DOI: 10.36036/MR.29.2.2020.116258
***Mushroom Research* 29 (2) : 181-194, 2020**

Protoplast fusion between *Pleurotus opuntiae* and *Pleurotus cystidiosus*

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ABSTRACT

Vanillin (0.05 %) and carbendazim (1 mM) were selected as dual biochemical markers for the PEG mediated protoplast fusion between *Pleurotus opuntiae* and *P. cystidiosus*. Three days old *P. cystidiosus* and four days old *P. opuntiae* recorded the maximum protoplast yield at five and four hours after incubation, respectively with 0.6 MKCl and 30 mg ml^{-1} of enzyme consortium. Eight fusant lines with varied mycelial characters were obtained. Among fusants, F6 and F8 did not segregate in the second generation whereas, F4 segregated. F6 and F8 recorded higher biological efficiency of 168.05 and 99.95 per cent respectively compared to the parental lines and other fusants. Sporocarp of F6 and F8 were morphologically similar to *P. cystidiosus* and *P. opuntiae* respectively; and F8 also exhibited low temperature adaptability.

Key word: PEG, protoplast fusion, *Pleurotus opuntiae, P. cystidiosus*

Advances in research on mushroom breeding and production is very limited when compared to other crops, due to a limited knowledge on genetics and breeding systems. Somatic hybridization through protoplast fusion is a useful technique that facilitates recombination of economically important traits by harnessing the natural genetic diversity from phylogenetically distant species (Hodge *et al*., 2010). In this context, protoplast fusion is carried out to introduce the important traits like high biological efficiency, low temperature tolerance and shorter cropping period from either of the *Pleurotus opuntiae* and *P. cystidiosus* into their fusant progeny.

MATERIALS AND METHODS

Strain improvement *via* protoplast fusion

Interspecific hybridization was carried out between the isolate of *P. cystidiosus* (PNC1) and *P. opuntiae* (PO1) using polyethylene glycol (PEG) mediated fusion/somatic hybridisation.

Barrage reaction between *P. cystidiosus* and *P. opuntiae*

Barrage reaction *via* formation of large contact zone was used as a presumptive evidence for the sexual compatibility between *P. cystidiosus* and *P. opuntiae* isolates, as suggested by Mallick and Sikdar (2015). The selected parental mycelia were inoculated at 2 cm distance (dual plate technique), on petri dish, containing PDA (pH 6.2) and maintained at 24 ± 1°C for 5 days, until two mycelia formed a large contact zone. The experiment was carried out in triplicate and macro-morphology of contact zone/barrage reaction *viz.*, thick barrages, line transects, fluffiness of growth and pigmentation were observed.

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Double selection strategy for somatic hybrids

In somatic fusion experiments, hybrids are identified by double selection strategy (Lalithakumari, 2000). *P. cystidiosus* and *P. opuntiae* were tested for their tolerance or sensitivity to the fungicide carbendazim at concentrations of 0.1, 0.5 and 1 mM. Also the potential of parental isolates to utilize vanillin, a degradation product of lignin, at concentrations of 0.01, 0.02 and 0.05 per cent were analysed. The experiment was carried out in triplicate and protoplast fusants were screened/ segregated from the parental self fusants, based on the selected, biochemical marker characteristics *viz.,* carbendazim tolerance and vanillin utilization. Thus, dual biochemical markers *viz*., carbendazim (1 mM) and vanillin (0.05%) were selected.

Optimization of protocol for protoplast isolation

Factors controlling the protoplast isolation of the *Pleurotus* spp. *viz*., osmotic stabilizer, concentration of lytic enzyme, mycelial age and duration of incubation of mycelia with lytic enzyme were standardised for the optimization of protoplast production.

Effect of mycelium age, osmotic stabilizers, enzymesandincubation of mycelia on protoplasts isolation

Mycelia of *P. cystidiosus* and *P. opuntiae* were harvested from the 100 ml potato dextrose (PD) broth at different days of incubation *viz*., 3, 4 and 5 days age of incubation. Keeping the different age of incubation constant, protoplasts were isolated, with different osmotic stabilizers *viz*., 0.6 M sucrose and 0.6 M potassium chloride (KCl) (prepared in 0.01 M sodium phosphate buffer pH 6.5), using different concentration of lysing enzyme *viz*., 20, 25 and 30 mg/ ml, at different duration of incubation of mycelia *viz*., 1, 2, 3, 3.5, 4.5 and 5 h. Protoplasts released were monitored, by removing aliquots of the reaction mixture aseptically at an interval of 30 min, examined under compound microscope (Carl Zeiss Primo star, Germany) and quantified using haemocytometer. Six experiments (for *P. cystidiosus*) and *P. opuntiae*), at 3, 4 and 5 days age of incubation, were separately carried out in CRD, with three replications for each treatment. Once the optimal conditions for maximum protoplast production were established, protoplast isolation of *P. cystidiosus* and *P. opuntiae* were performed using the standardised age of mycelium, osmotic stabilizer, enzyme consortium at the optimised concentration (mg/ml) and duration of incubation of mycelia as per the standard procedure.

Protoplast isolation

Protoplasts were isolated and purified from the mycelial cultures of parents *viz*., *P. cystidiosus* and *P. opuntiae*, as per the standard procedure described by Lalithakumari (2000). Mycelial bits (8 mm diameter) from actively growing cultures of the *Pleurotus* spp.(6 days old) were inoculated separately, in conical flasks (250 ml) containing sterile PD broth (100 ml) and incubated at 28 ± 2 °C on a conical flask shaker (Amstrong Biotech Research, Bangalore) at 120 rpm. The mycelia (100 mg) was harvested, at the standardised age of incubation, by filtration through Whatman filter paper no. 1, washed twice with sterile water and washed twice with the standardised osmotic stabiliser (prepared in 0.1 Msodium phosphate buffer, pH 6.5). The washed mycelium of *Pleurotus* spp. were aseptically and separately transferred to sterile centrifuge tubes, containing 2 *ml* of enzyme mixture (at the optimised concentration), dissolved in the osmotic stabilizer (prepared in 0.1 M phosphate buffer, pH 6.0) and incubated at 26 ± 2 °C, in a rocking shaker, with 50 rpm (Rockymax, Tarsons, Kolkatta). The protoplasts released, at the optimised incubation time were observed under compound microscope (Carl Zeiss Primo star, Germany) and quantified using haemocytometer. A consortium of commercial enzymes *viz*., â-glucanase, cellulase, protease, and chitinase named as the Lysing enzyme from *Trichoderma harzianum* (L1412 SIGMA

Glucanex-lyophilized powder), was used for the protoplast isolation. The enzyme consortium was prepared in the optimum concentration followed by filter-sterilization, using sterilized filter disc assembly.

