ORIGINAL ARTICLE

Protoplast fusion between *Pleurotus ostreatus* and *P. djamor*

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Abstract

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Protoplast fusion between *Pleurotus ostreatus* and *P. djamor* was carried out by isolating protoplasts from 4-day-old monokaryotic mycelia cultured on malt extract broth. The mycelia were then agitated at 100 rpm for 2 h with 9 mg Lysing Enzyme (Sigma L-1412) in 1 ml osmotic stabilizer (0.6 M $MgSO_4 \cdot 7H_2O$ in 0.05 M sodium maleate buffer,pH 5). The freshly prepared protoplasts were then mixed and incubated in 40% PEG (polyethylene glycol 6,000)/0.05 M $CaCl_2 \cdot 2H_2O$ for 20 min at room temperature. All protoplasts were regenerated on Regeneration Medium for 7-12 days. There were 412 regenerated colonies detected but only two of them were selected as fusants by posessing clamp connections on their mycelia. The fusants were proved to be "hybrids" of *P. ostreatus* and *P. djamor*. Their mycelia were significantly faster in growth and larger in size than the parental strains. They showed bands common to their parents when esterase was used for isozyme studies. The fruiting bodies of the fusants also showed recombined characteristics of the parental strains.

Key words : protoplast, *Pleurotus ostreatus, P. djamor*, single-spore isolation, monokaryon, polyethylene glycol, isozyme pattern, spore print

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บทคัดย่อ

พรรณี ฐิตาภิชิต และ ชนินันท์ พรสุริยา
การรวมโพรโตพลาสต์ระหว่างเห็ดนางรมและเห็ดนางนวล
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การรวมโพรโตพลาสต์ระหว่างเห็ดนางรมและเห็ดนางนวล มีขั้นตอนการทดลอง โดยเริ่มจากการแยกโพรโตพลาสต์โดยนำเส้นใยประเภทโมโนคาริออน (monokaryotic mycelia) ที่เลี้ยงอยู่ในอาหาร malt extract broth เป็นเวลา 4 วันมาทำการย่อยโดยการเขย่าในสารละลาย Lysing Enzyme (Sigma L-1412) ที่มีความเข้มข้น 0.9 มก./1 มล. ของสารละลายออสโมติกสเตบิไลเซอร์ ที่อุณหภูมิห้อง เป็นเวลา 2 ชั่วโมง ตามด้วยการนำโพรโตพลาสต์ที่เตรียมได้ใหม่ ๆ ของเห็ดทั้ง 2 ชนิดนี้มารวมกันโดยบ่มในสารละลาย PEG 6000 (polyethylene glycol 6000) ที่มีความเข้มข้น 40% ในสารละลายแคลเซียมคลอไรด์ 0.05 โมลาร์ ที่อุณหภูมิห้อง เป็นเวลา 20 นาที หลังจากนั้นย้ายโพรโตพลาสต์ที่บ่มในสารละลาย PEG ไปเลี้ยงใน Regeneration Medium เป็นเวลา 7-12 วัน ผลการทดลองพบว่ามีโคโลนีเกิดขึ้นทั้งสิ้น 412 โคโลนี แต่ในจำนวนนี้มีเพียง 2 โคโลนีที่พบว่ามีเส้นใยเชื่อม (clamp connection) เกิดขึ้นในเส้นใย จึงได้คัดไว้เป็นฟิวแซนต์ (fusant) ขั้นตอนต่อมาได้ทำการพิสูจน์ความเป็นลูกผสมในฟิวแซนต์ทั้งสอง ซึ่งพบว่าเส้นใยเจริญเร็วกว่าและมีขนาดใหญ่กว่าสายพันธุ์พ่อและแม่อย่างมีนัยสำคัญ รวมทั้งมีแบบแผนไอโซไซม์ (isozyme pattern) ของเอนไซม์ esterase ที่เหมือนของพ่อและแม่ และเมื่อเพาะเส้นใยของฟิวแซนต์ทั้งสอง พบว่าลักษณะของดอกเห็ดมีลักษณะ "ผสม" ระหว่างลักษณะของสายพันธุ์พ่อและแม่

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Protoplast fusion has been used as a method to create mushroom hybrids especially when conventional method cannot be achieved. As conventional hybridization, protoplast fusion can be performed intraspecifically (Kiguchi and Yanagi, 1985; Toyomatsu and Mori, 1987), interspecifically (Takehara *et al*., 1993; Matsumoto *et al*., 1997), intergenerically (Eguchi *et al*., 1993; Zhao and Chang, 1996) and even interheterogenerically (Eguchi and Higaki, 1995; Toyomatsu and Mori, 1987). However, for the same reason as that implied to the conventional method, the greater the distance in genetic relationship between the two mating isolates, the less successful protoplast fusion will be (Anne and Peberdy, 1976).

