Short Communication

Development of improved strain of *Pleurotus* species by interspecific hybridization

K.R. Jyothi* and Susha S. Thara

College of Agriculture, Kerala Agricultural University, Thiruvananthapuram 695 522, Kerala, India

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Abstract

Oyster mushroom (*Pleurotus* spp.) commonly called as Dhingri in India, is one among the commercially cultivated mushrooms throughout the world and stands second in world production. However, cultivated mushrooms face problems like loss of genetic diversity and strain degeneration. Hence, there is an increased demand for the development of new and improved strains with high production and better growth attributes. The present study was conducted to improve the strain of *Pleurotus* species by interspecific hybridization through crossing single spore cultures. *Pleurotus djamor* is an early yielding pink coloured oyster mushroom having leathery texture and low biological efficiency (BE). It was crossed with the high yielding, delicate *Pleurotus florida* and *Pleurotus ostreatus*, which required comparatively more days for primordial initiation. Two hybrids with blended characters of both the parents were developed by hybridization. The BE of the hybrids *viz., P. djamor* x *P. florida* and *P. djamor* x *P. ostreatus*, was improved by 34.38 and 48.48 per cent respectively over the parent, *P. djamor*. The days needed for primordial initiation were significantly reduced in *P. djamor* x *P. florida* and *P. djamor* x *P. ostreatus* by two days and three days respectively compared to the early yielding parent, *P. djamor*. Analysis of proximate content revealed that hybrids were superior in protein and fibre content. The genetic relatedness between parents and hybrids was evaluated using Random amplified polymorphic DNA (RAPD) and the hybrids exhibited more similarity to *P. djamor*.

Key words: Interspecific hybridization, Proximate content, RAPD analysis, Strain improvement.

High production along with good quality is always the principal goal for agriculturally important crops without the exception of mushrooms. Among the mushrooms, *Pleurotus* spp stands second in world production with 19 per cent contribution to total mushroom production (Sharma et al., 2017). It has high biological efficiency (BE) along with high nutritional values, therapeutic and biological properties.

For the study, cultures of *P. djamor*, *P. florida* and *P. ostreatus* obtained from the Mushroom Unit, Instructional Farm, College of Agriculture, Vellayani were selected. *P. florida* is a well-known mushroom which needed 22 days for primordial initiation and had 80.08 per cent biological efficiency (Chauhan, 2013). *P. ostreatus* required 21.4 days for pinhead initiation in paddy straw substrate (Khaliq et al., 2013) with BE of 66.33 per cent (Sharma and Sharma, 2014). Pinheads emerged within 16 days in pink coloured *P. djamor*, with BE of 43.05 per cent in paddy straw (Satpal et al., 2017; Jose, 2018). These species of *Pleurotus* could be exploited to aggregate the beneficial growth attributes, thus improving the strain with high BE and shorter duration.

Genetically improved strains not only increase the quality of cultivated mushrooms, but also reduce the cost of cultivation. They can also increase

*Author for correspondences : Phone: +91 9633742044, Email: jyothikr235@gmail.com

farmers' revenue in short term (Avin et al., 2012). Among the various methods used for strain improvement, hybridization method has been proved to be the best and most sustainable. Therefore, studies were carried out on interspecific hybridization among the given species of *Pleurotus* and the newly formed strains were evaluated.

The serial dilution method demonstrated by Bahukandi and Sharma (2002) was followed to get the monosporous cultures of the three species. Spore suspension was prepared from the spore print obtained by placing fresh mature sporocarp on a sterilized Petriplate. It was further diluted up to 10^{-4}, such that the spore concentration was as low as four to five spores when observed in Petri plate under low power microscope (100X magnification). The diluted spore suspension (1 mL) was pipetted out to the centre of sterile Petri plate. A thin layer of two per cent plain agar was then poured into the Petri plate and was uniformly mixed. The spores were marked under low power microscope and were allowed to germinate for two days. The marked germinated spores were then picked by a sterile inoculation needle and were grown in potato dextrose peptone agar (PDPA) media at 26+ 2° C.