Protoplast purification

Protoplasts isolated from *P. cystidiosus* and *P. opuntiae* were filtered from the hyphal debris, through a column of cotton wool packed up to the 0.5 *ml* mark of a 5 *ml* syringe. Protoplasts were then collected and sedimented from the filtrate by centrifugation at 2000 rpm for 15 min. Sedimented protoplasts were washed twice in the best osmotic stabiliser and suspended in 5 ml of the same osmotic stabilizer. The purified protoplasts were further used for the subsequent experiments.

Regeneration of protoplasts

Regeneration of protoplasts of *P. cystidiosus* and *P. opuntiae* were checked in solid medium *viz*., malt extract, yeast extract, glucose medium (MYG) (Mukherjee and Sengupta, 1987). The purified protoplasts were diluted with the standardised osmotic stabiliser to about 10^4 cells/ml and 0.1 ml of the diluted suspension was plated on petri dishes containing 25 ml of MYG with standardised osmotic stabilizer (prepared in 0.01 M sodium phosphate buffer pH 6.5), as per the pour plate method. The plates were incubated at 28 ± 2 °C for 3-4 days. Similarly, an equal volume of protoplast suspension was mixed with equal volume of distilled water and plated on the osmotically stabilised MYG medium, which served as control. In the control plate, all the protoplasts bursted and no mycelial colonies appeared on the plates, unless some mycelial fragments got included in the sample. Number of colonies which appeared on the regeneration medium was counted and regeneration frequency was assessed. Regeneration frequency referred to the fraction of protoplasts that regenerated a new cell wall and reverted to normal hyphal growth.

$$\text{Regeneration frequency} = \frac{\text{Number of colonies appeared on regeneration medium}}{\text{Number of protoplasts plated}}$$

PEG mediated fusion

The PEG-mediated fusion was carried out according to Anne and Peberdy (1975) with some modifications. The purified protoplasts of *P. cystidiosus* and *P. opuntiae* were separately, diluted to $1x10^4$ protoplasts / mL, in the best osmotic stabilizer (prepared in 0.01 M sodium phosphate buffer, pH 6.5). Freshly prepared, diluted protoplast suspension of the parents (1 ml each) was mixed in sterile centrifuge tube and 1 mL of fusion mixture was added to the purified parental protoplasts. The mixture was incubated at room temperature for 20 min, by shaking the tube every 5 min manually, for uniform mixing. Fusion mixture comprised of PEG (molecular weight 4000, 30 per cent), calcium chloride (0.05 M) and glycine (0.05 M). After uniform mixing, the mixture was centrifuged at 2000 rpm for 10 min and supernatant was decanted. Pelleted protoplasts were washed twice with the osmotic stabilizer and re-suspended in 5 ml of osmotic stabilizer. The fusion processes were examined by observing the aliquots of fusion suspensions (10 µl), under a high resolution, compound microscope (Carl Zeiss Primo star, Germany) at 40 and 100 magnifications.

Regeneration of fused protoplasts

100 µl of the PEG treated, presumptively fused protoplasts was plated onto petri dishes containing osmotically stabilised MYG amended with the dual biochemical markers *viz*., carbendazim and vanillin at the standardised concentrations. Petri dishes were incubated at 25°C, for the development of presumptive (putative) somatic hybrid colonies (macrocolonies). After 1-2 days, somatic hybrid colonies were formed on the surface of the amended MYG medium (containing dual biochemical markers) and were individually sub-cultured on fresh PDA slants, for

subsequent studies. The purity of the protoplast suspension was checked by maintaining a control plate, wherein, the 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. Fusion frequency was determined as the ratio of number of colonies which appeared on regeneration medium to the number of protoplasts plated. Fusion frequency of fusants was compared with the regeneration frequency of *P. cystidiosus* and *P. opuntiae* based on CRD experiment, with five replications for each treatment.

Confirmation and evaluation of protoplast isolates and fusants

Comparison of radial colony growth and hyphal width of protoplast isolates, fusants and parents

The protoplast isolates (PNCi and POi) and fusants (8 strains) were evaluated for the evidence of variability by comparing their radial colony growth and nature of mycelial growth, with the parental strains (PNC1 and PO1) on Potato dextrose peptone agar (PDPA). Width of the hyphae was also compared, using compound microscope (Carl Zeiss Primo star, Germany) under 10 X and 40 X magnifications. The experiment was carried out in CRD, with three replications for each treatment.

Comparative spawn production studies with protoplast isolates, fusants and parents

Spawns of protoplast isolates and fusants (8 strains) were compared with the parental strains, using paddy grains as the spawn medium. Observations on the time taken for spawn run, nature of mycelial growth and shelf life were recorded in case of spawn production for all the evaluated oyster mushroom isolates. The experiment was carried out in CRD, with three replications for each treatment.

Cultivation trials with protoplast isolates, fusants and parents

Cultivation trials with protoplast isolates, fusants (8 strains) and parents were conducted during the September-February season of 2018-2019, using bed substrate, rubber sawdust, as per the standard poly bag method, under indoor climatic conditions, in the growing rooms. The developmental morphology, total yield, total crop period, average weight of sporocarp, number of sporocarps and B.E were recorded during the process of mushroom production. The experiment was carried out in CRD, with three replications for each treatment.

RESULTS AND DISCUSSION

Strain improvement of *Pleurotus* spp.

Barrage reaction

Compatibility studies revealed a positive mating reaction between the crosses *viz.*, *P. cystidiosus* x *P. opuntiae*, indicated by the formation of a thick barrage of intermingled hyphae at the zone of contact (Fig 1). In line with the present findings, Rosnina

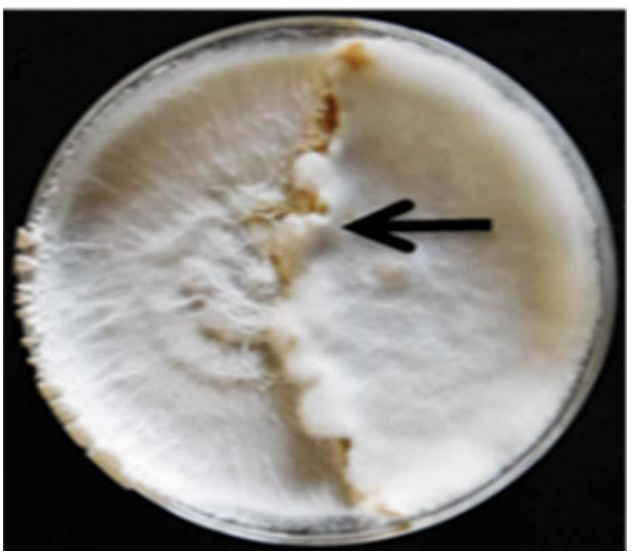

Fig. 1. Compatibility study between *Pleurotus* spp.

