



## Development of high yielding morphologically improved strains of *Pleurotus* through interspecific hybridization

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### ABSTRACT

The aim of hybridization is to combine desirable characters from different strains and create variability in the existing germplasm. Understanding of heterothallic nature of *Pleurotus* species makes the breeding programme a success. *Pleurotus florida* PAU-5 and *P. sajor caju* PAU-3 were cultivated to raise four and six spore prints which were germinated to isolate 28 and 56 monospores, respectively. Interspecific crosses (1568 no.) were conducted to develop 102 hybrid dikaryons which were shortlisted to 57 hybrids on the basis of endoglucanase activity and subjected to fruiting test. Thirty five hybrids were able to fruit. Maximum harvest of fruit bodies was observed in hybrid no. 42 with 97% biological efficiency (BE). Four hybrid cultures showed higher BE compared to *P. florida* PAU-5 while seven hybrids gave more yield than *P. sajor caju* PAU-3. Average weight of a fruit body ranged between 3.3g to 12.5g. Seven hybrids showed morphologically distinct characters. Based on yield and desirable characters, five hybrids were identified. Hybrid no. 8 and 37 showed morphology different from the parent strains while hybrid no. 16 and 46 were similar to the parent *P. sajor caju* PAU-3 but with higher whiteness index. Hybrid no. 42 was morphologically similar to *P. florida* PAU-5 but had a 51% higher BE. The strains developed with altered phenotypic expression, viz. pileus shape, colour etc. and better yield indicated usefulness of interspecific hybridization to improve yield and quality. This technique can be extended to improve other characters of *Pleurotus* namely sporeless strain, strains better suited to changing climate conditions, etc.

**Key words:** Biological efficiency, Interspecific hybridization, Monokaryons, *Pleurotus florida*, *P. sajor caju*

*Pleurotus* is a commercially important mushroom, widely cultivated throughout the world for their good taste, flavour, ease of cultivation and vast potential applications. It belongs to class basidiomycetes, order aphyllophorales, family Tricholomataceae with about 40 well defined species. *Pleurotus* attains tetrapolar and heterothallic nature which is controlled by two unlinked multiallelic loci. The fertile progeny is generated only when fusion involves haploids heteroallelic at both the loci. Understanding the genetic nature makes the breeding programme a success to improve quality of this mushroom.

*Pleurotus florida* PAU-5 and *P. sajor caju* PAU-3 have been recommended for cultivation in Punjab under natural environmental conditions during October to April (Khanna and Kapoor 2007). *P. florida* PAU-5 strain is high yielding strain with whitish to creamish, convex near margin but more or less depressed at centre and smooth pileus. Margins

are entire and incurved. Stipe is 2-7 cm long, eccentrically placed and white in color. In contrast, *P. sajor caju* PAU-3 is low yielding strain which has grayish, flat but more or less depressed near attachment and smooth pileus. Margins are incurved and entire but become straight at maturity. Stipe is 1-2 cm long, rudimentary and white in colour. White colour and high yield is a preferable character in *P. florida* PAU-5 while fruit body with rudimentary stipe of *P. sajor caju* PAU-3 is preferable.

Present study was aimed to develop high yielding morphologically improved strains through interspecific hybridization between *P. florida* PAU-5 and *P. sajor caju* PAU-3.

### MATERIALS AND METHODS

*P. florida* PAU-5 and *P. sajor caju* PAU-3 were procured from the culture collection bank, Department of Microbiology, Punjab Agricultural University, Ludhiana. The cultures were sub-cultured periodically after every 4 weeks and incubated at 25±1°C for 10 days on potato dextrose agar (PDA) slants and stored at 4°C.

The spawn was prepared on boiled wheat grains mixed

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with 2% CaCO<sub>3</sub> and 4 per cent CaSO<sub>4</sub> powders, autoclaved at 1.8 kg /cm<sup>2</sup> for 1.5 hr.

