韓 國 菌 學 會 誌 第 17 卷 第 3 號
Kor. J. Mycol. Vol. 17, No. 3, 137-144, 1989

Effects of Incompatibility on Protoplast Fusion between intra-and inter Species in Basidiomycete, *Pleurotus* spp.

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느타리버섯의 不和合性이 種內 및 種間 原形質體 融合에 미치는 影響

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ABSTRACT: Effects of incompatibility existing between intra-and interspecies in *Pleurotus* spp. on protoplast fusion, clamp formation of their fusants and fruitbody production were investigated.

Protoplast fusion between intra-and interspecies of the fungus was achieved by Poly ethylene glycol treatment. The fusion frequency between intraspecies was a little higher than that of interspecies. Fusion frequency between interspecies was not correlated with their similarities based on isozyme patterns. In case of protoplast fusion between intra-and interspecies, the fusants from the compatible isolates produced normal fruit bodies, while those from the incompatible isolates did not produce clamp connections and fruit bodies except those of a few isolates presumed as mutants.

KEYWORDS: Incompatibility, Protoplast Fusion *Pleurotus* spp.

Protoplast fusion has been attentioned as a tool for genetic manipulation and breeding in industrial microbiology (Peberdy, 1980). The improvement of edible mushroom spawn through protolast fusion is interesting not only for practical purposes but also for basic life science, because Basidiomycetes show the unique stage of dikaryotic hyphae and many incompatible mating combinations in one species. (Iijima, *et al.* 1986).

Basidiomycetes have unlinked incompatibility genes called A and B which have shown to control the expression of two distinct mycelial cell type occurring at different stage of the life cycle (Raper, 1966). A developmental process of the complexity of fruit body formation in Basidiomycetes must be controlled by the incompatibility sexual gene loci.

Hybridization through protoplast fusion could also provide information to aid our understanding of interspecies relationships and to supplement existing approaches based on morphological differences. (Anne *et al.* 1985) In fact, the taxonomy of *Pleurotus* species has not yet been established well and there is much confusion as to *Pleurotus ostreatus* complex. (Li *et al.* 1978) Namely, *P. ostreatus* and *P. florida* (Go *et al.* 1981). *P. ostreatus* and *P. columbinus,* and *P. pulmonarius* and *P. sajor-caju* (Toyamasu *et al.* 1987) were crossed well each other.

Therefore, the present works were performed to investigate effects of incompatibility on their fusion products based on relationship interspecies in *pleurotus* genus through protoplasts fusion.

Materials and Methods

Table I. The list of strain and species used for the experiments

Species	Strain No.	Auxotroph marker
P. ostreatus ASI 2018	18-50-4	Tyrosine
	18-50-35	Orinithine
	18-50-37	Biotin, Riboflavin
P. ostreatus ASI 2054	54-50-5	Adenine
	54-50-6	Tyrosine, Glutamine
	54-50-23	Phenylalanine, Biotin
P. ostreatus ASI 2097	97-60-1	Adenine, Thymidine
	97-60-3	Ornithine, Proline
	97-60-11	Inositol
	97-60-15	Tryptophan, $(NH_4)_2SO$
P. spodoleucus ASI 2010	2010-2	Lysine
	2010-3	Methionine
	2010-4	Folic acid, Riboflavin
P. florida ASI 2016	2016-4	Biotin
	2016-5	Guanine
	2016-8	Adenine, Hypoxanthine
	2016-22	Uracil
P. sajor-caju ASI 2070	70-60-4	Riboflavin
	70-60-17	Lysine
	70-60-71	Lysine, PABA
P. cornucopiae ASI 2011	2011-1	Pyridoxine
	2011-44	Biotin, Riboflavin
	2011-45	Guanine, Histidine
	2011-62	Serine
P. pulmonarius ASI 2091	2091-13	Arginine
	2091-17	Cystine
	2091-37	Orinithine
P. eryngii ASI 2126	26-1	Biotin
	26-5	Guanine
	26-10	Adenine, Biotin
	26-17	Hypoxanthine

Organisms

The dikaryotic lines of the species in *Pleurotus* were grown on potato dextrose agar (PDA) and stocked at 4°C refrigerator in the Agricultural Sciences Institute (ASI). The monokaryotic auxotrophs of the species were isolated from basidiospore suspension of each lines after UV-light treatment as previously described (GO. 1985) for using genetic marker. The auxotrophs of the species used in these experiments were listed in Table I. Incompatibility between intra-and interspecies of the monokaryotic mutants confirmed dependent upon whether clamp connections forming or not after crossing one another with full combinations.