Single spore cultures obtained were observed for their mycelial appearance and radial growth rate. Among the cultures, the fast growing, dense single spore cultures were selected from each strain. The single spore cultures of *P. djamor* were tested for compatibility in every possible combination with *P. florida* and *P. ostreatus* separately. Mycelial discs (five mm) of ten day old single spore cultures were placed two cm apart in a nine cm diameter Petriplate containing PDPA media and was incubated at 25°C for seven days.

Based on the prominent interaction and formation of thick tuft in the contact zone, compatible mating pairs were selected. A small portion of mycelium from the junction was cut and observed under microscope for clamp connection. Pairings were confirmed as compatible mating if clamp connections could be observed on the hyphae (Anitha, 1998). Then, a culture disc of five mm size was cut off from the confluence region on the Petri plate and sub-cultured to PDPA plate.The heterokaryotic mycelia thus produced were screened; fast growing and thick stranded cultures were selected. These were brought to mother spawn production and further cultivation trials. The spawn of the species of *Pleurotus* and their hybrids was prepared in paddy grains as per the standard procedure (Sinden, 1934). The fully grown spawn was used for cultivation in paddy straw as per the procedure described by Baskaran et al. (1978).

The experiment was laid out in completely randomized design (CRD) with five treatments. Four replications were maintained for each treatment. Each replication denoted five mushroom beds. The crop was laid in November, 2018.

Morphological observations, days needed for the completion of mycelial run on substrate bags, appearance of pinheads, days needed for harvest, average number of fruiting bodies and total crop period were recorded.The BE was worked out as follows:

$$\text{BE (per cent)} = \frac{\text{Total weight of fresh fruiting bodies}}{\text{Total dry weight of the substrate}} \times 100$$

The proximate contents present in parents and hybrids were analyzed and the results were compared. Nutrients like crude fibre, carbohydrate and protein were estimated by using the standard procedures as described by De (1965), Aminoff et al. (1970) and Bradford (1976).

Good quality genomic DNA was isolated from the hybrids and the parental species by using DNeasy plant mini kit (Qiagens).The PCR amplification reactions were carried out in a 25 µl reaction mixture containing 1 µl of 2 U of Taq DNA polymerase, 0.5 µl of 2.5mM $MgCl_2$, 2 µl of 2.5 mM dNTPs, 10 X PCR buffer 2.5 µl (100 mM TrisHcl), 100 ng DNA and 10 pmol of primer (OPT 5 GGGTTTGGCA). RAPD and the amplification condition was

optimized as initial denaturation at 94°C for 5 min followed by 35 cycles; denaturation at 94°C for 1 min, primer annealing at 61°C for 1 min and initial extension at 72°C for 2 min followed by final extension at 72°C for 10 min (Agarwal et al., 2013). Amplified product was subjected to electrophoresis on 1.2% agarose gel containing ethidium bromide in 0.5X TAE buffer at 75V for 1 h. Finally, the image was documented using Gel documentation system (Bio-Rad Gel DOC™ XR+) under UV light.The RAPD profiles were analyzed and Jaccard's similarity coefficient was generated. The matrix was used for grouping the mushroom samples based on the dendrogram constructed by UPGMA (Unweighed Pair Group Method with Arithmetic averages). All the numerical analysis was conducted using the software SPSS.

Basidiospores of species of *Pleurotus* were isolated based on serial dilution method demonstrated by Bahukandi and Sharma (2002). Twenty marked germinated spores from each isolate were picked by a sterile inoculation needle and were allowed to grow in PDPA medium. Ten fast growing, dense and cottony single spore cultures were selected from each species of *Pleurotus*. Selection of compatible pairs in hybridization studies was done based on study conducted on mycelial morphology by Gharehaghaji et al. (2007) and Guadarrama-Mendoza et al.(2014).

