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Strain Improvement Through Protoplast Fusion for Enhanced Coffee Pulp Degradation

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Abstract: *Pleurotus eous* colonies regenerated on plates containing complete medium overlaid with soft agar gave 0.28% regeneration efficiency, while that of *Pleurotus flabellatus* was slightly less (0.24%). In PEG (polyethylene glycol) - induced fusion experiments with *Pleurotus eous* and *Pleurotus flabellatus*, 0.18% of fusion frequency was achieved and four fusant colonies were recovered. The fusant No.4 could grow on regeneration minimal medium and minimal medium.

Key words:PEG · Complete medium · Minimal medium · Regeneration frequency · Fusion frequency · *Pleurotus eous* · *Pleurotus flabellatus*

INTRODUCTION

Protoplast fusion is of current interest because of its applications in pure and applied genetics. Protoplast fusion technology is applied for developing interspecific, intraspecific and intrageneric suprahybrids with higher potentiality than their parental strains.

Protoplast fusion has proved a feasible method for inter-specific and inter-generic hybridization for strain improvement among edible mushrooms [1]. Protoplasts have been studied and prepared in large number of edible mushrooms [1,2] but their protoplast yields have been found poor as compared to non-basidiomycetous fungi [3]. Induced protoplast fusion can overcome vegetative incompatibility and produce hybrids with the combined properties of both parents [4].

Hence the present investigation aims to evolve an improved strain involving the members of the best synergistic coculture through protoplast fusion with improved coffee pulp degrading capabilities.

MATERIALS AND METHODS

Organisms: Pure cultures of the oyster mushrooms *viz*., *Pleurotus eous*(Berk.) Sacc. (APK-1) and *Pleurotus flabellatus* (Berk & Br.) Sacc. (MDU2) were obtained from TamilNadu Agricultural University, Coimbatore, India. The cultures were maintained on Potato Dextrose Agar (PDA) slants and stored at 4°C and the slants were subcultured once a month.

Using the mixture of different mycolytic enzymes (commercial cellulase, chitinase, pectinase - each 1ml / study) were more efficient in effecting the yield of protoplasts from hyphae of 3 days - old cultures of *Pleurotus eous* and *Pleurotus flabellatus* using 0.6M KCl as osmotic stabilizer in phosphate buffer at pH 6.0 for an incubation period of 3 hours.

Regeneration of Protoplasts: Regeneration of protoplasts was checked by the agar overlay method [5]. For regeneration on solid medium, 1 ml of protoplast suspension ($1x10^3$ protoplasts / ml) was spread out on regenerating medium (PDA) containing 2% (w/v) agar and overlaid with PDA soft agar medium containing 0.5% agar. Regeneration of mycelial colonies was observed after 72h of incubation at 30°C. Regeneration frequencies were taken as the ratio of the number of colonies developed to the number of protoplasts added per plate as determined by haemocytometer count of diluted protoplast preparation. The purity of the protoplast suspension was always checked by using a control plate, on which a suspension of protoplasts initially lysed with distilled water, was plated in similar way. The absence of mycelial colonies on the control plate was taken as a measure of the purity of protoplast preparation.

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Protoplast Fusion: Protoplasts were fused following the method [6] in the presence of polyethylene glycol (PEG, mol.wt.4000, 30%), $CaCl_2$ (0.05 M) and glycine (0.05 M) at pH 7.5. To the purified protoplasts of the two test organisms in the osmotic stabilizer, an equal volume of PEG mixture was added and the mixture was shaken slightly for 10 min for fusion to take place. The sample was diluted with equal volume of osmotic stabilizer and a small volume was observed under the compound microscope for fusion. An aliquot (0.1ml) of the fused protoplasts sample was plated on non-selective medium (MYG, pH 5.5 with osmotic stabilizer) and checked for regeneration.

Aliquots (0.1 ml) were also plated on complete medium and minimal medium and incubated for 1 week. Protoplasts from the same strains were also fused as controls. Fusion frequency was determined as number of regenerated colonies in regeneration minimal medium (RMM) /number of regenerated colonies in regeneration complete medium (RCM). Each plate was sealed with parafilm to prevent contamination by other fungal strains. The isolation, regeneration and fusion of the protoplasts were observed and photographed using a photomicrographic equipment (Nikon Fluophot, HFx-II, Japan). (MYG medium g/l Glucose - 4.0, Yeast extract - 4.0, Malt extract - 10.0, Agar - 14.0,Distilled water-1000 ml; Regeneration Minimal Medium (RMM) g/l Asparagine - 2, Glucose - 20, Thiamine-HCl - 0.12, K_2HPO_4. 1, KH_2PO_4 - 0.46, $MgSO_4.7H_2O$ - 0.5, Bacto agar - 14,Distilled water - 1000 ml. For regeneration, minimal medium was supplemented with 0.6 M KCl. Regeneration Complete Medium (RCM) g/l Peptone - 2, Yeast extract - 2, Glucose - 20, Thiamine-HCl - 0.005, K_2HPO_4 - 1, KH_2PO_4 - 0.46, $MgSO_4.7H_2O$ - 0.5, Bacto agar - 14, Distilled water - 1000 ml. For regeneration, complete medium was supplemented with 0.6 M KCl.