(2017) reported a positive mating reaction between *P. pulmonarius* and *P. citrinopileatus*, identified by a flat and smooth mycelial mat at their junction zone.

Biochemical markers

P. opuntiae was insensitive to the fungicide carbendazim, at all three concentrations, but it couldn't utilize vanillin (0.05%), while *P. cystidiosus* was sensitive to carbendazim, at all three concentrations but could utilize vanillin, at all three concentrations. Thus, in PDA plates amended with both carbendazim (1 mM) and vanillin (0.05%), the growth of both parents was inhibited. But the fusant showed uniform growth rate in all the plate assays indicating that it had acquired the characters of both the parents. As it showed biparental morphology, it could be confirmed as a recombinant (Table 1).

Table 1. Selection of dual chemical markers for protoplast fusants

Sl. No.	Biochemical markers	*P. opuntiae**		*P. cystidiosus**	
1	Carbendazim 0.1 m*M*	7.00	+	-	-
2	Carbendazim 0.5 m*M*	7.33	+	-	-
3	Carbendazim 1 m*M*	7.67	+	-	-
4	Vanillin 0.01 %	9.33	+	6.90	+
5	Vanillin 0.02 %	12.33	+	8.00	+
6	Vanillin 0.05 %	-	-	8.67	+
7	Control	7.00	+	7.33	+

*Average of five replications+: Presence of growth-: Absence of growth

Protoplast isolation

Effect of mycelium age on protoplasts formation

Maximum release of protoplasts from *P. opuntiae*, was recorded from 3 days old mycelium ($7.87x10^7$ cells/ml) when 0.6 M KCl was used as osmotic stabiliser, at 4 hrs after incubation with 30 mg/ml of enzyme concentration. However, protoplasts of *P. opuntiae*, showed regeneration through budding, when 3 days old mycelium was used. Hence, the age of mycelium was optimised as 4 days old. Initially, the protoplasts gave rise to a chain of yeast-like cells which arose from a single growth point. As the regeneration proceeded, chain of cells lengthened and up to 20 cells were produced. Eventually the terminal cell produced a hypha and the newly formed hypha lengthened, branched and ultimately gave rise to a new mycelium. A proportional decrease in the size of protoplasts was also recorded with increasing age of mycelium, *i.e.*5.98 x 5.61 µm (at 3 days old mycelium) to 2.40 x1.65 µm (4 days old), with increasing granular residual hyphae and cell debris.

Maximum release of protoplasts from *P. cystidiosus* was recorded from 4 days old mycelium (5.85×10^7/ml). This fact would be related to the changes in the hyphal wall, which passing from the exponential phase of growth becomes more resistant to enzyme degradation. It might be also associated with the high number of growing tips when young and actively growing cultures are used for protoplast production (Farina *et al*., 2004). Parani and Eyini (2010) identified that, 3 days old cultures of *P. eous* and *P. flabellatus* gave the maximum release of protoplasts.

Effects of Enzymes on Protoplast Isolation

P. opuntiae recorded an increased release of protoplasts, with increased concentration of enzyme from 20 to 30 mg/ml, for all treatments, with 0.6 M KCl at 4 hrs of incubation of 3 and 4 days old mycelium. Similarly, *P. opuntiae* recorded increased protoplast release from 3 days and 4 days old mycelium with increasing enzyme concentration, with 0.6 M sucrose at 4 hrs of incubation. At low concentration of enzyme (15 mg/ml), the lysis of fungal mycelium was confined only to a small portion, whereas at high enzyme concentrations (35 mg/ml), the mycelium lysed effectively yielding large numbers of immature protoplasts, which bursted immediately after release and got disintegrated. Most fungi have a cell wall consisting largely of chitin and other

polysaccharides and the plasma membrane is surrounded by three layers of cell wall materials, therefore, the enzyme consortium should comprise at least the major mycolytic enzymes, which are able to degrade the chitin and other components of the fungal cell wall. Protoplast yield from *P. cystidiosus* and *P. opuntiae* was standardised at 30 mg/ml of L1412 SIGMA Glucanex-lyophilized powder. Mengesha (2013) identified the enzyme combinations of chitinase (5 mg/mL), â-1,3-glucanase (3 mg/mL), driselase (10 mg/mL) and lyticase (5 mg/mL) prepared in 0.01M phosphate buffer containing 0.6M sodium chloride (NaCl), pH 6, for optimum protoplast yields of *P. florida* strains *viz* Pf5 and FRD2; and *V. volvacea* (Vv12).

Effect of osmotic stabilizers

The osmotic stabilizer 0.6 M KCl gave the best results, which produced 5.85 x 10^7 and 5.93 × 10^7 protoplasts/ml for *P. cystidiosus* and *P. opuntiae*, respectively. Similar to the present study, 0.6 M KCl was used as osmotic stabiliser for isolation of protoplasts from *P. eous* and *P. flabellatus* (Parani and Eyini, 2010).

Time of incubation

At the optimised age of *Pleurotus spp.,* initial lysis of the protoplast was observed two hrs after enzyme treatment, followed by swelling and rounding up of cell content within 3 hrs. Complete lysis of