Single spore cultures of *Pleurotus* species are unfertile in nature, and only after mating with desirable homokaryons, produce fruiting bodies. They lack clamp connection which was further confirmed by observing under microscope (Jaswal et al., 2013).

The single spore cultures of *P. djamor* were tested for compatibility in every possible combination, with *P. florida* and *P. ostreatus* separately. Formation of thick strand at the interaction zone of the monokaryotic culture implied successful mating (Plates 1&2). Bahukandi and Sharma (2002) inferred that among the four basidiospores produced by *Pleurotus,* one type could only mate with one of the remaining three spores. Thus the sterile fertile ratio would be 75:25. Mating type in heterokaryotic

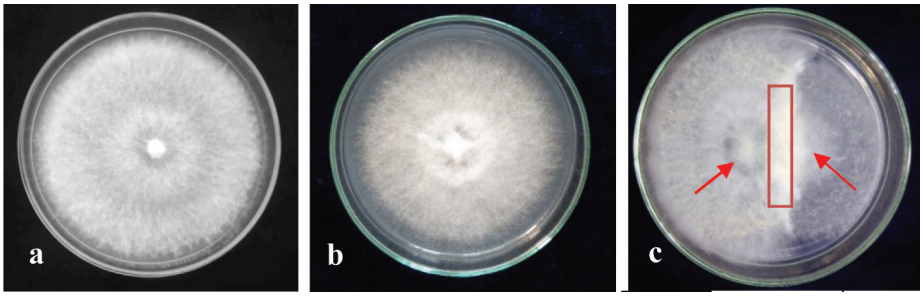


Plate 1. Single spore culture of *P. djamor*, *P. florida* and their dual culture
a. *P. djamor* **b.** *P. florida* **c.** Barrage formation in confluence region of *P. djamor* and *P. florida*

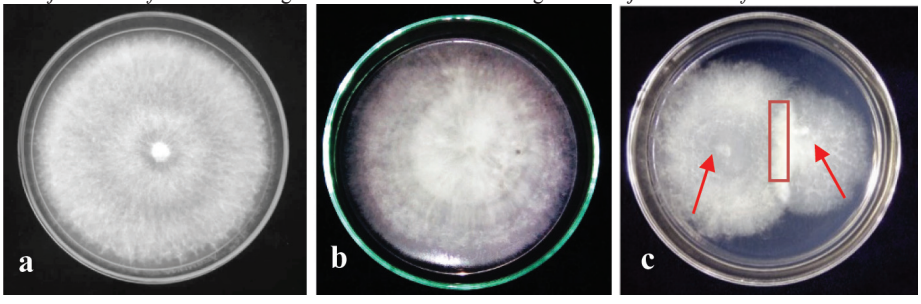


*Plate 2.*Single spore culture of *P. djamor, P. ostreatus* and their dual culture **a**. *P. djamor* **b**. *P. ostreatus* **c**. Barrage formation in confluence region of *P. djamor* and *P. ostreatus*

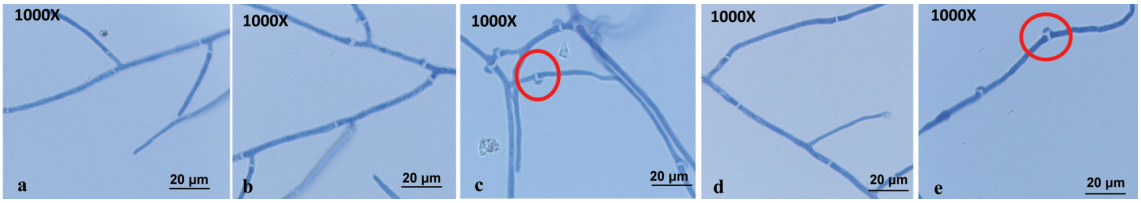


Plate 3. Mycelium of single spore culture of species of *Pleurotus* and their hybrids indicating absence/presence of clamp connection **a**. *P. djamor* **b**. *P. florida* **c**. *P. djamor* x *P. florida* **d**. *P. ostreatus* **e**. *P. djamor* x *P. ostreatus*

mushrooms like *Pleurotus* is controlled by two genetic loci named as A and B. The dikaryon was generated only when anastomosis involved haploids heteroallelic at both the loci (Jaswal et al., 2013).