Analyses of Fusion Products: Hyphal tips of regenerated colonies developing on RMM were transferred to minimal medium (MM). This procedure excluded the possibility of a dual culture. Only those progeny that continued to grow on MM were considered to be fusion hybrids [7]. The fusant genotypes were considered to be stable if after five successive transfers on appropriately supplemented MM, no loss of markers was observed [5]. The protoplast fusants screened, analyzed and characterized were subcultured and stored in PDA slants.

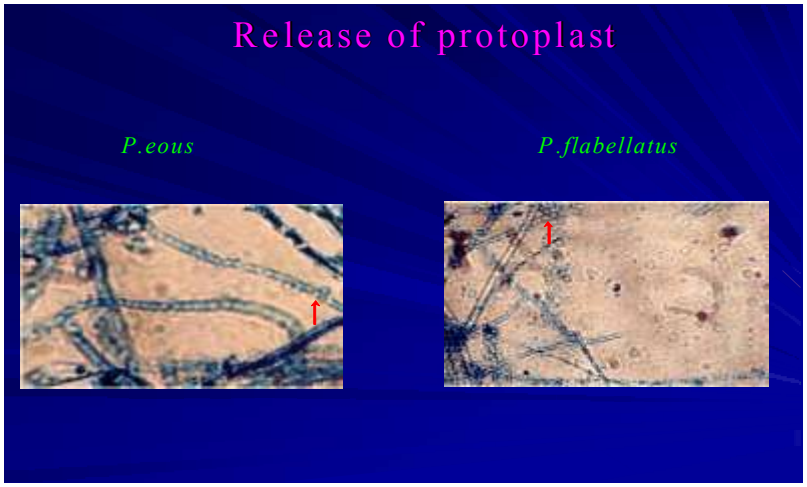
RESULTS AND DISCUSSION

Protoplasts have been isolated and regenerated by different methods from a large number of species belonging to all the major fungal groups [8,9]. 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 optimal conditions for high yields of protoplasts isolation and regeneration have to be established anew for each tested species of fungi (Plate 1).

Regeneration of Protoplasts: Regeneration frequency of fungal protoplasts was found to differ widely among the different groups and it seemed to depend upon the regeneration media, osmotic stabilizers and several other factors. Protoplasts for *Pleurotus eous* plated on regeneration complete medium overlaid with the same medium containing soft agar and incubated for 4 days at room temperature 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%. (Plate 2). Similar observations have been made that *Pleurotus florida* and *Pleurotus sajor-caju* required 3 and 4 days respectively and 30°C for regeneration [2]. They observed the highest regeneration rate (9.6%) in *P.florida* and much lower frequencies of 2.4% and 0.96% in *P. sajor-caju* and *Lentinus edodes* respectively.

The absence of nucleus in released protoplasts might result in variable and sometimes very low regeneration frequencies in filamentous fungi [10]. Similar views are expressed that the structure of hyphae may influence the yield of protoplasts and nuclear number contained in one protoplast [2].

Hyphal Regeneration: Two different modes of hyphal regeneration were shown by *P. eous*. In the first type a germ tube like hypha protruded from the spherical cell, that developed into a normal hyphal structure and in the second type, protoplasts seemed to develop into irregular chains of cells (Plate 2). *P. flabellatus* protoplasts showed the latter pattern of regeneration (Plate 2) and also showed regeneration forming hyphae directly from the irregularly shaped chains of cells. Similar observations have been made that all the three basic patterns of



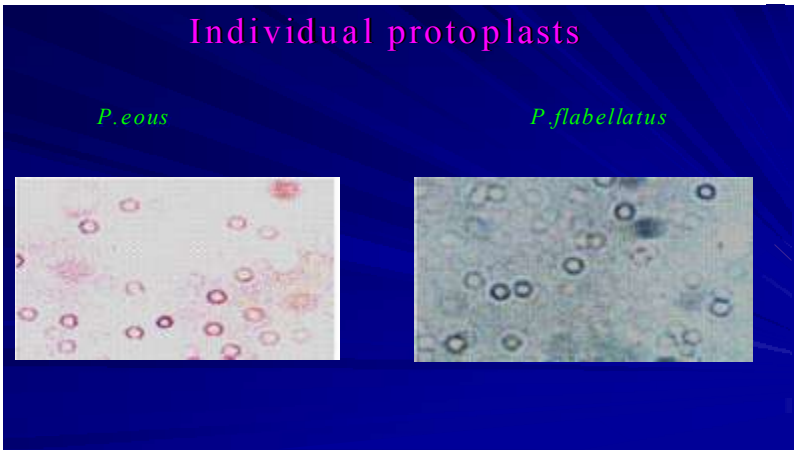
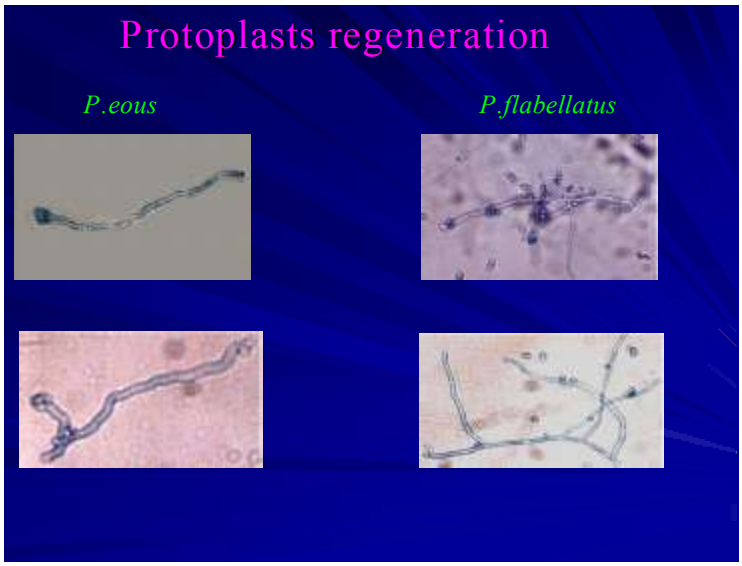