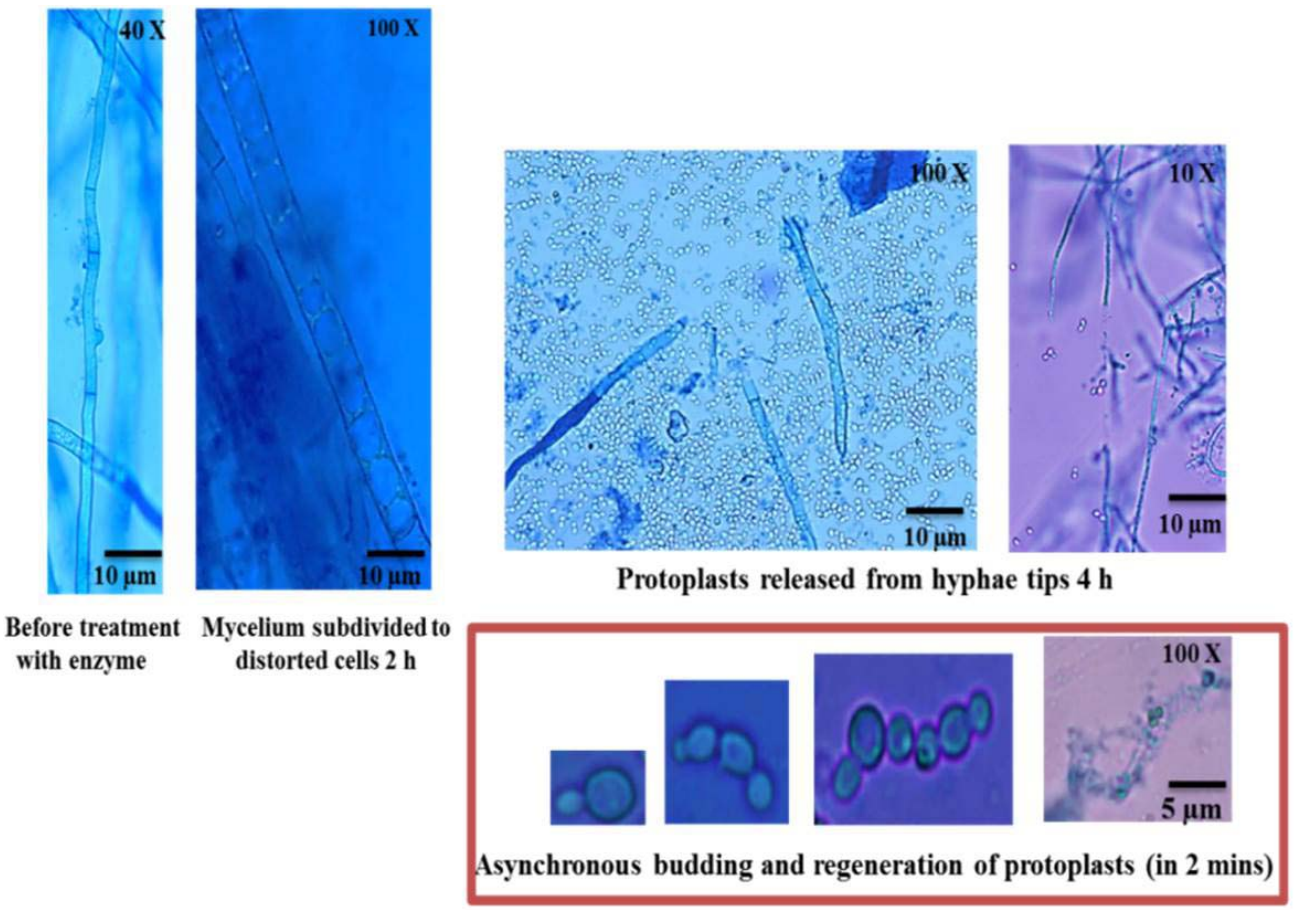

Fig. 2. Release of protoplasts from 4 days old *P. opuntiae* with 0.6 M KCl and 30 mg ml $^{-1}$ of enzyme

mycelium and release of protoplasts from mycelial tips was observed at 3.5 and 4.5 hours for *P. opuntiae* and *P. cystidiosus,* respectively. Maximum release of protoplast was recorded at 4 and 5 hours for *P. opuntiae* and *P. cystidiosus*, respectively. Also, bursting and considerable decrease in protoplasts was recorded, 5 and 6 hours after enzyme incubation, respectively. Thus, an optimized protocol involving 5 h hydrolysis of 3 day old mycelium of *P. cystidiosus* and 4 h hydrolysis of 4 day old mycelium of *P. opuntiae* was standardised, using 30 mg/ml of commercially available lysing enzyme from *Trichoderma harzianum* in a 1:1 (w/w) biomass: enzyme ratio, with 0.6 M KCl as osmotic stabilizer (Figure 2 and 3).

Micrographs showed that mycelium of *Pleurotus* spp. was first subdivided into small irregular fragments and there was some degree of cytoplasmic shrinkage at the beginning of incubation. Then, the fragments