Based on the prominent interaction and formation of thick tuft in the contact zone, ten such compatible mating pairs were selected. A small portion of mycelium was cut from the confluence region in the Petri plate and observed under microscope for clamp connection (Plate 3). Thick stranded fast growing six dikaryon cultures were used for spawn production and cultivation trials. Based on better growth attributes and stability over three generations, one hybrid from each cross was selected for comparative studies.

Hybrids and their parental species were cultured in PDPA media (Plate 4) and mother spawn was prepared. Mushroom beds were laid and hybrids along with parental species were compared for their growth performance (Table 1). Observation on number of days needed for spawn production revealed that both the hybrids, *P. djamor* x *P. florida* and *P. djamor* x *P. ostreatus* were significantly superior and completed the spawn production in minimum number of days (8.75 and 8.50) (Plate 5). Similarly, the number of days needed for complete spawn run, primordial initiation and first harvest were also minimum for these hybrids. The

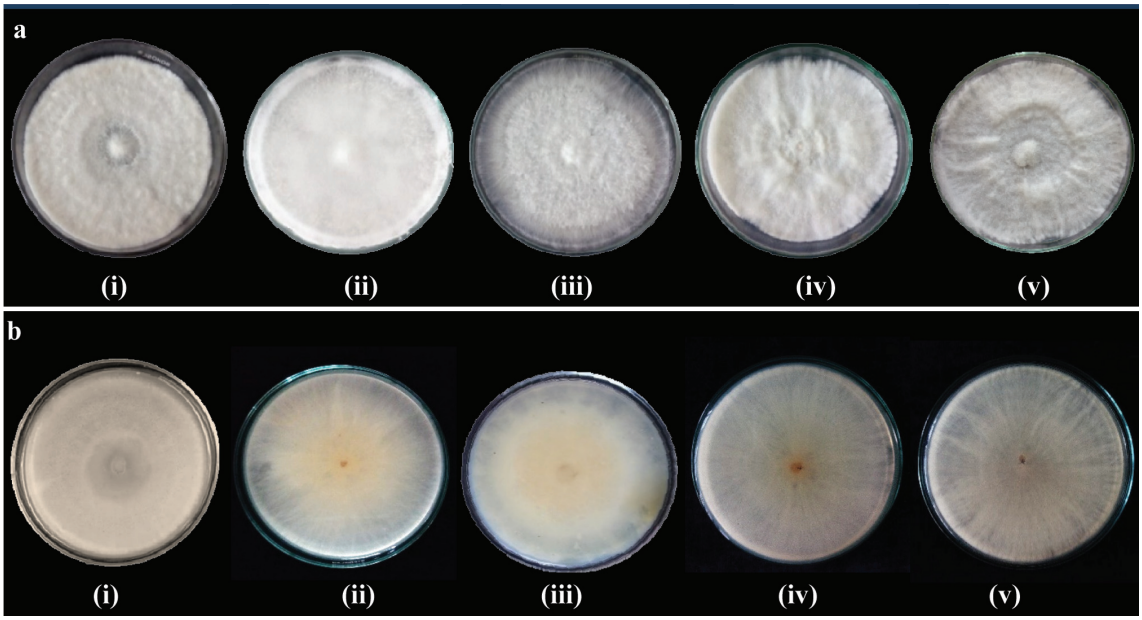


*Plate 4.*Colony morphology of species of *Pleurotus* and their hybrids in PDPA medium a.Upper side b. Rear side (i) *P. djamor* with concentric pattern (ii) *P. florida* with white fluffy mycelium (iii) *P. ostreatus* with greyish white mycelium (iv) *P. djamor* x *P. florida* with cottony cream mycelium (v) *P. djamor* x *P. ostreatus* with greyish white mycelium and radiating growth

