

Isolation, Regeneration and PEG-Induced Fusion of Protoplasts of *Pleurotus pulmonarius* and *Pleurotus florida*

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Inter-specific hybridization between *Pleurotus pulmonarius* and *P. florida* was attempted through PEG-induced protoplast fusion to select a fusant. The protocol for protoplast release, regeneration and fusion in these two *Pleurotus* species was standardized using the variables controlling the process. The mixture of mycolytic enzymes, *i.e.* commercial cellulase, crude chitinase and pectinase, KCl (0.6 M) as osmotic stabilizer, pH 6 of the phosphate buffer and an incubation time of 3 hours resulted in the maximum release of protoplasts from 3-day-old mycelia of *P. florida* ($5.3\text{--}5.75 \times 10^7$ protoplasts/g) and *P. pulmonarius* ($5.6\text{--}6 \times 10^7$ protoplasts/g). The isolated protoplasts of *P. florida* regenerated mycelium with 3.3% regeneration efficiency while *P. pulmonarius* showed 4.1% efficiency of regeneration. Polyethyleneglycol (PEG) - induced fusion of protoplasts of these two species resulted in 0.28% fusion frequency. The fusant produced fruiting bodies on paddy straw but required a lower temperature of crop running ($24 \pm 2^\circ\text{C}$) than its parents which could fruit at $28 \pm 2^\circ\text{C}$. The stable fusant strain was selected by testing for the selected biochemical markers *i.e.* Carbendazim tolerance and utilization of the lignin degradation product, vanillin.

KEYWORDS: Carbendazim, *P. florida*, *Pleurotus pulmonarius*, Polyethyleneglycol (PEG), Protoplast fusion

The fungal protoplast limited by the cell membrane can be isolated by mechanical or enzymatic removal of the cell wall. A viable protoplast is capable of cell wall regeneration, cell division, growth and it also reverts to the parent culture in an osmotically balanced medium. The isolation and regeneration of protoplasts has been considered a very useful tool in the biotechnological manipulations of higher plants and fungi (Peberdy, 1989). Protoplasts are also required for the genetic map studies using electrophoretic karyotype analysis as well as for the development of gene transformation systems and for the isolation of homokaryotic regenerates for their use in systematic breeding programme (Kaul, 2002). Protoplasts have been studied and prepared in a large number of edible mushrooms but their yields have been found poor as compared to non-basidiomycetes fungi (Zhao and Chang, 1993; Gupta *et al.*, 1997).

Protoplast fusion technology is applied for developing inter specific, intra specific and inter generic, intra generic supra hybrids with higher potentiality than their parental strains. Through protoplast fusion technique, improved strains with enhanced potential for the production of antibiotics, enzymes, useful myco products, and high yielding mushrooms could effectively be developed (Lalithakumari, 2000).

Oyster (*Pleurotus* sp.) mushrooms are commonly cultivated in Tamilnadu, India, as their cultivation technique is simple and as they can be grown under a wide range of

temperatures ($25\text{--}35^\circ\text{C}$). Strain improvement in mushrooms is carried out by induced mutations through UV irradiation or chemical mutagens. Protoplast fusion is yet to be developed as a successful strain improvement technique in mushrooms. This paper reports the standardization of the optimum conditions for protoplast release and regeneration in a native cultivated strain, *Pleurotus florida* and an exotic strain *Pleurotus pulmonarius* and for isolating a fusion product through PEG-induced protoplast fusion which showed the desirable characteristics of both the parents.

Materials and Methods

Fungal strains and growth conditions. A lyophilized culture of an exotic strain, *P. pulmonarius* (M42), procured from the National Institute for Agro-Environmental Sciences, Japan and a slant culture of native cultivated strain of *P. florida* (PF1) obtained from Tamilnadu Agricultural University, Coimbatore, India, were used in the present investigation. The cultures were maintained on Potato Dextrose Agar (PDA) slants at 15°C . Mating type compatibility between these two strains was checked by growing them as dual cultures in PDA plates with a distance of 4 cm between the two inocula and checking for the presence of a thick mycelial barrage at the zone of contact.

The parental strains were tested for their tolerance or sensitivity to the fungicide carbendazim in the range of concentration, 0.1 mM, 0.5 mM and 1 mM and for their

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potential to utilize vanillin, a degradation product of lignin, in the range of concentration, 0.01%–0.05%. The protoplast fusants were screened and segregated from the parental self fusants by the biochemical marker characteristics of carbendazim tolerance (Lalithakumari, 2000) and vanillin utilization (Cai *et al.*, 1993).

