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INTERSPECIFIC PROTOPLAST FUSION OF *PLEUROTUS* SPECIES

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Abstract: Mushrooms has been extensively used as food since ancient time due to nutritive and medicinal values. In view of the current energy crisis it has become most important to make an extensive breakthrough in the technology of food production to meet a serious food insufficiency condition. Although, many mushrooms still remain to be studied, insights into the biology and reproduction of commercially important mushrooms have opened new vistas for their genetic improvement to meet the requirements of both the producers and the consumers alike. Protoplast fusion is potentially important to develop mushroom hybrids, especially where the conventional methods cannot be achieved. A new method that has been used to create mushroom hybrids was protoplast fusion. However, for the same reason as that latent to the conventional method, the greater the distance in the genetic relationship between the two mating isolates, there will be limited successful protoplast fusion.

Index Terms **- Mushrooms, *Pleurotus*, interspecific, hybridization, protoplast fusion.**

I. INTRODUCTION

Mushrooms were used as a food even before man understood use of other micro organism. Mushrooms were often considered an exotic and luxurious food since ancient ages. The predictable number of mushroom species on earth is about 1, 40,000, out of which only as many as 22,000 are known species (Hawksworth, 2001). Among these known mushroom species 50% are considered to acquire varying degrees of edibility, more than 2000 are safe and about 700 species are identified to have considerable pharmacological properties (Wasser, 2002). However, it has been extensively used as food since ancient time due to nutritive and medicinal values. In view of the current energy crisis it has become most important to make an extensive breakthrough in the technology of food production to meet a serious food insufficiency condition. Although, a large number of mushrooms still remain to be studied, insights into the biology and reproduction of commercially important mushrooms have opened new vistas for their genetic improvement to meet the requirements of both the producers and the consumers alike. Protoplast fusion is potentially important to develop mushroom hybrids especially where conventional method cannot be achieved. As conventional hybridization, protoplast fusion be able to perform intraspecifically (Toyomatsu and Mori, 1987), interspecifically, intergenerically (Zhao and Chang, 1996) and inter-heterogenerically (Eguchi and Higaki, 1995).

Strain improvement by protoplasmic fusion assumed that the hybrid Pleurotus species is an important resource of food for mankind as it showed rich nutritive value with quick spawn run period, high yield and maximum biological efficiency than parental strains by utilizing agricultural waste. Among the biotechnological techniques, a new method has been used to create mushroom hybrids was protoplast fusion. The aim of the present study was to increase mushroom productivity, quality, spore-less strain, developing a high temperature stock and disease resistance variety using protoplast fusion technique to construct fusants of desirable characters.

II. MATERIALS AND METHOD

Four strains of *Pleurotus* species (*Pleurotus eryngii*, *Pleurotus florida*, *Pleurotus ostreatus* and *Pleurotus djamor* var. *roseus*) were used in this study. Pure cultures of *Pleurotus* species were maintained on potato dextrose agar (PDA) slants at 4°C, and sub-cultured fortnightly. Unless otherwise indicated PDA was used as a routine growth/maintenance media, a number of specialized media were used for performing different types of experiments.

2.1 Culture preparation for protoplast isolation

Agar blocks (8 mm) from actively growing 6-day-old cultures of the selected fungi were inoculated individually in Erlenmeyer flasks (250 ml) containing sterile potato dextrose broth (PDB) (100 ml) and incubated at 28 ± 2ºC in a rotary shaker (Remi, India) at 120 rpm for 3 days. The mycelia were harvested by filtration through sterile muslin cloth and washed twice with sterile water (Lalithakumari, 2000). Protoplasts were separated from the mycelia using the modified method of Santiago (1982).

2.2 Isolation of protoplasts

By transferring 100 mg of harvested mushroom mycelium (*P. eryngii, P. florida, P. ostreatus* and *P. djamor* var. *roseus*) was aseptically transferred to a test tube containing 1ml of osmotic stabilizer and the sterilized lytic enzyme mixture (3ml) containing Glucanex (a lytic enzyme from *T. harzianum*). The mycelium in the enzyme mixture was incubated in a rotary shaker at 120 rpm at room temperature (28 ± 2ºC) for 3 hours. After the incubation period, the hyphal fragments were removed by filtration through a column of cotton wool packed in a 5 ml syringe upto the 0.5ml mark. Protoplasts of all the four mushroom strains were collected from the filtrate by centrifugation at 1400 rpm for 10 min. Pellets were washed twice in 0.6 M KCl buffer and then suspended in the same osmotic stabilizer.