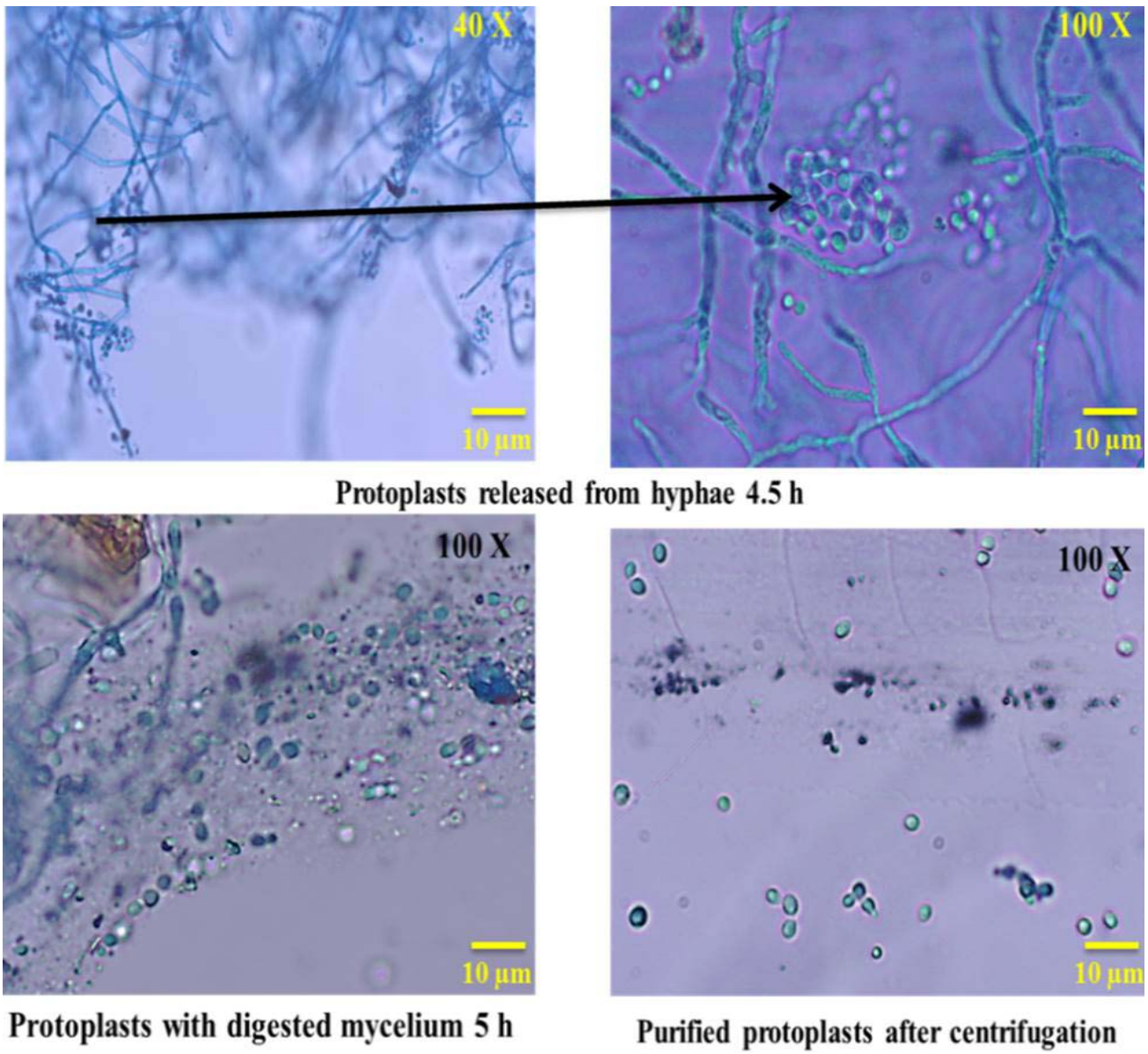

Fig. 3. Release of protoplasts from 3 days old *P. cystidiosus* with 0.6 M KCl and 30 mg ml^{-1} of enzyme

were transformed into distorted cells, and at the end of the incubation, almost all the cells were converted into protoplasts. At early stages, protoplasts were mostly emerging from the hyphal tips but, as cell wall degradation progressed, protoplasts emerged from other regions in addition to the tips. The protoplasts released from *Pleurotus* spp. initially were smaller in size but later they enlarged to spherical structures. A constant increase and subsequent decline in yield of protoplasts was observed with increasing time of incubation of *Pleurotus* spp. Microscopical observation revealed the successive release of small non-vacuolated and large vacuolated protoplasts, reflecting differences in susceptibility of the different regions of the hyphae to the lytic enzymes, *i.e*. newly synthesized wall at the hyphal tip get more readily degraded than the wall in older regions. Mallick and Sikdar (2015) recorded that, cell wall degrading enzymes released protoplasts from *P. florida* (1.5 × 107 protoplast / g tissue) and *L. squarrosulus* (9.6 × 10^6 protoplast / g tissue) after 12 and 10 h of incubation, respectively.

Regeneration of protoplasts

After 48 h of incubation, at 24°C protoplasts developed in to micro colonies on MYG media and were observed under stereo microscope. *P. cystidiosus* recorded significantly more number of colonies (3200.80), compared to *P. opuntiae* (2220.00), with fusion frequencies of 0.54 and 0.38, respectively (Fig 4; Table 2). Regenerated colonies were separately, transferred to PDPA. The germinating protoplasts soon developed into filamentous mycelia and exhibited no variation in mycelial morphology from the parent. Reversion to mycelial form is the starting point for downstream genetic manipulation.

Table 2. Regeneration frequency of protoplast isolates and protoplast fusants on regeneration medium

Sl. No.	Protoplast isolates/ fusants	Number of colonies regenerated on RM*	
		Counts	Regeneration/ Fusion frequency
1	*P. opuntiae* isolate (POi)	2220.00[b]	0.38[b]
2	*P. cystidiosus* isolate (PNCi)	3200.80[a]	0.54[a]
3	Fusants	1270.60[c]	0.21[c]
	SE m (±)	557.22	0.095
	CD (0.05)	0.974	0.001

*Average of five replications, Means followed by similar superscripts are not significantly different at 5% level

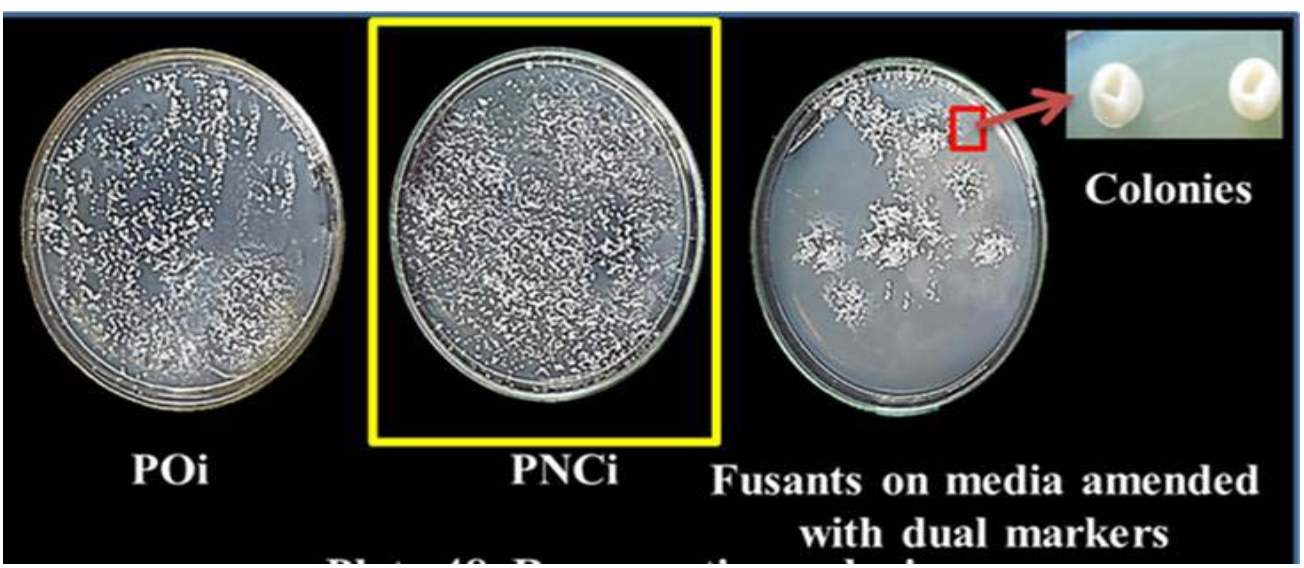

Fig. 4. Regeneration of colonies on MYG

PEG mediated fusion and regeneration

Protoplasts of *P. cystidiosus* and *P. opuntiae* at the optimized condition, recorded sizes of 1.50 x 1.35 µm size and 2.40 x1.65 µm size, respectively. With the addition of PEG solution, their sizes increased to 2.14 x 1.50 µm and 3.07 x 2.71 µm, respectively, in 5 minutes. Protoplasts were attracted and adhered to each other in 15 minutes, accompanied by increase in sizes to 2.84 x 1.99 and 7.92 x 6.72, respectively. In next 5 min, size increased from 13.06 x 11.18 µm and 43.73 x 31.07 µm to 55.66 x 53.56 µm and 102.37 x 113.72µm, respectively, followed by their fusion to form an elongated, oval cell of size 24.68 x11.49 µm. During, next 4 minutes, germination was observed through formation of one or two germ tubes. Self-fusion and multiple of protoplasts was also observed.