Pleurotus ostreatus and *P. djamor* together with many other species of the genus *Pleurotus* are widely industrially cultivated in many parts of the world including Thailand. They are rich in nutrition and are claimed to have medicinal value (Gunde-Cimerman, 1999).

The obvious differences between *P. ostreatus* and *P. djamor* are that *P. ostreatus* has creamish white colour both in the caps and spore prints while *P. djamor* has pink colour in both parts of the fruiting body. The shape of fruiting bodies of these two species are also different.

The aim of this study was to construct fusants which may show recombined characteristics of their parents, *P. ostreatus* and *P. djamor.*

Materials and Methods

Spawns of *P. ostreatus* was kindly provided by the Center of Operation Training and Conveying Technology, Department of Agriculture of Thailand, while *P. djamor* was from Aranyik Mushroom Farm, Nakhon Pathom Province, Thailand. The lytic enzyme solution contained 9 mg Lysing Enzyme (Sigma L-1412, sterilized by filtration through an autoclaved 0.45 µm millipore membrane) in 1 ml of the osmotic stabilizer which contained 0.6 M $MgSO_4 \cdot 7H_2O$ in 0.05 M sodium maleate buffer, pH 5.

Cultivation for Fruiting Bodies of *P. ostreatus* and *P. djamor*

The spawns of *P. ostreatus* and *P. djamor* were cultured to fruit in sawdust plastic bags by the following general commercial method. Mecelia of each species on MEA (malt extract agar) slant were subcultured onto boiled millet seeds and incubated at room temperature until they grew over the seeds. Ten seeds were sprayed on the ready-prepared mushroom compost and incubated in a moist area until fruiting bodies occurred. The shape and colour of the friuting bodies including the colour of their spore prints were determined and photographed.

Spore Prints Preparation and Single Spore Isolation

Spore prints were prepared by cutting a small piece (about 2.5×2.5 cm) of a fresh mushroom cap and sticking it gills-down on the sterilized lid of a Petridish with paraffin or sticky tape which was then put on top of a sterilized beaker lined with 1×1 cm of sterilized filter papers. The spores were allowed to discharge onto the pieces of paper for 3-10 h before the lid was replaced with a new sterilized one. The beaker with spore prints on pieces of paper had been kept in a refrigerator until single basidiospore isolations were performed. Single spore isolates which were monokaryons (n) and without clamp connections (clamps) on their hyphae were prepared using the method described by Petersen and Ridly (1996).

Protoplast Isolation

Four day-old monokaryotic mycelia of *P. ostreatus* and *P. djamor* were transferred onto 50 ml of malt extract broth plus 20 glass beads (0.50 cm in diameter) in a 250 ml Erlenmeyer flask placed on a shaker with the agitation speed of 200 rpm, at 25ºC for 4 days. Protoplasts were separated from the mycelia using a method modified from Hashiba (1992) by transferring 0.3 g of the mycelia onto 3 ml of the sterilized lytic enzyme solution in a test tube and shaking it at 100 rpm at room temperature for 2 h. The mycelial remnants were removed by filtration through a sintered glass filter and the suspended protoplasts were precipitated at 1000 × g for 10 min. The protoplasts were then washed twice with the osmotic stabilizer and were finally suspended in 5 ml of the osmotic stabilizer. Protoplasts obtained in *P. ostreatus* and *P. djamor* were counted using a haemacytometer.