Paddy straw (cut 3-5" length) was spread on cemented floor, wetted with clean, fresh water for 16-20 hr to attain 60-70% moisture content, inoculated with spawn @ 10% (w/w) of dry straw using thorough spawning method and was then filled in polythene bags of size 18"×12" to 2/3<sup>rd</sup> of its capacity (1.6 kg wetted straw in each polythene bag). The top of bags was tied with gunny thread and incubated at 25±1°C for 25 to 35 days for complete impregnation. The bags were cut opened and watered daily twice using misty spray. The mushroom started appearing in flushes up to 35-40 days. Fruit bodies were harvested when the margin start rolling upwards or downwards. The number and weight of fruit bodies from each bag were recorded. Percent biological efficiency (BE) was calculated by the formula:

$$BE (\%) = \frac{\text{Fresh wt. of mushrooms}}{\text{Dry wt. of substrate}} \times 100$$

Basidiospores of *P. florida* PAU-5 and *P. sajor caju* PAU-3 strains were collected in a specially constructed glass apparatus designed by Pal (1986). The spore print was preserved at 25°C for further use.

A small bit of spore coated paper was cut and aseptically transferred into 10 ml saline solution. Serial dilution was made to get approximately 500 spores per ml using haemocytometer. 0.1 ml of spore suspension was spread on PDA agar plate and incubated at 25±1°C for germination. After 3-4 days of incubation, single spore isolates (monokaryons) appeared as small colony heads which were carefully transferred on to the PDA slants and incubated at 25±1°C for 7-10 days. These monospore cultures were confirmed as monokaryons, showing no clamp connections under the microscope at 45x, and were maintained on PDA slants at 4°C (Fig 1A).

Interstrain cross hybridization was done between monokaryons of *P. florida* PAU-5 and *P. sajor caju* PAU-3. The mycelial bits of both strains, each of 4 mm diameter, were placed on sterilized PDA plate with the mycelium of the bits almost touching each other as well as agar medium

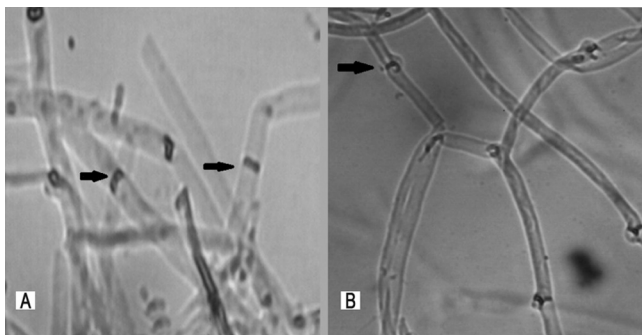


Fig. 1 (A) Monokaryotic mycelium without clamp connection, (B) Dikaryotic mycelium showing hook shaped clamp connection.

of the petriplate. These plates were incubated at 25±1°C for 4-5 days. Depending on their compatibility, the dikaryons were screened out by picking the part, where the mycelium of the two showed fluffy growth at the junction. This cottony fluffy growth indicated the putative dikaryon formation which were examined microscopically (45X) for presence of clamp connections to confirm dikaryotic nature and stored as above (Fig 1B).

The hybrid dikaryons were grouped on the basis of least significant difference of endoglucanase activity between hybrid dikaryons in broth culture. From each group at least three hybrids were randomly selected for cultivation. Further selection was made on the basis of morphological characters and yield potential of hybrid dikaryons.

### RESULTS AND DISCUSSION

#### Collection and germination of basidiospores

*P. florida* PAU-5 and *P. sajor caju* PAU-3 were cultivated to collect six spore and nine spore prints, respectively. Viability of the spores was estimated to calculate their germination efficiency which was in the range of 10 to 18% (Table 1). Out of six PF-5 spore prints, four spore prints

Table 1 Percentage germination efficiency of basidiospores and number of single spore isolates of *Pleurotus* species