The fused protoplasts plated on MYG medium started regenerating in two days with a regeneration frequency of 0.21%, followed by development of mycelium in three days (Table 2). Parani and Eyini (2010) recorded a regeneration efficiency of 0.28 and 0.24% for *P. eous* and *P. flabellatus*, respectively. Regeneration of protoplasts requires optimum hypertonic culture media where cell wall regeneration takes place. Selvakumar *et al.* (2015) standardised protoplast fusion between *P. ostreatus* var. *florida* and *P. djamor* var. *roseus.* They standardised 40 g PEG in 100 mL of 0.05 M calcium chloride dihydrate ($CaCl_2.2H_2O$) as optimum mixture for protoplast fusion.

Confirmation and evaluation of strains/hybrids

Selection on the basis of radial growth

Eight protoplast fusant lines were obtained *viz*., F1, F2, F3, F4, F5, F6, F7 and F8, of which F6 took the minimum time for completion of mycelial growth (4.01 days), showing white, thick, fluffy and concentric growth, with initiation of primordial. Some putative hybrid fusants failed to grow in the subsequent sub-culturing processes, due to unstable genotype or loss of genotype. Two lines were also developed as protoplast isolates *viz*., POi (protoplast isolate of *P. opuntiae*) and PNCi (protoplast isolate of *P. cystidiosus*). Clamp connection was found in the

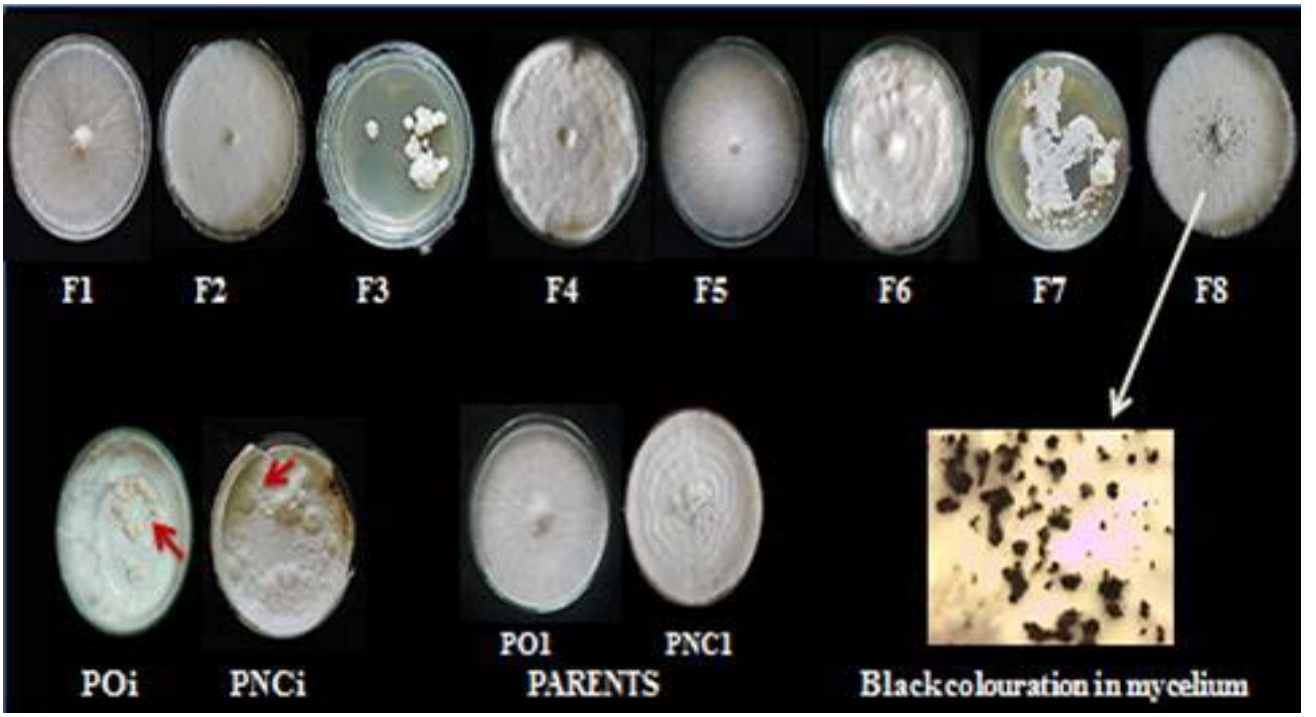

Fig. 5. Mycelial characters of parent isolates and protoplast isolates/fusants

Table 3. Mycelial characters of parent isolates and protoplast isolates/ fusants on PDPA

Sl. No.	Isolates	Nature of mycelial growth	Days for complete growth *	Hyphal width (µm)*
1	POi	Pure white, thin cottony, radiating with primordia	5.44^{cde}	1.38^{h}
2	PNCi	Pure white, thick cottony, radiating with primordia	5.01^{def}	2.43^{e}
3	F1	White, light cottony, stranded, radiating from centre	6.33^{c}	1.70^{g}
4	F2	White, thick cottony, suppressed, stickiness of mycelium on medium	5.44^{cde}	2.67^{b}
5	F3	White, dense, suppressed, radiating into discrete patches	22.00^{a}	3.13^{a}
6	F4	White, thick, fluffy	5.34^{cde}	1.93^{f}
7	F5	White, thin, radiating	6.44^{c}	1.20^{i}
8	F6	White, thick, fluffy, concentric with primordia	4.33^{ef}	1.97^{f}
9	F7	Slimy, sticky, scattered, crust like, actinomycete pattern	11.33^{b}	-
10	F8	White, thick cottony, suppressed, with black colourations, primordia and stickiness of mycelium on medium	4.00^{f}	2.61^{c}
11	PO1	Pure white, thick and fluffy with smooth margin	6.33^{c}	1.66^{g}
12	PNC1	Pure white, thick cottony with concentric zonations	6.00^{cd}	2.49^{d}
		SE m (±)	1.436	0.182
		CD (0.05)	1.132	0.048

*Average of three replications, Means followed by similar superscripts are not significantly different at 5 % level, Treatments without observation not taken for statistical analysis

parent lines, hybrids and protoplast isolates (Figure 5, Table 3). Gharehaghaji *et al*. (2007) suggested that, mycelia of hybrids produced faster and thicker mycelial mat than those of parent cultures. This is one of the most important adaptive characteristic that determines suitability towards neo-physiological condition and high biological efficiency. Similar finding was observed by Chauhan (2014). Rosina (2017) screened out two hybrid cultures *viz*., P1XC9 and P3XC8 which exhibited thicker mycelium, high colony density and faster growth rate (8.5, 8.2 mm/day) compared to *P. pulmonarius* and *P. citrinopileatus*.