Table 1. Comparative performance of species of *Pleurotus* and their hybrids developed through hybridization followed by three generations of selection in paddy straw substrate

Species of *Pleurotus* and their hybrids	Days taken for spawn production	Days taken for complete spawn run	Days taken for pinhead formation	Days taken for first harvest	Total yield* (g-fresh weight)	Crop period (days)	Sporocarp weight (g-fresh weight)	No. of sporocarps	BE (%)
P. djamor	15.75±0.50^{b}	9.75±0.50^{b}	14.75±0.50^{c}	17.75±0.50^{b}	406.25±15.84^{e}	41.00±0.82^{b}	6.03±1.15^{c}	97.75±8.34^{bc}	40.63
P. florida	20.75±0.96^{a}	19.75±0.95^{a}	25.75±0.96^{a}	28.75±0.96^{a}	703.75±17.40^{b}	53.50±1.29^{a}	11.47±2.10^{a}	85.50±5.20^{c}	70.38
P. ostreatus	20.50±0.58^{a}	19.00±0.82^{a}	24.25±0.50^{b}	28.25±0.50^{a}	828.25±49.68^{a}	54.50±1.29^{a}	10.72±2.03^{a}	125.50±16.42^{a}	82.83
P. djamor x *P. florida*	8.75±0.50^{c}	7.75±0.50^{c}	13.00±0.82^{d}	16.00±0.82^{c}	546.00±42.43^{d}	43.25±1.89^{b}	7.93±0.65^{b}	92.00±4.96^{bc}	54.60
P. djamor x *P. ostreatus*	8.50±0.58^{c}	7.75±0.50c	12.25±0.50^{d}	15.25±0.50^{c}	603.25±27.97^{c}	43.50±2.64^{b}	7.69±0.81^{b}	102.75±5.50^{b}	60.33
CD (0.05)	1.128	1.039	1.039	1.039	50.919	2.597	1.081	13.959	
SE (m) ±	0.371	0.342	0.342	0.342	16.74	0.854	0.541	4.589	

*Total yield from three harvest. Values are mean ± SD of four replications. Values followed by similar superscripts are not significantly different at 5 % level

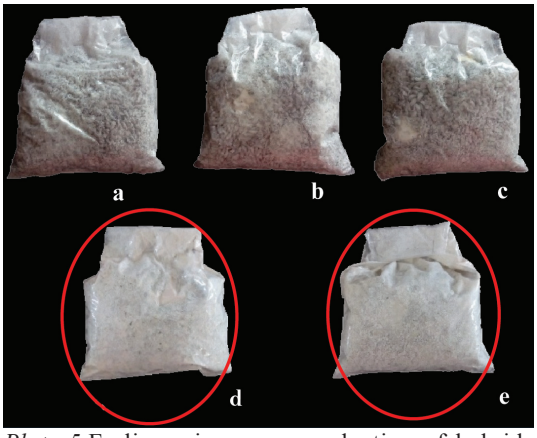


Plate 5.Earliness in spawn production of hybrids compared to their parental species of *Pleurotus* on 9th day of culture inoculation a. *P. djamor* b. *P. florida* c. *P. ostreatus* d. *P. djamor* x *P. florida* e. *P. djamor* x *P. ostreatus*