Isolation and purification of protoplasts. 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 (100 ml) and incubated at $28 \pm 2^\circ\text{C}$ on 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).

The culture filtrates of 3-day-old *Trichoderma harzianum* grown in Czapek broth substituted with chitin or pectin as the sole carbon source instead of sucrose were used as the source of lytic enzymes (Benitez *et al.*, 1975). The culture broth was filtered successively through glass wool and Geena glass filter (G_3) to remove the hyphal fragments. The lytic enzyme mixture (3 ml/study) used in the protoplast fusion studies contained equal volumes of chitinase and pectinase enzyme extracts and commercial cellulase (Celluclast, India). Potassium chloride (0.6 M) in 0.1 M phosphate buffer (pH 6.0) was used as the osmotic stabilizer.

The harvested mycelium (100 mg) was aseptically transferred to 1 ml of osmotic stabilizer (in 0.1 M phosphate buffer, pH 6.0) and the enzyme mixture (3 ml) was added to it. The mycelium in enzyme mixture was incubated on a rotary shaker (120 rpm) (Remi, India) at room temperature ($28 \pm 2^\circ\text{C}$) for 3 h (Lalithakumari, 2000). After the incubation period, hyphal fragments were removed by filtration through a column of cotton wool packed up to the 0.5 ml mark of a 5 ml syringe. Protoplasts were collected from the filtrate by centrifugation (1400 g, 10 min). Pellets were washed twice in 0.6 M KCl and then suspended in the same osmotic stabilizer. Protoplast yield was determined with a haemocytometer.

Effect of physico-chemical parameters. The different factors studied to standardize the protocol for protoplast isolation and regeneration were: different enzymes (Celluclast-commercial cellulase, crude pectinase, crude chitinase and their combination), osmotic stabilizers of different molarities (MgSO_4 , KCl, sorbitol - 0.2 to 1 M), age of the culture (3, 5, 7 days), pH of incubation medium (pH 4, 6, 8) and incubation time (1–4 h).

Regeneration and growth of protoplasts. Regeneration of protoplasts was checked in solid medium (Mukherjee and Sengupta, 1986). For regeneration, 0.1 ml of suitably diluted protoplast suspension (about $10^4/\text{ml}$) was plated on

MYG medium (malt extract, yeast extract, glucose medium) containing 0.6 M KCl as an osmotic stabilizer. The plates were incubated at room temperature ($28 \pm 2^\circ\text{C}$) for 3 to 4 days. The regeneration frequency was calculated as the ratio of number of protoplasts regenerated to the number of protoplasts incubated in the regeneration medium. Regeneration in liquid phase was checked by suspending aliquots of protoplasts in the regeneration medium both at $28 \pm 2^\circ\text{C}$ and observed under microscope at different intervals.

PEG-induced protoplast fusion. Protoplasts were fused following the method of Stasz *et al.* (1988) in the presence of polyethyleneglycol (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 (0.1 ml) of the fused protoplasts sample was plated on non-selective medium (MYG-malt extract 10 g/l; yeast extract, 4 g/l; glucose 4 g/l; agar 14 g/l, pH 5.5 with osmotic stabilizer) and checked for regeneration. Protoplasts from the same strains were also fused as controls. Fusion frequency was determined as the ratio of the number of regenerated colonies in regeneration minimal medium (RMM) to the number of regenerated colonies in regeneration complete medium. The isolation, regeneration and fusion of the protoplasts were observed and photographed using photomicrographic equipment (Nikon, Japan). Hyphal tips of regenerated colonies developing on RMM were transferred to minimal medium (MM-asparagine 2 g/l; glucose 20 g/l; thiamine - HCl 0.12 g/l; K_2HPO_4 1 g/l; KH_2PO_4 0.46 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g/l; agar 14 g/l. RMM contained in addition the osmotic stabilizer). This procedure excluded the possibility of a dual culture. Only those progeny that continued to grow on MM were considered to be fusion hybrids.

Selection of fusion products. The parent and fusant strains were subjected to fruiting trials using paddy straw (100 g with 70% moisture content) as the substrate. The fusant strains were selected by testing for sensitivity/resistance to the fungicide carbendazim and for the ability to utilize vanillin, a lignin derived phenolic compound.