Mother spawn production trials withthe protoplast isolates and fusants

F6 took the minimum time for mother spawn production (8.32 days). Thicker and fluffy growth was observed in F4, F6, F7, protoplast isolate of *P. opuntiae* and protoplast isolate of *P. cystidiosus* (Table 4). Kaur and Kapoor (2014) reported that, hybrid strain PFPS-131 completely impregnated the wheat straw in 19 days of incubation compared to parental strains *viz*., *P. sajor-caju*-3 (35 days) and *P. florida*-5 (33 days).

Cultivation trials with the protoplast isolates, fusants and second generation

The protoplast isolates of *P. cystidiosus* and *P. opuntiae* failed to produce fruiting bodies in rubber wood sawdust. Maximum fresh weight of sporocarps and biological efficiency was recorded with F6 (1677.30 g, 167.80 %). F8 took the minimum time for primordial initiation (16.40) with maximum number of sporocarps (2541.20). Fruiting body of F8 was similar to that of *P. opuntiae*, with 5.5x4.6 cm pileus size and 1.14 stipe length. Fruiting body of F6 was similar

Table 4. Comparative performance of parent isolates and protoplast isolates/fusants for mother spawn production in paddy grains

Sl. No.	Isolates	Days for mother spawn production*	Nature of mycelial growth
1	POi	10.62[i]	++++
2	PNCi	8.62[k]	++++
3	F1	19.62[d]	+++
4	F2	12.35[g]	+++
5	F3	26.35[a]	++
6	F4	13.35[f]	++++
7	F5	17.40[e]	+++
8	F6	8.32[l]	++++
9	F7	22.07[c]	++++
10	F8	10.30[j]	+++
11	PO1	23.68[b]	+++
12	PNC1	11.13[h]	+++
	SE m (±)	1.808	
	CD (0.05)	0.114	

++++: Thicker and fluffy growth +++ : Thick growth ++ : Poor growth

*Average of three replications, each replication denotes 5 spawns Means followed by similar superscripts are not significantly different at 5 % level

to that of *P. cystidiosus* with 14.82x11.79 cm pileus size and 1.50 cm stipe length. Fruiting body of the fusant line F4, showed recombined characteristics of the parental strains, with 9.61x9.10 cm pileus size and 0.81 cm stipe length (Table 5,Figure 4).

Thus, three isolates *viz*., F4, F6 and F8 gave normal, distinct fruiting bodies. Hence, cultivation trials with these were further carried out. F8 took the minimum time for spawn run and first harvest (16.30, 18.05 days) with maximum number of sporocarps (2787.00). Maximum fresh weight and biological efficiency was recorded with F6 (1680.35 g, 168.05%). Second generation of F6 and F8 line showed similar morphology to the original fusant lines, with 15.90x12.88 cm pileus size, 2.70 cm stipe length and 5.50x4.50 cm pileus size, 1.13 cm stipe length, respectively, with comparable biological efficiencies of 168.05 and 99.95%, respectively. However the fusant line, F4 showed segregation in the recombined character and reverted back to the morphology of its parent *P. opuntiae*, with 5.90 x 5.00 cm pileus size and 1.36 cm stipe length (Table 6; Figure 6).

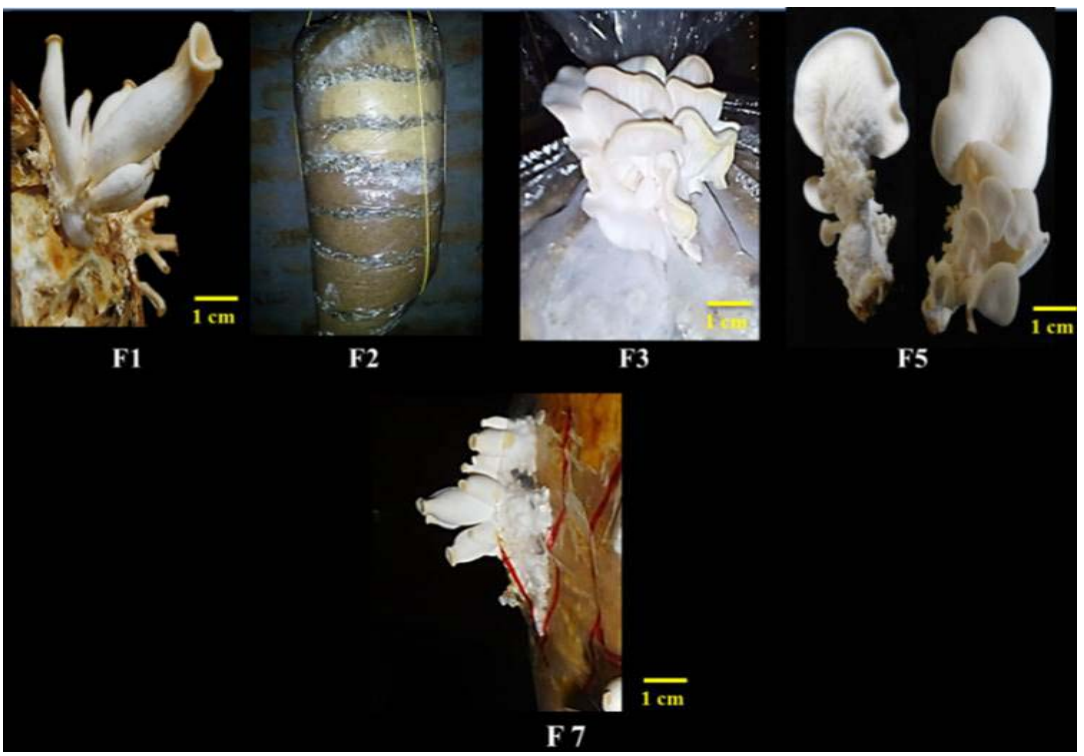

Fig. 6. Sporocarps of first generation

Table 5. Comparative performance of parent isolatesand protoplast isolates/fusants in rubber wood sawdust