2.3 Protoplast viability testing

Isolated and purified protoplasts were used for viability testing prior to regeneration and fusion experiments. The viability of the fresh protoplast was examined by Fluorescein diacetate (FDA) test (Widholm, 1974). The viable intact protoplasts appeared as fluoresced yellow-green while the damaged protoplasts were fluoresced red. The number of viable yellow-green fluoresced protoplasts was counted with the help of a haemocytometer.

Percentage of viability was calculated by the following formula:

$$\frac{\text{Average number of viable protoplasts}}{\text{Average number of total protoplasts counted}} \times 100$$

2.4 Protoplast yield and their fusion

The yields of freshly prepared protoplasts from selected monokaryotic cultures were determined by using a haemocytometer. Protoplasts preparations were finally diluted to l x $l0^6$/ml by an osmotic stabilizer. Isolated and purified protoplasts were used for viability testing prior to regeneration and fusion experiments. The viability of the fresh protoplasts was examined by the FDA test (Widholm, 1974). The viable intact protoplasts fluoresced yellow-green in colour while the damaged protoplasts were fluoresced red.

For fusion experiments 1.0 ml of the two final protoplast preparations were mixed together in a test tube and centrifuged at 1000 rpm for 10 min. The supernatant was discarded and equal amount of sterilized fusogen was added to the pellet containing protoplasts and incubated at room temperature for 10 min by shaking the tube every 2 minutes. After incubation, 9 ml of the osmotic stabilizer was added and subjected for centrifugation at 1000 rpm for 10 min. The supernatant was discarded and the mixed protoplasts were washed twice with the osmotic stabilizer. The fusion process was examined under the microscope by taking 10µl aliquot of fusion mixture and at 40X magnification.

2.5 Regeneration and selective isolation of fusants product

The fusion mixture was diluted to $1x10^5$ protoplasts / ml. The suspension of 0.1 ml was used for regenerating the fusant protoplasts (heterokaryons) by culturing it on plates of Non-Selective media or Minimal media (MM). The regenerated colonies were transferred to Regeneration Minimal Media (RMM) according to Kiguchi and Yanagi (1985).

2.6 Fusants Selection

Only those progeny that continued to grow on MM were considered to be fused hybrids or fusants. 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 sub-cultured in MEA medium and the plates were incubated at 25°C until colonies developed for counting and transfer to PDA slants for further studies.

2.7 Proof of Hybrids

Confirmed Fusants were sub-cultured on PDA plates and incubated at room temperature for 10 days. Subsequently, the fusants were confirmed by the characteristic features of mycelial growth and hyphal size compared with their parental strains. This experiment was designed using a completely randomized design. Data were then analyzed statistically.

2.8 Determination of Esterase Isozyme Pattern

Isozyme patterns of mycelia of all fusants possessing clamps along with their parental strains were studied by the method modified from that of Pasteur *et al.,* (1988). Mycelium of each strain was 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 mortar and pestle. The grinded mycelia were subsequently transfered into a micro centrifuge 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 which contains the tank buffer (7.2g of boric acid and 15.75g of boric / L). The gel was then taken up and stained with substrate solution of enzyme esterase and the electrophoretic pattern was photographed.

III. RESULTS AND DISCUSSION

3.1 Isolation of protoplast

The yield of protoplasts depends on the age of the mycelium. Each method has been designed to isolate and regenerate protoplasts of specific species or strains of fungi. Because of the diversity of cell wall structure among fungi, no common technique can be applied to all groups. Therefore, the best condition (age of strain) for high yield of protoplasts and regeneration have to be established a new for each tested species of fungi. When hyphae of various ages were used for protoplast isolation, the highest number of protoplasts was derived from the hyphae of 2-5 days old culture. The maximum yield of protoplasts was observed in the mycelium of *P. djamor* var. *roseus* (16.8×10^3) followed by *P. florida* (14.8×10^3), *P. ostreatus* (12×10^3) and the minimum protoplast was found in the mycelium of *P. eryngii* (9.2×10^3) respectively.

According to Dhitaphichit and Pornsuriya (2005), the amounts of protoplasts obtained in *P.ostreatus* and *P. djamor* var. *roseus* were 4.59×10^6 and 3.36×10^6 protoplasts/ml. The research also concludes that the change in composition and thickness of hyphal cell wall was associated with aging process.