Plate 1:



Plat 2:

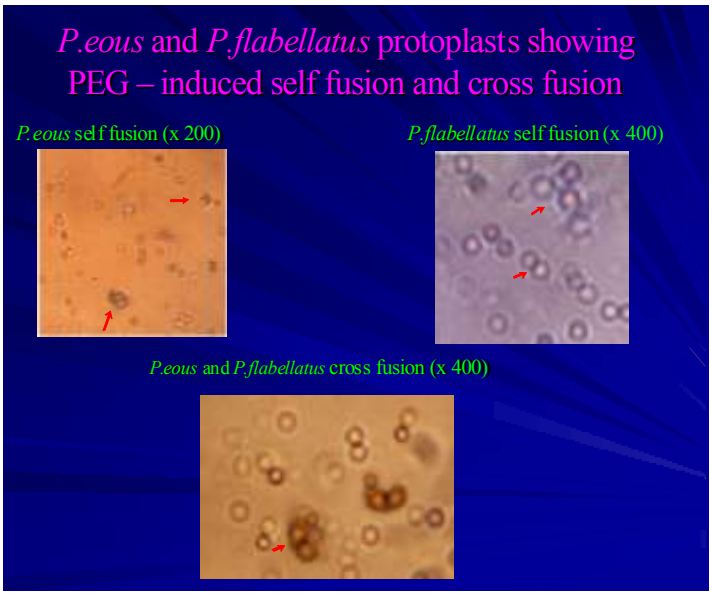


Plate 3:

Plate 4:

regeneration were observed in *Venturia inaequalis*, *Pyricularia oryzae* [10-12] and also observed considerable variation in regeneration of Trichoderma *harizianum* protoplasts using different regeneration media [13].

Fusion Frequency: The protoplast fusion between *Pleurotus eous* and *Pleurotus flabellatus* resulted in fusion frequency of 0.18% (Plate 3). Out of the four fusant colonies retrieved, only fusant No.4 could be retrieved by transfer through hyphal tips on minimal medium (MM) and it was stable even after five successive subculturings (Plate 4). By the definition [7], the fusant No.4 should be a fusion hybrid between *Pleurotus eous* and *Pleurotus flabellatus* as it could continue to grow on minimal medium and exhaustively worked on protoplast fusion on filamentous fungi and successively developed several intergeneric and interspecific protoplast fusants for strain improvement [10,14]. In intraspecific fusion in *Trichoderma*, obtained fusion frequency between 0.002% and 0.02% [15]. Inter specific fusion frequencies of 0.20% to 1.41% have been reported in non basidiomycetous fungi. When compared to lower fungi, however protoplast fusion in higher fungi was not very successful [16].

A fusion frequency of 0.23% was obtained [13] by exposing *Trichoderma longibrachiatum* protoplasts to 30% PEG for 15 min. The results of protoplast fusion between *Pleurotus eous* and *Pleurotus flabellatus* carried out under similar conditions are in agreement with this study. Several workers obtained very low fusion frequencies of 0.0036 to 0.007% in intergeneric fusion between *P. ostreatus* and *S. commune.* But they obtained

higher fusion frequencies 16.7% to 50% and 6.9% to 8.4% in intraspecific fusion of *C. cinereus* and *S. commune* respectively [2,4,7,17].

While interspecific hybridization between *Volvariella volvaceae* and *Volvariella bombycina* gave a much higher percentage of 0.032%-0.33% [7,17]. Strain improvement by protoplasmic fusion for enhancement of xylanase production had been achieved through interspecific recombination of *Aspergillus indicus* and *A.wentii* [18]. Employing the fungal strains, which show synergistic associations in coculture for the development of protoplast fusants with the desirable characters of both the parents will be a viable option for efficient biodegradation and bioconversion of lignocellulosic substrates.

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