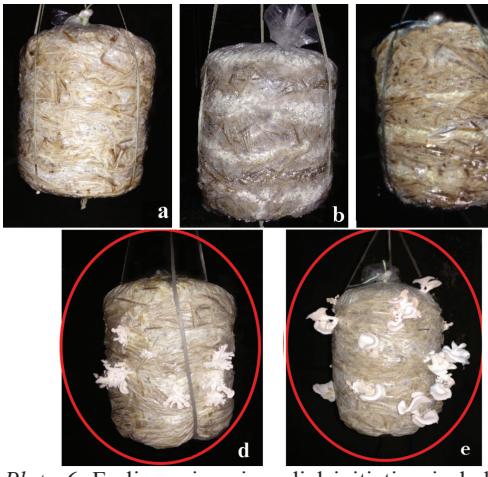


Plate 6. Earliness in primordial initiation in hybrids compared to their parental species of *Pleurotus* on 12th day after spawning a. *P. djamor* b. *P. florida*

days required for primordial initiation were reduced in hybrid of *P. djamor* x *P. florida* by two and 13 days respectively than the parents; and three and 12 days in hybrid of *P. djamor* x *P. ostreatus*, compared to its respective parents (Plate 6). The faster radial growth rate of mushroom mycelia and better colonisation were the morphological markers of a worthy mushroom strain (Gupta et al., 2011). The faster substrate colonization leading to rapid completion of the crop cycle, would accelerate the time to initiate primordial formation (Yang et al., 2013). The thicker mycelium mat provided better ability to colonize vast agricultural lignocellulosic wastes, as Abdulgani et al. (2017) observed in the hybrid of *P. pulmonarius* and *P. citrinopileatus*, which completed its spawn run earlier compared to the two parental strains.

The average sporocarp weight of the hybrids was intermediate to their parents. Total crop period of the two hybrids (*P. djamor* x *P. florida* and *P. djamor* x *P. ostreatus*) was reduced and was comparable with *P. djamor*. The BE of the hybrids *viz., P. djamor* x *P. florida* and *P. djamor* x *P. ostreatus* was improved by 34.38 and 48.48 per cent respectively over the parent, *P. djamor* (Plates 7&8). Prasad (2008) reported that all the hybrids formed from *P. sajor-caju* x *P. djamor*, *P. sajor-caju* x *P. florida*, *P. sajor-caju* x *P. flabellatus* and *P. florida* x *H. ulmarius* had significantly superior yield as compared to their respective high yielding parents.

The hybrids and their parents were studied for the

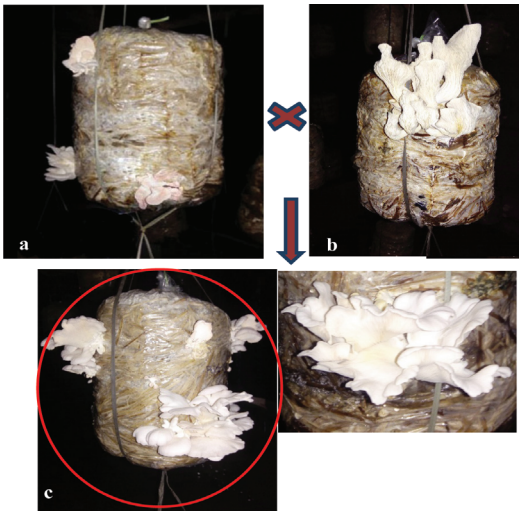


Plate 7. Enhanced yield in hybrid of *P. djamor* and *P. florida* compared to *P. djamor* **a**. *P. djamor* **b**. *P. florida* **c**. *P. djamor* x *P. florida*

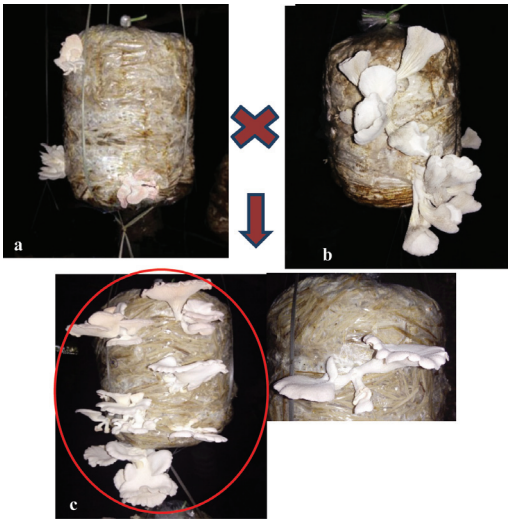


Plate 8. Enhanced yield in hybrid of *P. djamor* and *P. ostreatus* compared to *P. djamor* **a**. *P. djamor* **b**. *P. ostreatus* **c**. *P. djamor* x *P. ostreatus*