Protoplast Fusion and Protoplast Regeneration

One milliliter of each of the freshly prepared protoplasts (diluted to be 1×10^6 protoplasts/ml) of *P. ostreatus* and *P. djamor* was mixed in a test tube and centrifuged at 1000 × g for 10 min. The supernatant was rinsed off and 1 ml of sterilized PEG (40 g PEG in 100 ml 0.05 M $CaCl_2 \cdot 2H_2O$) was added to the protoplasts in the test tube and incubated at room temperature for 20 min by shaking the tube every 5 min. Another 9 ml of the osmotic stabilizer was then added to the tube before centrifugation at 1000 × g for 10 minutes. The supernatant was rinsed off and the "mixed protoplasts" were washed twice with the osmotic stabilizer. The protoplast solution was then diluted to 1×10^4 protoplasts/ml and 0.1 ml of the suspension was used for protoplast regeneration by culturing it on a plate of Regeneration Medium which contained 30 g agar, 20 g malt extract, 20 g glucose, 1 g peptone and 1000 ml of 0.6 M sucrose followed by overlaying with the same kind of medium but with a concentration of agar of only 5 g. The plate was incubated at 25ºC until colonies occurred. Each colony was isolated day by day onto a MEA slant.

Fusant Selection

The colonies were screened by examining microscopically for clamp connections on their hyphae. The colonies with clamps on mycelia were selected as "fusants" and were individually subcultured on MEA slants.

Proofs of Hybrids

The fusants were further studied for evidence of hybridization by checking the following characteristics in comparison with their parental strains. The characteristic studies were mycelial growth, hyphal size, isozyme patterns and

morphology of fruiting bodies.

Determinations of Mycelial Growth and Hyphal Size. Inocula of mycelia from each fusant and the parental strains were subcultured on MEA plates and incubated at room temperature for 9 days followed by determining the diameter of each colony for at least 10 replications to represent mycelial growth, while hyphal width was measured microscopically as the hyphal size using a calibrated eyepiece micrometer for 100 replications. The results were analysed statistically.

Determination of Isozyme Patterns. Isozyme patterns of mycelia of all fusants possessing clamps along with their parental strains were studied followed a method modified from that of Pasteur *et al*. (1988). Mycelia of each strain were cultured on malt extract broth, pH 7 at 25ºC for 20 days. The mycelia were filtered with two layers of muslin cloth which were then washed twice with sterilized distilled water followed by grinding with liquid nitrogen in a motar. The mycelia were then picked up into a microcentrifuge tube in which extraction buffer for enzyme extraction was added. The tube was then centrifuged at 12000 × g at 4ºC for 30 min and the supernatant was kept at -20ºC. Electrophoresis analysis was performed by mixing 15 µl of the supernatant with 5 µl of the sample buffer (pH 6.8, 0.6 M Tris-HCl, 10% glycerol and 0.025% bromphenol blue) before loading the liquid of each strain into each slit on the acrylamide gel in the electrophoresis set. The gel was then taken up and stained with substrate solution of 3 enzymes namely esterase, malate dehydrogenase, and alcohol dehydrogenase. Each enzyme electrophoretic pattern was then photographed.

Morphology of Fruiting Bodies. Mycelia of each fusant possessing clamps cultured on MEA slant were cultivated to fruit as the method already described for the parental strains. The same characteristics of fruiting body studied in their parental strains were determined in all fusants and the results were compared to those of the parents.

Results and Discussion

The Amount of Protoplast Isolation

The amounts of protoplasts obtained in *P. ostreatus* and *P. djamor* were 4.59×10^6 and 3.36×10^6 protoplasts/ml, respectively. The results obtained agree to some extent with those published by Kitamoto *et al*. (1988). They reported that when protoplast isolations were performed in various filamentous fungi from all fungal subdivisions by using the enzyme prepared from *Trichoderma harzianum*, Basidiomycotina including *P. ostreatus* gave the highest productions of protoplasts with the values of 1.3×10^7 - 8.4×10^7 and 3.2×10^7 protoplasts/ml, respectively. Production of protoplast, however, varies with the factors used in the isolation process e.g. species and age of fungal mycelia (Yamada *et al*., 1983), type and condition of the lytic enzyme (Peberdy, 1989) and of the osmotic stabilizer (Peberdy and Fox, 1993).

Table 1. Mycelial growth and hyphal size of Fu4 and Fu5 compared to those of the parental srains, *P. ostreatus* and *P. djamor*. All mycelia were cultured on MEA at 25ºC for 9 days.