Protoplast formation

The organisms were inoculated on the sterile cellophan membrane placed on media of mushroom complete medium (MCM) in a petridish and incubated at 25°C for 2 to 7days. As the mycelia grew, the cellophan including the mycelia was moved from the media into new sterile petridish and lytic enzyme solution was poured to submerge the mycelia. Novozyme 234 (Novo Industry in Denmark) was used solely as a lytic enzyme. 5 mg of the lytic enzyme was solved in 1 m*l* of 0.6 M sucrose osmotic stablizing solution. The mixture were incubated in a gentle shaking (80 rpm) incubator at 28°C for about two hours. The number of protoplasts was counted by haemocytometor under the microscope

Similarity

A similarity between interspecies in *Pleurotus* spp. were determined based upon isozyme patterns of esterase and peroxidase by electrophoretic analysis as previously described. (Park *et al.* 1988) For the electrophoresis, each dikaryotic line of the species was grown in submerged culture for 10days and the mycelium was ground in a mortar dish in presence Tris-HCl buffer (pH 7.5) after harvesting the mycelium. The mycelial suspension was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was collected as used for the electrophoretic sample.

The similarity was assessed by following formular, similarity (%) = number of same bands/total number of bands × 100. Based on these similarities, a dendrogram was drawn according to single linkage method.

Protoplast fusion

The general procedure of protoplast fusion were following the manuals of Pebery (1980). About 10^7 protoplast of each parents were combined in a fusion tube and centrifused at 2,000 rpm for about 7 min. The pellet of protoplasts was resuspended in 1 m*l* at 30% polyethylene glycol (M.W. 4,000) solution containing 0.1 M $CaCl_2 \cdot 2H_2O$ and 0.05 M glycine and adjusted pH to 8.0. After incubation for 5 min at 30°C, the suspension was diluted with 5 m*l* of 0.06 M sucrose. Serial dilution of the protoplasts were plated onto hypertonic mushroom minimal medium (MMM) and MCM as a control. The plates were incubated at 25°C for 10-15days. The fusion frequency was assessed by determination of the ratios of colonies that arising as result of nutritional complementation on MMM compare to the number of colonies growing on MCM.

Characteristics of fusion products

Clamp connections of the fusion products were observed under 400 × microscope. Assessment of the mycelial growth of fusion isolates was made in length on MCM at 25°C for 7days growing. Whether development of primordia and sporophore growing from the vegetative stage were investigated using sawdust substrates preparing as previously described. (Go *et al.* 1986).

Results and Discussions

Intraspecies protoplast fusion

Protoplast fusion between intraspecies were achieved not only between compatible parents, but also incompatible one. Whether compatible or not the fusion frequency were almost same with more or less 20%, although there were except a few cases of *P. ostreatus* ASI 2097, *P. florida* (ASI 2016) and *P. pulmonarius* (ASI 2091) which showed higher rates in compatible fusion than those in incompatible case. While *P. eryngii* showed higher rates in case of incompatible fusion than those in compatible one (Table II.)

However, clamp connections were formed mostly on the mycelium of compatible fusion products, not on the incompatible one. But there were a few exceptions in case of *P. florida* and *P.sajor-caju* which showed clamp connections on the incompatible fusion products even though low rates of 15 and 16 percent, respectively.

Mycelial growth of the fusion products were seemed to be good not only compatable case but also incompatible one in case of limiting examination of five isolates each. But the primordia were developed only in isolates from the compatible fusion and did not developed in isolates from incompatible fusion.