Table 2. Biometric observations of sporocarp of species of *Pleurotus* and their hybrids cultivated in paddy straw substrate

Species of *Pleurotus* and their hybrids	Colour	Texture	Pileus length (cm)	Pileus breadth (cm)	Stipe length
*P. djamor**	Pink	Fibrous and leathery	4.80±0.60^{c}	6.57±0.65^{c}	0.92±0.16^{d}
*P. florida**	White	Delicate	6.46±1.11^{b}	7.58±1.45^{b}	3.33±0.44^{b}
*P. ostreatus***	Pale white	Delicate	7.40±1.23^{a}	8.27±0.69^{a}	5.58±0.66^{a}
P. djamor x *P. florida**	Light pink	Slightly delicate	5.28±0.66^{c}	7.35±0.47^{b}	1.53±0.23^{c}
P. djamor x*P. ostreatus**	Light pink	Slightly delicate	6.69±0.37^{b}	7.03±0.42^{bc}	1.64±0.27^{c}
CD(0.05)			0.627	0.603	0.289
SE(m) ±			0.222	0.213	0.102

Values were recorded on 3rd* and 4th day** after primordial initiation. Values are mean ± SD of four replications. Values followed by similar superscripts are not significantly different at 5 % level

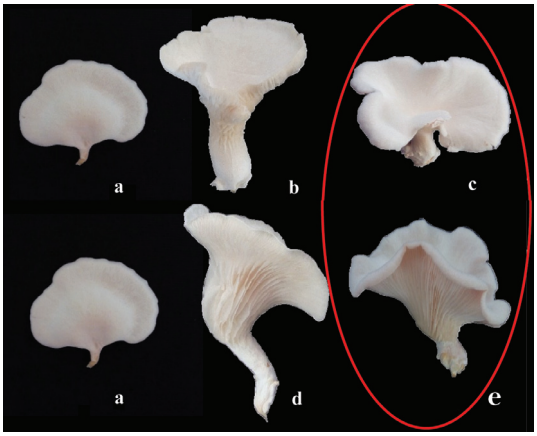


Plate 9. Blended morphological characters of species of *Pleurotus* in their hybrids a. *P. djamor* b. *P. florida* c. *P. djamor* x *P. florida* with increased pileus size and

morphological characteristics (Table 2; Plate 9). Hybrids produced light pink coloured slightly delicate fruiting bodies which were intermediate to pink coloured leathery *P. djamor* and white coloured delicate *P. florida* and *P. ostreatus*. The pileus dimension of the hybrids was also intermediate to their parents. Similarly, the length of the stipe increased in both the hybrids over *P. djamor*. This is in accordance with the study of Bahukhandi and Sharma (2002) who observed the blended characters in hybrids when crossing was done between *P. sajor-caju* and *P. cornucopiae*. The shape and size of the fruiting body was similar to *P. sajor-caju* while the colour was white, resembling *P. cornucopiae*. Sawashe and Sawant (2005) also observed that the average size of the hybrid fruiting bodies formed by crossing *P. eous* with *P. florida* was intermediate

of both the parents, while the stipe length and average weight increased with respect to *P. eous* but was on par with *P. florida.* Abdulgani et al. (2017) conducted interspecific hybridization between *P. pulmonarius* and *P. citrinopileatus* and the resultant hybrid had superior sporophore features of *P. pulmonarius* like large fleshy pileus with rigid stipe and cluster-type growing pattern of *P. citrinopileatus*.