Results and Discussion

Parental characteristics. The parental strains, *P. pulmonarius* and *P. florida* showed compatibility in dual culture plates with a thick barrage of intermingled hyphae at the zone of contact. In PDA plates amended with different

concentrations of carbendazim (0.1~1.0 mM), the growth rate of *P. florida* was not affected, while the growth of *P. pulmonarius* was significantly inhibited at the higher concentration. In PDA plates amended with different concentrations of vanillin (0.01~0.05%), the growth rate of *P. florida* was significantly inhibited at the higher concentration, while the growth of *P. pulmonarius* was not affected indicating its potential to utilize the lignin break-down product, vanillin (Table 1). The biochemical marker characters of carbendazim tolerance and vanillin utilization were used to characterize the parents and to identify the fusion products as suggested by Lalithakumari (2000) who reported that when wild - type parent strains were used as fusion partners, some type of selective medium must be employed to ensure that only fusion products regenerate into colonies.

Table 1. Radial growth (cm) of 6-day-old cultures of *P. florida* and *P. pulmonarius* on PDA plates amended with vanillin/carbendazim (values are mean \pm S.E of three replicates)

PDA medium Amendments	<i>P. florida</i>	<i>P. pulmonarius</i>
Control (PDA)	3.8 \pm 0.2	4.5 \pm 0.3
PDA + Vanillin (0.01%)	2.4 \pm 0.2	3.6 \pm 0.3
PDA + Vanillin (0.03%)	2.2 \pm 0.2	3.6 \pm 0.2
PDA + Vanillin (0.05%)	1.4 \pm 0.2	3.5 \pm 0.3
PDA + Carbendazim (0.1 mM)	3.6 \pm 0.3	3.6 \pm 0.3
PDA + Carbendazim (0.5 mM)	3.6 \pm 0.3	2.5 \pm 0.3
PDA + Carbendazim (1.0 mM)	3.4 \pm 0.2	1.3 \pm 0.1

Release of protoplasts. The mixture of lytic enzymes (commercial cellulase, crude pectinase, and crude chitinase) was more efficient in the release of maximum yield

Table 2. Effect of various factors on the protoplast yield ($\times 10^7$) of *Pleurotus florida* and *Pleurotus pulmonarius* (values are mean \pm S.E of three replicates)

Factors	Protoplast yield ($\times 10^7$)	
	<i>P. florida</i>	<i>P. pulmonarius</i>
1. Enzyme		
Commercial cellulase (3 ml)	4.40 \pm 0.08	4.83 \pm 0.36
Crude pectinase (3 ml)	3.10 \pm 0.16	2.80 \pm 0.16
Crude chitinase (3 ml)	4.66 \pm 0.20	4.90 \pm 0.20
Celluclast + pectinase + chitinase (1 ml + 1 ml + 1 ml)	5.42 \pm 0.08	6.00 \pm 0.16
2. Culture Age (days)		
3	5.30 \pm 0.16	5.80 \pm 0.16
5	3.42 \pm 0.38	4.66 \pm 0.22
7	2.50 \pm 0.16	2.08 \pm 0.22
9	1.80 \pm 0.16	0.90 \pm 0.16
3. Incubation time (h)		
½	---	---
1	2.40 \pm 0.21	2.82 \pm 0.26
1 ½	2.68 \pm 0.23	3.48 \pm 0.29
2	3.16 \pm 0.22	4.00 \pm 0.14
2 ½	4.66 \pm 0.22	5.66 \pm 0.22
3	5.40 \pm 0.16	5.80 \pm 0.16
4	2.70 \pm 0.12	1.80 \pm 0.15
4. pH of incubation medium		
4.0	4.25 \pm 0.28	3.91 \pm 0.30
6.0	5.30 \pm 0.16	5.60 \pm 0.08
8.0	4.75 \pm 0.28	4.66 \pm 0.22
5. Osmotic stabilizers (0.6 M)		
MgSO ₄	4.66 \pm 0.08	4.40 \pm 0.22
KCl	5.75 \pm 0.33	5.66 \pm 0.22
Sorbitol	5.41 \pm 0.25	4.75 \pm .17
6. Concentration of osmotic stabilizers (M)		
0.2	3.08 \pm 0.08	3.83 \pm 0.22
0.4	4.60 \pm 0.22	4.33 \pm 0.22
0.6	5.50 \pm 0.28	5.90 \pm 0.22
0.8	3.91 \pm 0.16	4.91 \pm 0.22
1.0	1.58 \pm 0.16	2.81 \pm 0.08