Sl. No.	Isolates	Days for primordial initiation*	Days for first harvest*	Number of sporocarps*	Total yield per bed from three harvests (g)*	BE (%)*	Stipe length (cm)*	Pileus diameter (l cm x b cm)*
1	PNCi	-	-	-	-	-	-	-
2	POi	-	-	-	-	-	-	-
3	F1	25.25^{d}	-	-	-	-	-	-
4	F2	-	-	-	-	-	-	-
5	F3	29.35^{c}	34.25^{c}	200.00^{c}	97.95^{f}	9.70	0.40	3.70 x 2.50
6	F4	18.25^{f}	21.30^{d}	165.55^{f}	485.20^{e}	48.50	0.81	9.61 x 9.10
7	F5	17.20^{g}	20.30^{e}	266.30^{b}	90.25^{g}	9.00	2.53	2.40 x 3.66
8	F6	36.35^{b}	41.10^{b}	180.20^{d}	1677.30^{a}	167.80	1.50	14.82 x 11.79
9	F7	22.20^{e}	-	-	-	-	-	-
10	F8	16.40^{h}	18.20^{g}	2541.20^{a}	911.25^{c}	91.10	1.14	5.50 x 4.60
11	PNC1	51.15^{a}	55.30^{a}	107.95^{g}	1558.30^{b}	155.80	2.89	14.20 x11.20
12	PO1	16.65^{h}	19.40^{f}	170.10^{e}	659.40^{d}	65.90	2.45	8.00 x 6.30
	SE m (±)	3.878	5.351	337.544	242.53			
	CD (0.05)	0.417	0.452	0.646	0.524			

* Average of four replications, each replication denotes 5 beds
Means followed by similar superscripts are not significantly different at 5 % level
Treatments without observation not taken for statistical analysis

Table 6. Comparative performance of protoplast isolates of parents and fusants (second generation) in rubber wood sawdust

Sl. No.	Second generation	Days for primordial initiation*	Days for first harvest*	Number of sporocarps*	Total yield per bed from three harvests (g)*	BE (%)*	Stipe length (cm)*	Pileus diameter (l cm x b cm)*
1	F4	19.40^{c}	22.25^{c}	902.90^{b}	326.40^{e}	32.41	1.36	5.90 x 5.00
2	F6	38.25^{b}	41.65^{b}	67.75^{e}	1680.35^{a}	168.05	2.70	15.90 x 12.88
3	F8	16.30^{e}	18.05^{e}	2787.00^{a}	1000.25^{c}	99.95	1.13	5.50 x 4.50
4	PNC1	51.35^{a}	55.50^{a}	103.20^{d}	1555.30^{b}	155.59	2.81	14.10 x 11.00
5	PO1	17.20^{d}	21.30^{d}	169.90^{c}	654.35^{d}	65.10	2.46	8.10 x 6.28
	SE m (±)	6.988	7.243	518.522	258.366			
	CD (0.05)	0.381	0.648	0.969	0.484			

* Average of four replications, each replication denotes 5 beds,
Means followed by similar superscripts are not significantly different at 5 % level

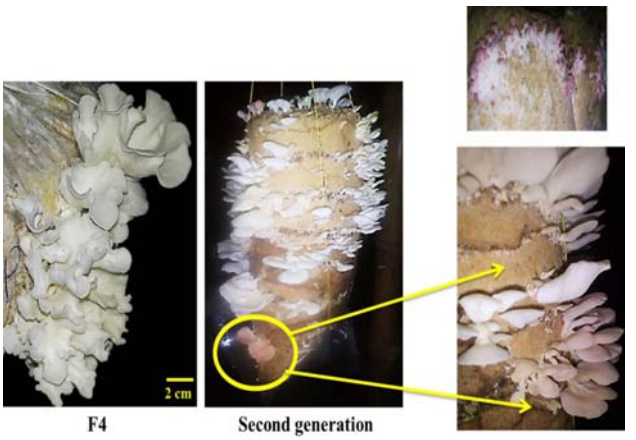

Fig. 7. Fruiting body characteristics of fusant lines

Morphological variations among the hybrid lines proved that the genome constitution of the independent hybrid lines was not equal and their differences in morphology resulted from gene recombination. Fruiting body of F4 was morphologically in between those of parents, whereas, F6 and F8 was similar to those of parents *viz*., PC1 and PO1, respectively. F6 and F8 were stable in their morphological characters; and recorded improved biological efficiency over the parents in both generations tested. However, F4 was not stable and segregated in the second generation. Whether this is the result of the original cytoplasmic variation or of some procedure during the protoplast technique, such as the selection of vital cells, is unknown and needs further investigation (Magae *et al*., 1985).

Rosina (2017) recorded that, sporocarps of somatic hybrids between *P. pulmonarius* and *P. citrinopileatus viz*., P8XC7 and P13XC7 were lung shaped similar; and was morphologically similar to *P. pulmonarius*. On the contrary, pileus of P19XC5 different from both the parental strains and produced fleshy and thicker sporophore with improved biological efficiency and durable shelf life. Sporocarps of the third generation belonging to P19XC5 showed consistency in morphological features. The somatic hybrids obtained through this study are not the end product and they could serve as resource material for further studies to give us insight about the basic

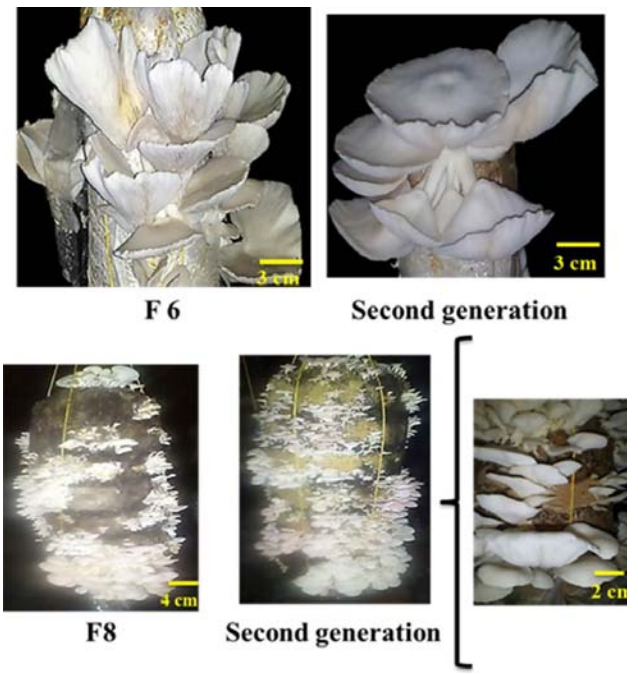

Fig. 8. Fruiting body characteristics of fusant lines

genetics of basidiomycetes mating type genes, clamp formation, mode of sexuality and mushroom improvement programmes Mallick and Sikdar (2015).

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