Spore print	Germination efficiency (%)	Single spore isolates	Total
<i>Pleurotus florida</i> PAU-5			
FA	10	FA1, FA2, FA3, FA4, FA5	5
FB	13	FB1,FB2, FB3, FB4, FB5, FB6, FB7	7
FC	12	FC1, FC2, FC3, FC4, FC5, FC6, FC7	7
FD	18	FD1, FD2, FD3, FD4, FD5, FD6, FD7, FD8,FD9	9
FE	0	No isolate recovered	0
FF	0	No isolate recovered	0
		Total number of isolates	28
<i>Pleurotus sajor caju</i> PAU-3			
SA	17	SA1, SA2, SA3, SA4, SA5, SA6, SA7, SA8, SA9, SA10	10
SB	11	SB1,SB2, SB3, SB4, SB5, SB6	6
SC	16	SC1, SC2, SC3, SC4, SC5, SC6, SC7, SC8, SC9, SC10	10
SD	0	No isolate recovered	0
SE	16	SE1, SE2, SE3, SE4, SE5, SE6, SE7, SE8, SE9, SE10, SE11	11
SF	15	SF1, SF2, SF3, SF4, SF5, SF6, SF7, SF8	8
SG	0	No isolate recovered	0
SH	10	SH1, SH2, SH3, SH4, SH5, SH6	6
SI1	10	SI1, SI2, SI3, SI4, SI5	5
		Total number of isolates	56

Table 2 Total number of confirmed hybrids on the basis of clamp connection

Assigned code no.	Hybrid culture	Assigned code no.	Hybrid culture	Assigned code no.	Hybrid culture
1	FB5 × SA2	35	FA3 × SA3	69	FB7 × SH1
2	FB4 × SC10	36	FB5 × SE4	70	FC1 × SA4
3	FB6 × SI1	37	FA4 × SA1	71	FC5 × SC4
4	FB4 × SI1	38	FA2 × SA9	72	FC5 × SF1
5	FD1 × SA9	39	FB1 × SB2	73	FB3 × SB1
6	FA2 × SC5	40	FA3 × SB1	74	FA1 × SA1
7	FB2 × SI3	41	FA2 × SH1	75	FA2 × SC1
8	FD4 × SA3	42	FB3 × SI3	76	FD1 × SF6
9	FA4 × SC10	43	FA5 × SC7	77	FB1 × SA3
10	FD9 × SH5	44	FD4 × SF2	78	FB3 × SF2
11	FB1 × SF6	45	FB1 × SE5	79	FC1 × SI3
12	FD4 × SC5	46	FA1 × SF6	80	FD8 × SF4
13	FB3 × SA7	47	FB3 × SC5	81	FD4 × SH4
14	FB3 × SB3	48	FB3 × SA3	82	FC5 × SB5
15	FA5 × SF8	49	FA3 × SI3	83	FD5 × SA1
16	FB5 × SE7	50	FB6 × SI5	84	FD7 × SC1
17	FA2 × SB2	51	FD1 × SI1	85	FC5 × SH4
18	FB3 × SE6	52	FC1 × SA6	86	FA5 × SA2
19	FD1 × SF3	53	FB7 × SE7	87	FA5 × SF6
20	FA4 × SH6	54	FD4 × SF6	88	FD8 × SE5
21	FB7 × SE9	55	FC1 × SE7	89	FA4 × SA3
22	FA3 × SC5	56	FD2 × SA8	90	FA4 × SA5
23	FB6 × SC4	57	FB7 × SE6	91	FA4 × SA8
24	FB7 × SF2	58	FB1 × SA2	92	FA4 × SB6
25	FB2 × SA10	59	FA5 × SA1	93	FA1 × SA5
26	FB7 × SE8	60	FA2 × SB6	94	FA1 × SA10
27	FB7 × SF1	61	FD7 × SE1	95	FA1 × SC5
28	FB2 × SC10	62	FC5 × SE6	96	FB6 × SA1
29	FD4 × SE1	63	FD6 × SA2	97	FB6 × SC10
30	FA4 × SH1	64	FD6 × SA3	98	FB6 × SA9
31	FA5 × SA3	65	FB1 × SI3	99	FA5 × SH4
32	FB2 × SI5	66	FC1 × SF8	100	FA5 × SH6
33	FB6 × SA8	67	FC2 × SB5	101	FA2 × SA10
34	FA1 × SE8	68	FB7 × SF6	102	FA2 × SB1

showed germination of spores, which were named as FA, FB, FC and FD to give 5, 7, 7 and 9 monospore cultures, respectively. Nine spore prints of PSC-3 designated as SA to SI gave 56 monospore cultures (Table 1). However 2 spore prints SD and SG did not show germination of any spore.

Shnyreva and Shtær (2006) isolated monokaryons and dikaryons based on presence and absence of clamp connections for mon-mon and di-mon matings of *P. pulmonarius* and *P. ostreatus*. Hernandez and Salmenes (2008) had isolated 20 monokaryons from each strain of *Pleurotus ostreatus*.