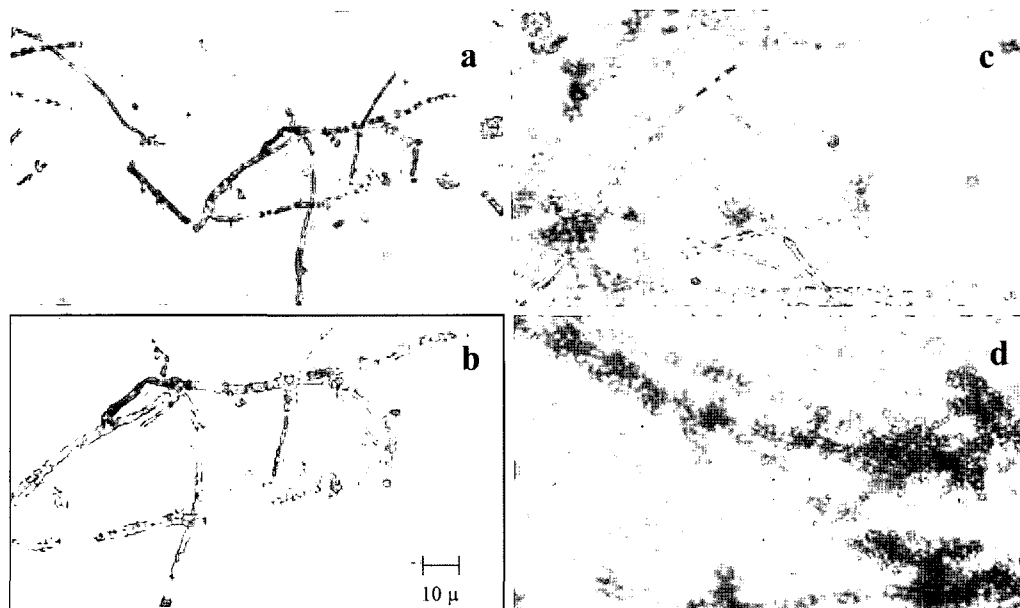


Fig. 1. Time course of protoplasts release from the hyphae of *P. pulmonarius* and *P. florida*. a. Rounding up of protoplasts with the hyphae ($\frac{1}{2}$ h), b. Protoplast emerging from hyphal tips (1 h), c. Protoplast with digested mycelial debris ($1\frac{1}{2}$ h), d. Chain of protoplast after complete digestion. Experimental conditions as in materials and methods.

of protoplasts from the cultures of *P. pulmonarius* and *P. florida* mycelia (6.0×10^7 and 5.42×10^7 protoplasts g^{-1} respectively) than the individual enzyme. 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 (Table 2, Fig. 1).

Potassium chloride (0.6 M) used as the osmotic stabilizer was the best to yield the maximum 5.75×10^7 protoplasts g^{-1} in *P. florida* and 5.66×10^7 protoplasts g^{-1} in *P. pulmonarius* mycelia respectively. Several other osmotic stabilizers like sucrose (Kim *et al.*, 2000), magnesium sulphate (Gupta *et al.*, 1997), potassium chloride (Iijima and Yanagi, 1986), mannitol (Wakabayashi *et al.*, 1985) and sorbitol (Vijaya palani, 1995) had been successfully used for maximum protoplast yield in fungi.

pH of the incubation medium was found to affect protoplast yield. Phosphate buffer at pH 6.0 was more effective in releasing the maximum number of protoplasts from the cultures of *P. florida* and *P. pulmonarius* (5.3×10^7 and 5.6×10^7 protoplasts g^{-1} mycelium respectively) than at other pH values. Several workers had recorded the optimum pH of the incubation medium for the maximum release of protoplasts in different white rot fungi *i.e.* citrate buffer at pH 4.0 for *Agaricus bisporus* (Gupta *et al.*, 1997) and pH 5.0 for *Lentinus lepideus* (Kim *et al.*, 2000); malate buffer pH 5.5 for auxotrophic mutants of *Coprinus macrorrhizus* (Kiguchi and Yanagi, 1985).

