보다 증가했다..

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EXPLANATION OF FIGURES

Fig. 1. 10-14 days cultures developed from a fusion mixture of *P. ostreatus* and *P. florida* plated on (1) stabilized MM and (2) stabilized CM.

Fig. 2. Morphological variants obtained as fusion products from *P. ostreatus* and *P. florida* following protoplast fusion.

Fig. 3. Basidiocarps of the fusion products on sawdust medium were morphologically distinguishable from the wild type of *P. ostreatus* 2018 and *P. florida* 2016 parents.

Fig. 4. Basidiocarps of the sporeless strains and the wild type parents in bottle culture containing sawdust medium.