#### Interspecific hybridization between *P. florida* and *P. sajor caju*

The monospore cultures isolated from *P. florida* PAU-5 (28 no.) and *P. sajor caju* PAU-3 (56 no.) were intercrossed

on PDA agar medium to produce hybrid dikaryons. One thousand five hundred and sixty eight crosses were conducted to develop 102 hybrid dikaryons with hybridization efficiency of 6.5% (Table 2). The hybrid dikaryons were confirmed microscopically to possess clamp connection up to 3-5 subculturing.

Gharehaghaji *et al.* (2007) conducted 289 crosses between monokaryons of different strains of *Pleurotus ostreatus* to develop 27 hybrid dikaryons with hybridization efficiency of 9.3%. Interspecies hybridization between *P. florida* and *P. eous* monokaryons was conducted by Sawashe and Sawant (2005) to develop 9 hybrids with hybridization efficiency of 12.5%.

#### Selection of hybrid dikaryons

The hybrid dikaryons were subjected to production of

Table 3 Grouping of hybrid dikaryons on the basis of endoglucanase activity using critical difference

Group no.	Endoglucanase activity ( $\mu\text{g/ml/min}$ )	Culture code*
1	2.3-7.04	<b>14, 3, 18, 44, 63, 32, 47</b>
2	7.2-11.6	48, 65, 99, <b>78, 62, 79, 15, 29</b>
3	12.8-16.7	45, 69, 100, <b>04, 54, 43</b>
4	19.5-24.1	84, <b>42, 86, 101, 94, 13, 17, 20, 30</b>
5	24.6-29.1	<b>22, 59, 102, 70, 49, 64</b>
6	31.0-34.5	50, <b>34, 56, 26, 51, 83, 95</b>
7	36.1-36.8	<b>61, 1, 75</b>
8	41.0-45.7	<b>52, 77, 9, 89, 96, 7</b>
9	45.9-47.4	<b>71, 19, 76, 82, 41, 38</b>
10	56.0-58.3	10, <b>31, 25, 39, 33, 60, 28</b>
11	63.8-97.0	<b>16, 2, 21</b>
12	69.0-73.6	<b>72, 81, 40, 90, 93, 68</b>
13	74.9-76.9	53, <b>58, 24, 11</b>
14	79.9-83.4	<b>35, 57, 5, 23</b>
15	86.0-89.1	<b>27, 91</b>
16	91.9-96.6	<b>12, 98, 92, 73, 80</b>
17	97.5-98.7	66, <b>37, 97, 74, 85</b>
18	103.4-107.8	<b>67, 36</b>
19	124.3-125.0	<b>87, 88</b>
20	131.0	<b>6</b>
21	140.2-144.5	<b>55, 8</b>
22	178.0	<b>46</b>

CD (P = 0.05) = 4.74

\*Selected cultures are given in bold.

endoglucanase enzyme in modified Mushroom Minimal Broth (g/l of L-asparagine-1.6, D-glucose-20,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -0.5,  $\text{KH}_2\text{PO}_4$ -0.46,  $\text{K}_2\text{HPO}_4$ -1.0, Thiamine hydrochloride-0.125). A wide variation in enzyme activity ranging from 2.3 to 178  $\mu\text{g/ml/min}$  was observed. The hybrids were grouped into 22 classes on the basis of least significant difference in enzyme activity between hybrid dikaryons (Table 3). Number of hybrids per group varied from 1 to 9. At least three hybrid dikaryons were randomly selected giving 58 cultures for further study.

Bahukhandi and Sharma (2002) categorized hybrid dikaryons into nine groups based on size, shape, colour, yield etc. From each group, one type sample having high yield, early fruiting and light colour was selected for further studies.

#### Cultivation of hybrid dikaryons

Fifty eight hybrid dikaryons were subjected to fruiting test to confirm dikaryotic nature of mycelium. Thirty five hybrids were able to fruit. The observations were recorded for the days of spawn run, number of fruit bodies, total yield and average weight of fruit bodies. A complete spawn run was observed from 26 to 65 days of incubation. Six hybrids showed earlier spawn run compared to parent *P. florida* PAU-5 while 2 hybrids with respect to *P. sajor caju* PAU-3 (Table 4).