The yield of protoplasts depended on the age of the mycelium. When hyphae of various ages were used for

protoplast isolation, the highest number of protoplasts was derived from the youngest hyphae of *P. florida* and *P. pulmonarius* (3-day-old cultures) which yielded 5.3×10^7 protoplasts g^{-1} mycelium and 5.8×10^7 protoplasts g^{-1} mycelium respectively. The results are in accordance with these of various researchers who observed high yield of protoplasts from 2-day-old cultures of *Volvariella volvaceae* and in several mushrooms and other fungal cultures which are 3–6 days old (Gupta *et al.*, 1997; Kim *et al.*, 2000). The four-day-old cultures of *P. florida* and *P. pulmonarius* showed a drastic decrease in the yield of protoplasts. Zhao and Chang (1993) related the decrease in protoplasts yield from older mycelia to the changes in composition and thickness of hyphal cell wall associated with aging process.

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 h and their release through the hyphal tips at 2.5 h. The highest yield of protoplasts from *P. florida* and *P. pulmonarius* were obtained after 3 h of incubation with the complete digestion of the mycelium (Fig. 1) using the mixture of lytic enzymes (5.4×10^7 protoplasts g^{-1} *P. florida* mycelium and 5.8×10^7 protoplasts g^{-1} *P. pulmonarius* mycelium). Prolonged incubation resulted in a decrease in number of protoplasts from both the mushroom fungal mycelia (Table 2). Different white rot fungi were found to differ in the duration of incubation time required for maximum yield of protoplasts. A minimum incubation time of 2–3 h was required by *Volvariella volvaceae* (Mukherjee and Sengupta, 1988) but *Lentinus lepideus* exceptionally

needed 6 h of incubation for maximum release of protoplasts (Kim *et al.*, 2000).

Regeneration of protoplasts. Protoplasts of *P. florida* and *P. pulmonarius* showed a regeneration frequency of 3.3% and 4.1% respectively. Similar observations have been made by Zhao and Chang (1993) who noted that *P. florida* and *P. sajor-caju* required 3 and 4 days respectively and 30°C for regeneration. 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 *L. edodes* respectively. Two types of regeneration *i.e.* a chain of cells or a normal hypha directly emerging from the protoplasts were observed (Fig. 2). Lalithakumari (2000) reported similar patterns of hyphal regeneration (including a third type of a germ tube like hypha germinating from the protoplasts) in several filamentous fungi.

Isolation of fusion products. The protoplast fusion between *P. florida* and *P. pulmonarius* resulted in fusion frequency of 0.28%. Self fusants of the parents, *P. florida* and *P. pulmonarius*, in the control experiments showed nearly the same fusion frequency of 0.26% and 0.29% respectively. Six fusant colonies were isolated after eliminating self fusants by their growth on PDA plates amended with carbendazim (1 mM) and vanillin (0.05%). Only fusant No. 2 could be retrieved by transport through hyphal tips on minimal medium (MM) and on PDA plates with the amendments carbendazim and vanillin. It was sta-

ble even after five successive subculturings. By the definition of Zhao and Chang (1996), the fusant No. 2 was not a dual culture but should be a fusion hybrid of *P. florida* and *P. pulmonarius* as it could continue to grow on minimal medium. Lalithakumari (2000) reported that colony morphology can be used to identify interspecific and intergeneric fusion products especially if the species differ greatly in colony morphology. The fusant No. 2 showed mixed morphology acquired from its parents (*P. pulmonarius* with stringy hyphae and *P. florida* with fluffy hyphae) on PDA plates amended with carbendazim and vanillin (Fig. 3: c3).

Zhao and Chang (1996, 1997) obtained very low fusion frequencies of 0.0036 to 0.007% in intergeneric fusion between *P. ostreatus* and *S. commune*, while interspecific hybridization between *Volvariella volvaceae* and *V. bombycina* gave a much higher percentage of 0.032%–0.33%.