Strain	Diameter of colony (cm)	hyphal width (µm)
P. ostreatus	8.83b	2.43b
P. djamor	3.24c	2.24b
Fu4	9.00a	2.76a
Fu5	9.00a	3.00a

[a,b,c] **Values in a row followed by the same letter do not differ at P≤0.05**

Fusant Selection

The number of colonies isolated after protoplast fusion of *P. ostreatus* and *P. djamo*r was 412. Among these, only 2 of them possessed clamps and thus were chosen as fusants and named as Fu4 and Fu5.

Mycelial Growth and Hyphal Size

Mycelial growth and hyphal size of Fu4 and Fu5 were significantly different from those of the parental strains (Table 1) which are relevant to the theories that fusants which are dikaryontic (n+n) grow faster (Toyomatsu and Mori, 1987) and have larger hyphae (Abe *et al*., 1982) than the monokaryotic parental strains.

Determination of Isozyme Patterns

For the three enzymes tested (Figure 1), only esterase verifies the hybridization relationship as the fusants showed band(s) common to either of the parental strains or to both. Furthermore, the non-parental new bands observed in the esterase isozyme patterns of the fusants indicates that there was an occurrence of interaction between the two parental genomes (Toyomatsu *et al*., 1986). The same esterase isozyme patterns of the two fusants also indicates that the two fusants coincidentally had the same esterase gene. However, more

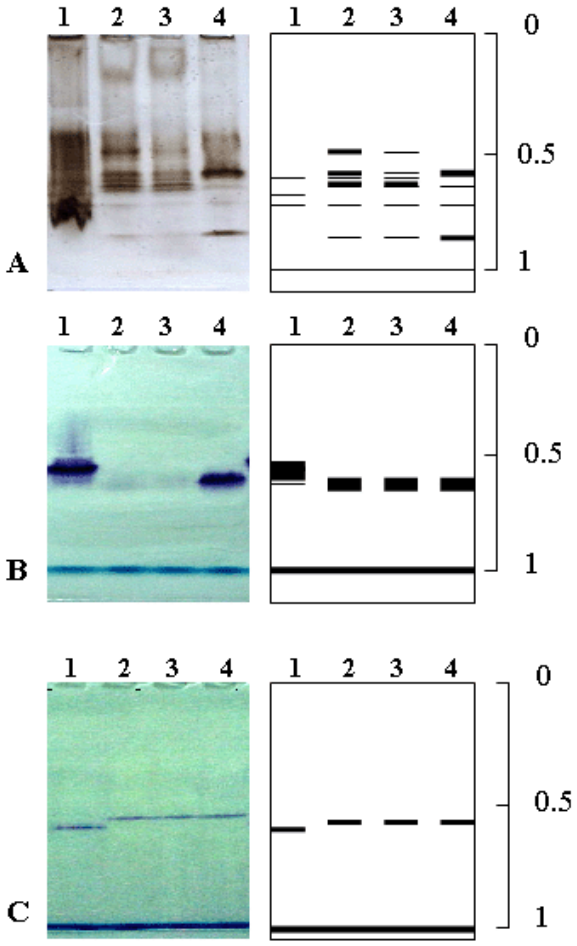

Figure 1. Isozyme patterns of *Pleurotus djamor* (Lane 1), Fu4 (Lane 2), Fu5 (Lane 3) and *P. ostreatus* (Lane 4).
A. Esterase, B. Malate dehydrogenase, C. Alcohol dehydrogenase