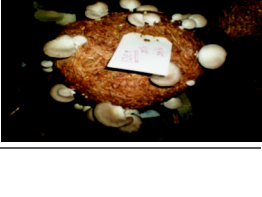
Table 4 Cultivation of hybrid dikaryons

Sr. no.	Culture code	Spawn run	No. of fruit bodies/q dry straw	Yield* (%BE)	Av. wt. of a fruit body (g)
1	1	35	5 121	30	5.8
2	2	40	5 555	47	8.5
3	4	55	1 891	13	6.9
4	6	35	6 067	63	10.4
5	8	45	7 879	39	5
6	12	55	5 200	34	6.6
7	14	35	7 327	55	7.5
8	15	53	3 545	31	8.7
9	16	32	7 958	48	6
10	18	56	2 836	35	12.5
11	22	55	10 636	54	5.1
12	23	40	9 218	41	4.5
13	24	55	6 618	28	4.2
14	25	55	6 973	59	8.5
15	26	42	9 691	55	5.7
16	27	65	6 382	44	6.9
17	31	40	2 994	17	5.8
18	32	39	3 073	14	4.7
19	33	40	1 891	20	10.6
20	35	30	14 655	84	5.7
21	36	50	3 309	12	3.8
22	37	40	8 273	43	5.1
23	40	32	9 848	55	5.6
24	41	29	19 382	73	3.8
25	42	31	14 418	97	6.7
26	43	29	11 188	56	5
27	46	26	7 091	65	9.1
28	52	39	10 636	69	6.4
29	54	37	6 736	58	8.6
30	71	32	9 139	62	6.8
31	75	55	6 145	33	5.4
32	78	55	3 427	23	6.7
33	79	36	15 364	80	5.2
34	80	40	6 855	22	3.3
35	82	28	10 873	64	5.9
	**PF-5	34	7 583	64	8.4
	***PSC-3	31	6 295	60	9.5
	CD (5%)	2.0	52.8	3.7	0.1

Dry straw per bag – 550 g; spawn rate – 10% of dry weight of straw; date of spawning – 6 November 09; date of termination – 1 Feb 2010; crop season – November – March; \*kg/q dry straw, \*\**P. florida* PAU-5, \*\*\**P. sajor caju* PAU-3.

The yield data indicated maximum harvest of fruit bodies in hybrid no. 42 showing 97% biological efficiency (BE) while minimum was observed in hybrid no. 36 (12% BE). Four hybrids (35, 42, 52, 79) showed higher % BE in comparison to that of the parent *P. florida* PAU-5 while seven hybrids (35, 41, 42, 46, 52, 79, 82) yielded higher than the parent *P. sajor caju* PAU-3. Four hybrid cultures (6, 46, 82, 71) showed yield at par with the parent *P. florida* PAU-5 while four hybrid cultures (6, 25, 54, 71) showed yield at

Table 5 Morphological description of cultivated hybrid dikaryons

Culture code	Morphological description	Image
8	Pileus 3-7 cm broad, flat, brittle, light gray color, surface is rough with depression lines radiating towards centre. Margin incurved and streaked. Stipe is 1-1.5 cm, white in color, lateral in position. Gills white, decurrent and distantly placed.	
16	Pileus 4-9 cm broad, flat, smooth, often imbricate, white with light gray tints at margin. Margin entire, straight becomes upturned at maturity. Stipe was 1-1.5 cm, rudimentary, white in color, lateral in position. Gills white, decurrent, membranous and not crowded at all.	
37	Pileus 2.5-6 cm broad, white centrally depressed convex near margin. Margin dentate and incurved. Stipe 2-4 cm long, white, eccentric in position. Gills decurrent, white and distantly placed.	
42	Pileus 3-9 cm broad, white to cremish color, smooth, infundibuliform to centrally depressed. Margin upturned and entire. Stipe 3-8 cm long, eccentric and white in color. Gills white, decurrent and not crowded at all.	
46	Pileus 4-8 cm broad, smooth, light gray color, often imbricate, flat but more or less depressed towards attachment. Margin entire and straight becomes upturned and curled at maturity. Stipe 1-1.5 cm long, rudimentary and white in color. Gills white, decurrent, membranous and not crowded at all.	
<i>P. florida</i> PAU-5	Pileus 4-9 cm broad, white to cremish, smooth, convex near margin while more or less depressed centrally. Margin entire and incurved. Stipe eccentrically placed, short (2-7 cm long) and almost equal in diameter throughout. Gills white, decurrent, membranous and not crowded at all.	
<i>Pleurotus sajor caju</i> PAU-3	Pileus 5-8 cm broad, gray color which lessens towards stipe attachment, flat but more or less depressed towards attachment. Margin incurved and entire but becomes straight at maturity. Stipe 1-2 cm long, rudimentary and white in color. Gills white, decurrent, membranous and not crowded at all.	