The fusant could not fruit at the crop running temperature required by its parents ($28 \pm 2^\circ\text{C}$) but produced fruiting bodies at a lower temperature of $24 \pm 2^\circ\text{C}$ (data not shown). Fusants of mushroom strains of compatible mating types produce fruiting bodies but those of incompatible mating types are sterile as they are either aneuploids or heteroploids. There are reports on the fusants exhibiting novel nutrient and biochemical characteristics even

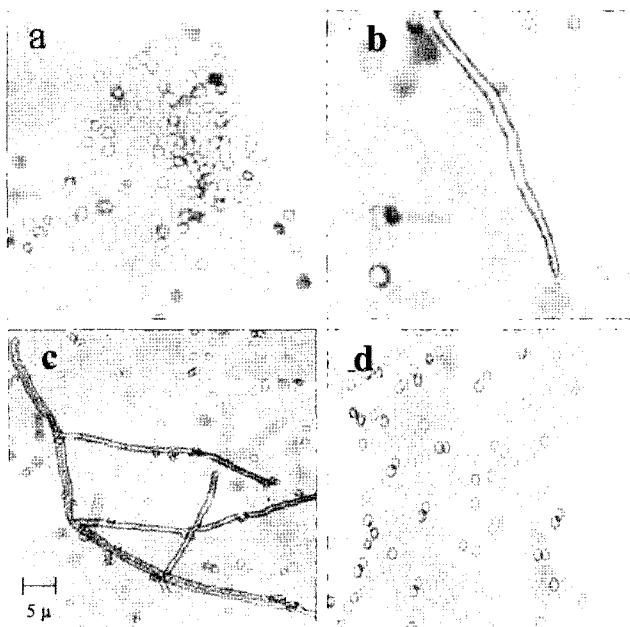


Fig. 2. Isolation regeneration and fusion of protoplasts *P. pulmonarius* and *P. florida*. a. Isolated protoplast, b, c. Regeneration of protoplasts into chain of cells and hyphae; d. PEG-induced protoplast fusion. Experimental conditions as in materials and methods.

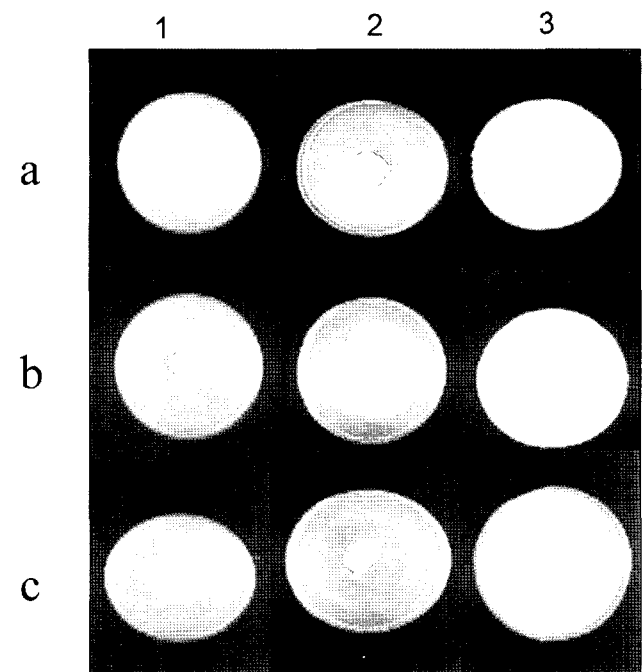


Fig. 3. Selection of fusion product of *P. pulmonarius* and *P. florida*. a. Vanillin tolerant, Carbendazim sensitive *P. pulmonarius*, b. Vanillin inhibited, Carbendazim resistant *P. florida*, c. Vanillin tolerant, Carbendazim resistant fusant on 1. Vanillin (0.05%) + PDA, 2. Carbendazim (1 mM) + PDA, 3. Vanillin (0.05%) + Carbendazim (1 mM) + PDA.

though they resembled any one or both the parents morphologically (Zhao and Chang, 1996). Non-parental interspecific fusion products were also obtained in *Pleurotus* (Go et al., 1989). Yoo et al. (1987) mentioned that the induced markers like auxotrophic and drug resistant mutants were often unstable and resulted in slow growth, while the naturally occurring property of tannin tolerance was stable and did not inhibit the growth rate. Specific tolerance to tannin and the ability to utilize vanillin, a lignin derived phenolic compound had been used as selective markers for mushrooms like *L. edodes* in protoplast fusion experiments (Cai et al., 1993). Among the parent strains, *P. pulmonarius* was sensitive to the fungicide carbendazim (1 mM) but it showed the ability to utilize vanillin (0.05%), while *P. florida* showed resistance to carbendazim (1 mM) and growth inhibition in the presence of vanillin (0.05%). 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 (Fig. 3). As it showed biparental morphology (Fig. 3: c3), it could be a recombinant as suggested by Lalithakumari (2000). Further work is in progress to study the ligno cellulolytic potential and the nutrient use efficiency of the protoplast fusant.

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