3.2 Release and Regeneration of protoplast

The release of protoplasts was observed to start with the lysis of hyphal walls at 1.5 hours of incubation followed by the rounding up of the protoplasts at 2 hours and their release through the hyphal tips at 2.5 hours. The highest yield of protoplasts from *P. djamor* var. *eous* followed by *P. florida, P. ostreatus* and *P. eryngii* were obtained after 3 hours of incubation with the complete digestion of the mycelium (Figure 1). Prolonged incubation resulted in a decrease in number of protoplasts from the mushroom fungal mycelia. The percentages of viable cells were shown in Figure 2. It has been reported that in most of the fungi, the regeneration frequency of protoplasts to be less than 0.1% to 50% (Peberdy, 1991). The observations similar to this study were reported by Lalithakumari (1996) who observed the maximum release of protoplasts in *Venturia inaequalis* using a mixture of enzymes containing cellulase, chitinase, pectinase and β-glucuronidase.

The regeneration frequency of the fungal protoplasts was found to differ widely among different groups and it found to depend upon the regeneration media, osmotic stabilizers and several other factors. Protoplast of *P. djamor* var. *roseus* showed a regeneration frequency of 12.6%, while *P. florida* showed 8.7%, *P. ostreatus* showed 0.5% required 5 days for regeneration and *P. eryngii* showing a lower regeneration frequency of 0.25% under the same conditions (Figure 3). In consistent with Parani and Eyini (2010) showed a regeneration frequency of 0.28%, while *Pleurotus flabellatus* under the same conditions required 6 days for regeneration showing a lower regeneration frequency of 0.24%.

3.3 PEG-induced fusion of protoplasts

The isolated protoplasts from the strains of *Pleurotus* were fused by PEG and it was examined under a microscope (Plate 16). To the purified protoplasts of the test organisms in the osmotic stabilizer, an equal volume of PEG mixture was added and shaken slightly for fusion. The sample was diluted with equal volume of osmotic stabilizer and observed under the compound microscope for fusion. An aliquot (0.1 ml) of the fused protoplasts were plated on RMM and checked for regeneration. Fusion frequency was determined as the ratio of the number of colonies regenerated. The isolation, regeneration and fusion of the protoplasts were observed and photographed using photo-micrographic equipment (Nikon, Japan). Based on the microscopic observation, viability of isolated protoplasts after FDA treatment was surprisingly higher with 90% viability (Figure 4). According to Uttara Chakraborty and Samir Ranjan Sikdar (2010), the viability of the protoplasts observed after FDA staining was found to be 95.26 ± 3.33% in *Pleurotus florida* and 88.51 ± 2.82% in *Volvorella volvacea.*

3.4 Selection of Fusants

After 4 days of incubation, small and round mycelial growths were observed. Hyphal tips of regenerated colonies developing on RMM were transferred to MM. Only those progeny that continued to grow on MM were considered as fusion hybrids (Plate18**)**. No Visible mycelial growth on RMM when parental genotypes were inoculated as controls. The number of colonies grown on RMM after protoplast fusion of *P. eryngii* and *P. florida* was 22 colonies, 32 colonies were obtained by the fusion of *P. eryngii* and *P. ostreatus* and 31 colonies were observed by the fusion of *P. eryngii* and *P. djamor* var. *roseus* (Figure 5). The progeny candidates were selected as fusants and named as FST 1, FST 2 and FST 3 after were grown on MM for 2-3 weeks.

3.5 Determination of Isozyme Patterns

For the three enzymes tested (Figure 6), only esterase documented the hybridization relationship as the fusants showed bands 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 indicate that there was an occurrence of interaction between the two parental genomes. The same esterase isozyme pattern of the two fusants also indicates that the two fusants coincidentally had the same esterase gene.

3.6 Mycelial Growth and Hyphal Size

Mycelial growth and hyphal size of dikaryotic strains (fusants) were significantly different from those of the parental strains. All the mycelia were cultured on MEA plates for 6-7 days at 25°C to find out the difference in mycelial growth and hyphal size. The fusants of FST 3 showed 10.98cm of growth and 2.59 µm in size, FST 2 showed 10.19 cm growth and 3.24 µm in size and FST 1 showed 9.86 cm growth and 2.73 µm in size whereas parental strains *P. eryngii* shows 6.82 cm of growth and 2.04 µm in size, *P. florida* shows 8.21 cm of growth and 1.50 µm in size, *P. ostreatus* shows 8.53cm of growth and 1.93 µm in size and *P. djamor* shows 9.20 cm of growth and 1.28 µm in size (Table 3). These results are reliable to the previous studies indicating that fusants dikaryotic (n+n) grows faster (Toyomatsu and Mori 1987) and have larger hyphae (Abe *et al.*, 1982) than the monokaryotic parental strains (Dhitaphichit and Pornsuriya, 2005). Then all the parental strains as well as the fusants were transferred to PDA Medium for further studies in order to obtain more precise evidence of the hybridization.