par with the parent *P. sajor caju* PAU-3. Twenty seven hybrids in comparison to *P. florida* PAU-5 and 24 hybrids with respect to *P. sajor caju* PAU-3 revealed low yield potential (Table 4).

Maximum average weight of fruit body was observed in hybrid no. 18 (12.5 g) while hybrid no. 80 showed minimum average weight of 3.3 g. Six hybrid cultures (6, 15, 18, 33, 46, 54) showed higher average weight compared to parent *P.*

*florida* PAU-5 while three hybrids (6, 33, 18) have higher average weight compared to *P. sajor caju* PAU-3.

A wide variation has been observed in number of fruit bodies (1891-19382) in hybrid cultures. Thirteen hybrid cultures in comparison to PF-5 and 20 hybrid cultures in comparison to PSC-3 gave higher number of fruit bodies while remaining showed less number of fruit bodies to that of both parents (Table 4).

Suwannee and Wichain (1996) reported 40 combinations of monokaryons of *P. ostreatus* with clamp connection, out of which only 24 were able to produce fruit bodies. Hybrid cultures with high yield as well as lower yield compared to that of parent has been reported in literature. Nattaya and Wichain (1997) reported yield of 4 hybrid dikaryons higher than that of parents, Florida type oyster mushroom while other hybrids showed lower yield potential. Five hybrid dikaryons were developed by Kaur *et al.* (2008) among monokaryons of *Pleurotus florida* PAU-5, out of which PFJ 11 out yielded the parent while average weight of fruit bodies was higher in PFJ 13 (9.9 g) as compared to parent (9.6 g). Spawn run was faster in PFJ 11 (39 days) and PFJ 14 (41 days) with respect to that of the parent (48 days).

#### Morphological characterization

The hybrid dikaryons were characterized for their morphological characters as observed from pileus, stipe and gills of each hybrid in order to compare with the parents. Out of 35 hybrid dikaryons, 20 resembled with the parent *P. florida* PAU-5 while 8 were close to *P. sajor caju* PAU-3 with a little variation in pileus from hybrid to hybrid. Seven hybrid dikaryons (8, 16, 25, 37, 42, 46, 71) showed morphologically distinct characters. Hybrid no. 25 showed gray color pileus with upturned and curled margin which was not a desirable character. Whereas hybrid no. 42 and 71 were morphologically similar to PF-5. On comparing yield of these hybrids with parent *P. florida* PAU-5, hybrid no. 42 revealed 35 kg higher yield than hybrid no. 71. Based on the yield and morphological characters, five hybrid dikaryons (8, 16, 37, 42, 46) were identified for future characterization. The detailed morphological description of these hybrid dikaryons has been tabulated along with the parents in Table 5.

A change of morphology of hybrid cultures compared to parents has been reported in literature. Bahukhandi and Sharma (2002) obtained a specific hybrid by mating of *P. sajor caju* and *P. cornucopiae* having shape and size of fruit

body similar to *P. sajor caju* with white color resembling *P. cornucopiae*. Kaur (2007) developed funnel shaped fruit bodies, lateral fruit bodies with wavy margins and gray color fruit bodies by intraspecific hybridization *P. florida* PAU-5.

Thus interspecific hybridization proved a successful technique to improve yield and quality of *Pleurotus* and can be used to develop efficient substrate utilizing strains, sporeless strains, strains better suited to changing climate, etc. In future, the hybrids will be evaluated for texture, color, enzyme potential and will be characterized at molecular level.

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