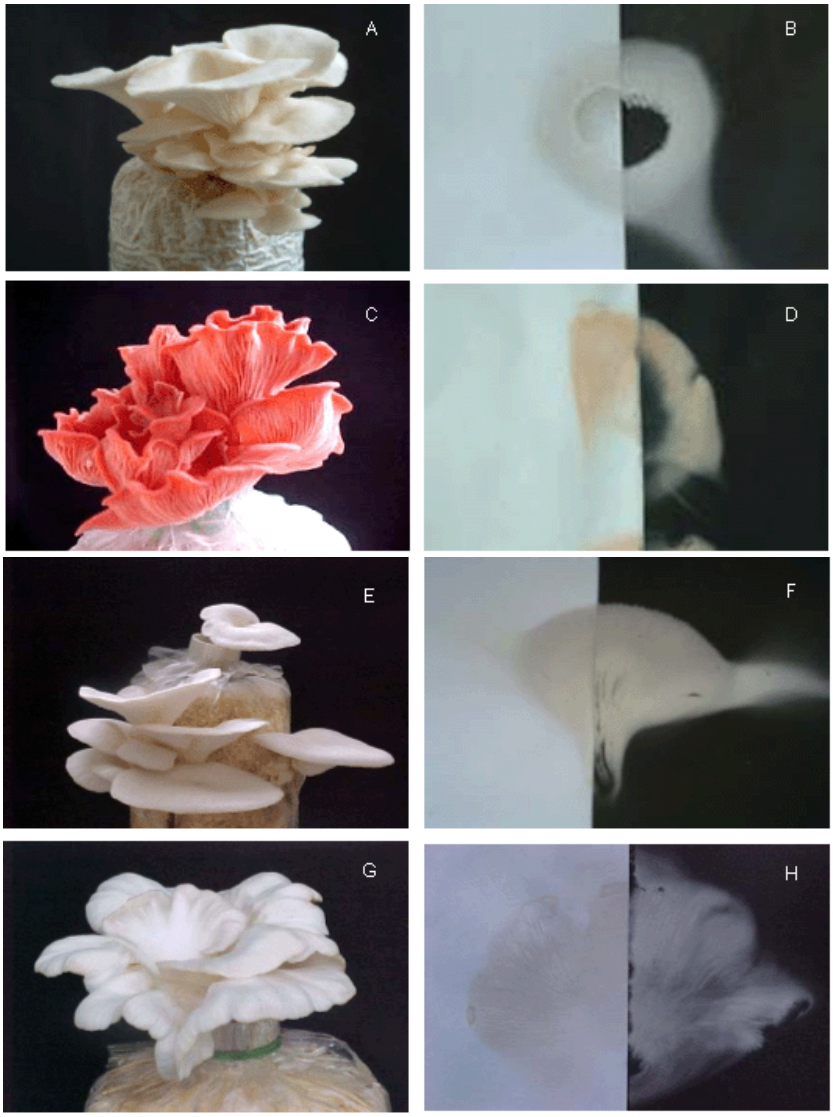

Figure 2. Fruiting body and spore print, respectively, of *Pleurotus ostreatus* (A, B), *P. djamor* (C, D), Fu4 (E, F) and Fu5 (G, H).

enzymes should have been tried and DNA fingerprinting should be further studied in order to obtain more precise evidence of the hybridization.

Morphology of Fruiting bodies

The morphology of fruiting bodies of both Fu4 and Fu5 (Figure 2) were creamish white in colour which was similar to that of *P. ostreatus* but the shape of Fu4 was more similar to *P. ostreatus* while Fu5 was similar to *P. djamor*. However, the spore print of Fu4 possessed two colours in different parts,viz, greyish pink (similar to *P. djamor*) at the outer part (wider area) of its gills and creamish white (similar to *P. ostreatus*) in the inner part of the gills while the spore print of Fu5 was creamish white which was similar to that of *P. ostreatus*. These results indicate that the whole genomes of Fu4 and Fu5 were not identical and that the differences in their morphology resulted from gene recombination. The allele (gene)

controlling creamish colour in the fruiting body, to some extent, was completely dominant to that of the pinkish colour, while those controlling shape of pileus (cap) and spore print probably belonged to other types of dominance.

Therefore, the reasons that Fu4 and Fu5 were similar in esterase isozyme patterns (Figure 1) but different in morphology (Figure 2) could be due to their whole genomes not being identical but havig only some genes (e.g. esterase) in common.

Conclusion

Two fusants possessing clamps, Fu4 and Fu5, were selected and proved to be hybrids of *P. ostreaus* and *P. djamor* by comparing their isozyme patterns and morphology of fruiting bodies. This experiment confirmed other previous works (e.g.Toyomatsu *et al*., 1986 and Takehara *et al*., 1993) that protoplast fusion is an appropriate method in creating hybrids of interspecific hybrization in mushrooms especially in the genus *Pleurotus*.

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