The sporphores of isolates from compatible protoplast fusion which have clamp connections on their mycelium were normally grown and matured although there were exception isolates

Table II. Effect of incompatibility on protoplast fusion between intraspecies and characteristics of their fusion products

Combinations[1]	Incompatibitlity	Fusion rate(%)	Clamp formation	Primodia[3] formation	Developing[4] fruibody
POS1 4×37	+	25.9	100	PF	FD
4×35	–	25.3	0	NF	ND
POS2 5×23	+	25.2	100	PF	FD
5×6	–	24.2	0	NF	ND
POS3 1×15	+	28.1	100	PF	FD
3×11	–	13.6	0	NF	ND
PCO4 44×45	+	22.4	100	PF	FD
44×62	–	20.1	0	NF	ND
PFL 4×5	+	32.0	100	PF	FD
5×8	⚠	34.0	30	PF	ND
5×22	–	23.6	15	NF	ND
PSA 4×71	+	29.4	100	PF	FD
4×17	–	33.4	16	FF	FD*
PPU 37×17	+	19.1	43	PF	FD
37×13	–	14.5	0	NF	ND
PER 5×17	+	17.4	100	PF	FD*
5×10	–	36.3	0	NF	ND

1) POS1; *P.ostreatus* ASI 2018 POS2; *P. ostreatus* ASI 2054 POS3 *P.ostreatus* ASI 2097
PCO *P. cornucopiae* ASI 2011 PSP *P. spodoleucus* ASI 2010 PFL *P. florida* ASI 2016
PSA *P. sajor-caju* ASI 2070 PPU *P. pulmonarius* ASI 2091 PER *P. eryngii* ASI 2126
2) +; compatibility –; incompatibility ⚠; common pairings
3) PF; primodia formation NF; non primodia formaiton
4) FD; fruitbody developed ND; non fruitbody developed FD*; abnormal fruitbody

of *P. florida* and *P. eryngii* which grown abnormally even from compatible fusions.

Incompatibility was a barrier to fuse between isolates in the traditional crosses. Therefore, the mycelium can be fused only in the case of compatible. The protoplast fusion was achieved by PEG treatment. It seems the recognition system of incompatibility to exist on the cell wall (Yamaguchi *et al.* 1984) which also regarded as an obstacle to cytoplasmic fuse. Therefor, by removal of the cell wall protoplasts can be fused and exchanged cytoplasmic materials each other. The fusion ratios between incompatible combination was same to those of compatible in *Pleurotus* spp. These data suggest that the mechanism of fusion and stability of fused protoplasts are not affected by mating characters. (Maraz, 1978) However, clamp connections and fruit bodies were formed mainly on mycelium of compatible fusion products, not on the incompatible productions even they were fused cytoplasmically. Because those incompatibility gene exists on the chromosome. A few species of *P. florida* and *P. sajor-caju* showed exception which formed clamp connections although the rates were quite low even in case of incompatible fusion. This resulted in a cause of mutation occurred during either protoplast forming, fusion, and regeneration as reported by Hamamoto *et al.* (1986).

Even only one case of *P. eryngii* produced abnormal fruit body, it meant that a mutant

Table III. Clamp formations of pairing between interspecies in full combinations

Species[1)]	POS 1	POS 3	POS 2	PPU	PFL	PSP	PSA	PCO	PER
POS 1		+	+	−	+	−	−	−	−
POS 3			+	−	+	−	−	−	−
POS 2				−	+	−	−	−	−
PPU					−	−	−	−	−
PFL						−	−	−	−
PSP							−	−	−
PSA							−	−	−
PCO									−
PER									

1) Indicated in Table II 2) +; clamp formation −; not clamp formation

Table IV. Similarity of the species based on isozyme patterns in *Pleurotus* spp.

Species[1)]	POS 1	POS 3	POS 2	PPU	PFL	PSP	PSA	PCO	PER
POS 1		81.4	81.4	56.7	46.0	43.2	25.0	13.9	23.2
POS 3			70.6	63.3	50.0	43.2	27.8	16.9	18.1
POS 2				60.0	49.0	28.5	22.2	19.4	26.7
PPU					51.6	37.7	55.3	33.3	28.8
PFL						57.9	44.4	33.3	20.7
PSP							57.9	57.9	29.2
PSA								26.2	20.6
PCO									
PER									

1) Indicated Table 2

could be produced through protoplast fusion ether while processing of inducing auxotroph by U.V. or protoplast fusion.