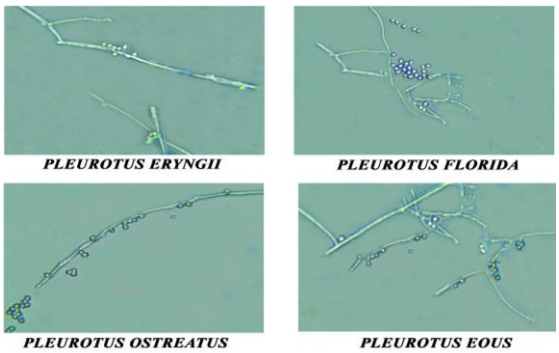

Figure 1: Yield of Protoplast from mycelia of *Pleurotus species*

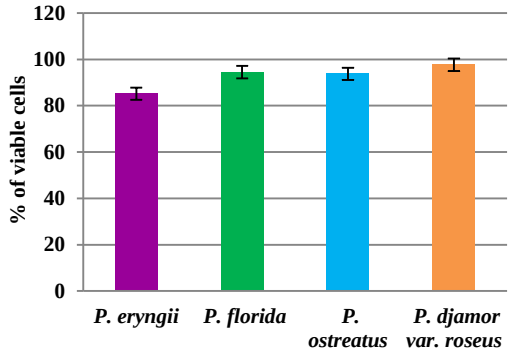

Figure 2: Percentages of viable Protoplasts

Figure 3: Regeneration of Protoplasts in Non-selective medium

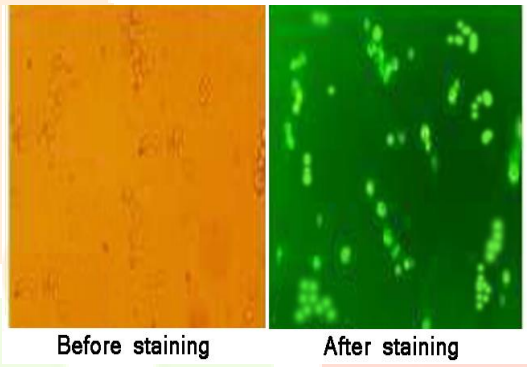

Figure 4: FDA stained viable protoplasts

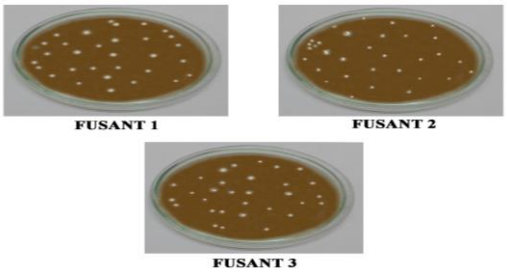

Figure 5: Regeneration of Fused Protoplasts in Minimal Medium

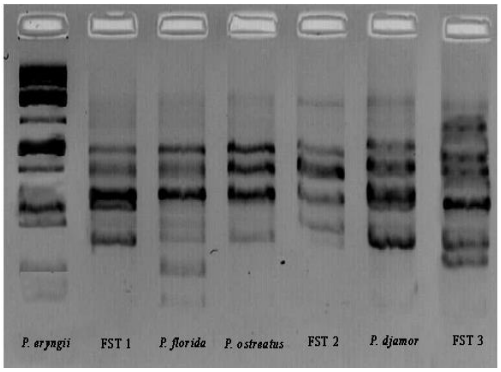

Plate 20: Determination of Esterase Isozyme pattern

IV. SUMMARY

The present study was planned to develop an interspecific fusant between bigger, rich antioxidant, high nutritional value and especially longer storage shelf life species *P. eryngii* and fast growing and higher productivity with rich nutritional value possessing species *P. florida, P. ostreatus* and *P. djamor* var. *roseus* belonging to same generic group. Interestingly all the desirable characters from parent strains were inherit to the fused strains (FST 1, FST 2 and FST3). The somatic hybrids obtained through this study were used to serve as resource material for further studies that would give us insight about the basic genetics of fusants and in future it could be exploited as a commercial strain for cultivation throughout the year in tropical countries of the world.

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