Relationships interspecies

In mating between each line of monokaryotic mycelium, *P. ostreatus* and *P. florida* made dikaryon successfully each other as shown in Table III. However, mycelial mating between monokaryontic line of *P. ostreatus P. pulmonarius, P. sajor-caju, P. spodoleucus, P. cornucopae* and *P. eryngii* was not achieved, each other.

Based on isozyme patterns of esterase and peroxidase by electrophorestic analysis, similarity between inter species were shown as in Table IV. and Fig. 1. Between *P. ostreatus* 1 (ASI 2018) from local field and *P. ostreatus* 3 (ASI 2097) from Italy showed the highest rates of similarity and followed by *P. ostreatus* 2 (ASI 2054) from Denmark. To these *P. ostreatus* species, *P. pulmonarius* had much affinity than others. However, *P. eryngii* and *P. cornucopae* showed the lowest similarity with *P. ostreatus* species.

To determine incompatibility existing between inter species and their relationship, mating were carried out. A monokaryotic mycelium of *P. ostreatus* could be crossed only with *P. florida* and made clamp connections to become a dikaryon. This was agreed with previous results (Li *et al.* 1978, Go *et al.* 1981). However, between monokaryotic mycelium of others showed a sexual incompatibility each other. They didn't form clamp connections to

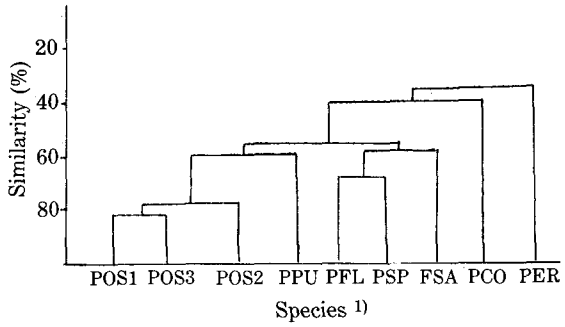

Fig. 1. Genetic similarty dendrogram among isolates of *Pleurotus* spp. based on isozyme variation.
1) see in Table II.

dikaryon when they were placed for somatic crossing.

Biochemical properties such as isozyme patterns of the fungi were analysised to find out their relationship with the sexual incompatibility and effects on following protoplast fusion. As shown in Table IV and Fig 1, there were neither reliable relationship with sexual incompatibility, nor correlations with fusion frequency. Even the isozyme bands could be used for genetic hybridization marker. (Royse *et al.* 1982).

Interspecies fusion

Protoplast fusion between interspecies

Table V. Protoplast fusion rates between interspecies in *Pleurotus* spp. (%)

species[1)]	POS 1	POS 3	POS 2	PPU	PFL	PSP	PSA	PCO	PER
POS 1		10.0	7.6	6.2	8.8	11.3	18.0	22.0	6.5
POS 3			13.5	11.7	10.1	5.5	9.8	6.3	13.8
POS 2				11.1	14.3	10.1	5.9	14.3	14.2
PPU					8.6	10.2	4.3	12.5	12.7
PFL						10.8	13.5	18.6	11.9
PSP							4.9	18.8	4.8
PSA								14.7	5.3
PCO									6.4
PER									

1) see in Table II.

Table VI. Clamp formation and fruitbody development of protoplast fusion products

	Species[1)]	Clamp formation[2)]								
		POS 1	POS 3	POS 2	PPU	PFL	PSP	PSA	PCO	PER
Development of fruitbody[3)]	POS 1		+	+	−	+	−	⚠	−	−
	POS 3	FD		+	−	+	−	⚠	−	−
	POS 2	FD	FD		−	+	+	−	−	−
	PPU	ND	ND	ND		−	−	−	−	−
	PFL	FD	FD	FD	ND		+	−	−	⚠
	PSP	ND	ND	ND	ND	FD		+	−	−
	PSA	ND	ND	ND	ND	ND	ND		−	−
	PCO	ND	ND	ND	ND	ND	ND	ND		−
	PER	−	ND	FD	ND	ND	ND	−	ND	

1) see in Table II
2) +; clamp formation −; not clamp formation ⚠; psudoclamp formation
3) FD; Fruitbody development ND; not fruitbody development

were achieved by treatment of PEG. The interspecies between *P. ostreatus*, *P. Pulmonarius*, *P. florida*, *P. spodoleucus*, *P. cornucopae* and *P. eryngii* were fused successfully one and another. The fusion frequencies were varied as shown in Table V and lower than those of intraspecies. The fusion rates were more or less 10% with a few case of exceptions. The fusion rates even between protoplasts which showed low rates of similarity were almost same to those between protoplasts having higher rates of similarity. The fusants didn't show mycelial segrigation even at low similarity.

However, the fusion products of interspecies showed various characteristics. The clamp connections formed in the products of all case of between intra-and interspecies of *P. ostreatus* and *P. florida*. Also a few clamps were found in other cases of between *P. spodoleucus* and *P. ostreatus* 2 (ASI 2054) *P. florida* and *P. spodoleucus*, *P. spodoleucus* (ASI 2054) and *P. sajor-caju*.

Primodia forming and sporophore developing were successed only in a few cases of protoplasts fusion among intra and interspecies combinations of *P. ostreatus* and *P. florida*, *P. ostreatus* 2 (ASI 2054) and *P. eryngii*, and *P. florida* and *P. spodoleucus*.

To improve mushroom strain, the ability of primodia sporophore development is essential. In these experiments the fusion products formed clamp connections and produced fruit bodies in a combination between *P. ostreatus* and *P. florida* which can mated by the conventional means of mycelial anastomosis. And fusants from between *P. ostreatus* 2 (ASI 2054) and *P. erygii*, *P. florida* and *P. spodoleucus* developed fruitbody, even they were incompatible each other. The results were quite agreeable to those reported by Toyamasu *et al.* (1987) with a few exceptions. But any basic differences between protoplast fusion and sexual hybridization were not reported. It was not clear why all of the fusant didn't form fruitbodies except in cases of compatable combinations between *P. ostreatus* 2 (ASI 2054) and *P. eryngii* and *P. florida* and *P. spodoleucus*. The formation of fruitbodies from incompatible fusants between *P. ostreatus* 2 (ASI 2054) and *P. eryngii*, *P. florida* and *P. spodoleucus* showed the possiblities to use as a tool for improving strains among incompatible relations. In the case of fusion between *P. ostreatus* and *P. eryngii* the fusion products didn't have clamp connection even though the fusants formed fruitbodies. It might be due to mutation during either auxotroph making or protoplast fusion. On the other hand, in most cases of the protoplast fusion between incompatible isolates neither formed clamps connections, nor developed fruitbodies. Even though interspecific combinations which exsit the incompatibility gene on chromosome protoplast fusion were achieved by PEG treatment. Therefore, it might be neccessary to enforce additional treatment for fruiting from between incompatibility isolates.

摘 要

느타리버섯의 種內 및 種間 原形質體 融合을 Polyethylene Glycol (P. E. G. M. W. 4, 000) 處理로 成功하였다. 種內 原形質體 融合에서 和合性 菌株間 融合으로 얻은 菌株는 clamp를 形成하였으며 正常的인 子實體가 發育되었으나 不和合成 菌株間 融合으로 얻은 菌株는 大部分 clamp 形成 및 子實體 發育이 이루워 지지 않았다. 種間 原形質體 融合率은 10% 內外로 種內 融合率보다 낮았다. 融合率과 同位酵素 band에 依한 生化學的 類似度와는 서로 相關이 없었다. 種間 原形質體 融合의 경우도 菌絲體 融着에 의한 交配에서 和合性을 보인 原形質體間 融合에서 얻은 融合 菌株는 正常的인 子實體가 發育되었으나 不和合性 菌株間 融合에서 얻은 菌株는 變異로 추측되는 몇 가지 경우를 除外하고 大部分이 clamp 形成 및 子實體 發育이 이루워 지지 않았다.

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Accepted for Publication 4 July 1989