The selected monokaryons might have desirable gene or genes from their parents.Thus mycelial mating of monokaryons incited the recombinant strain in the expression of phenotypic characters which were not present in the parental dikaryon (Guadarrama-Mendoza et al., 2014).

Based on the comparative performance of hybrids with their parents, *P. djamor* x *P. ostreatus* was found to be promising, with increased BE over *P. djamor* with reduced crop period than the parents. Hybrids were found to be superior to their parents with improved pileus size and slightly delicate texture favouring good transit, packaging and consumer preference.

Carbohydrate content in *P. djamor* x *P. florida* (50.44 %) and *P. djamor* x *P. ostreatus* (51.68 %), was intermediate to parents. Protein content was recorded significantly high in *P. djamor* x *P. florida* (26.54 %) followed by *P. djamor* x *P. ostreatus* (25.14%) and *P. djamor* (23.33 %). The results are in agreement with Selvakumar et al.(2015) who reported that maximum protein content was found for the hybrid of *P. ostreatus* var. *florida* and *P. djamor* var. *roseus*, compared to the parents. Similarly, crude fibre content was also high in hybrids compared to parents. Thus the hybrids were found superior in nutritional aspects too (Table 3). DNA of the parents, *P. djamor*, *P. florida*, *P. ostreatus* and their hybrids *P. djamor* x *P. florida*, and *P. djamor* x *P. ostreatus* was isolated using DNeasy plant mini kit (Qiagens). The isolated DNA of parents and hybrids were amplified using RAPD primer OPT 5 (GGGTTTGGCA), which was selected based on the earlier reports of Agarwal et al. (2013).

The primer OPT 5 amplified a total of 23 scorable bands and the size of the amplified products varied from 200 bp to 1000 bp. Out of total amplicons produced, three amplicons of size 980 bp, 938 bp and 500 bp were monomorphic between parent, *P. florida* and *P. djamor* x *P. florida*, while four amplicons of size 957 bp, 938 bp, 907 bp and 500 bp were monomorphic between parent, *P. djamor* and *P. djamor* x *P. florida*. All the other bands were polymorphic. Four amplicons of size 938 bp, 907 bp, 738 bp and 500 bp were monomorphic between *P. djamor* and *P. djamor* x *P. ostreatus*, whereas two amplicons of size 980 bp and 500 bp were monomorphic between *P. ostreatus* and *P. djamor* x *P. ostreatus*. All the other bands were polymorphic (Plate 10). The bands common in hybrids and either of the parent are especially important marker to identify the true hybrid (Akhare et al., 2008). Almost

Table 3. Analysis of proximate constituents in species of *Pleurotus* and their improved strains developed through hybridization and gamma irradiation followed by three generations of selection in paddy straw substrate

Species of *Pleurotus* and improved strains	Moisture* (%)	Carbohydrate** (%)	Protein** (%)	Fibre** (%)
P. djamor	85.97±1.29^{c}	45.79±1.66^{d}	23.33±1.04^{bc}	9.73±0.68^{cd}
P. florida	92.16±1.12^{a}	52.42±1.32^{bc}	21.61±1.21^{c}	8.38±0.52^{d}
P. ostreatus	89.14±1.05^{b}	55.77±0.77^{ab}	22.31±1.06^{c}	11.54±1.38^{b}
P. djamor x *P. florida*	87.81±1.17^{bc}	50.44±1.94^{c}	26.54±1.05^{a}	14.07±1.07^{a}
P. djamor x *P. ostreatus*	88.31±1.18^{bc}	51.68±1.60^{c}	25.14±1.24^{ab}	13.35±0.59^{a}
CD (0.05)	2.977	3.802	1.958	1.608
SE(m) ±	0.956	1.220	0.629	0.516

*Fresh weight basis; ** Dry weight basis. Values are mean ± SD of four replications. Values followed by similar superscripts are not significantly different at 5 % level

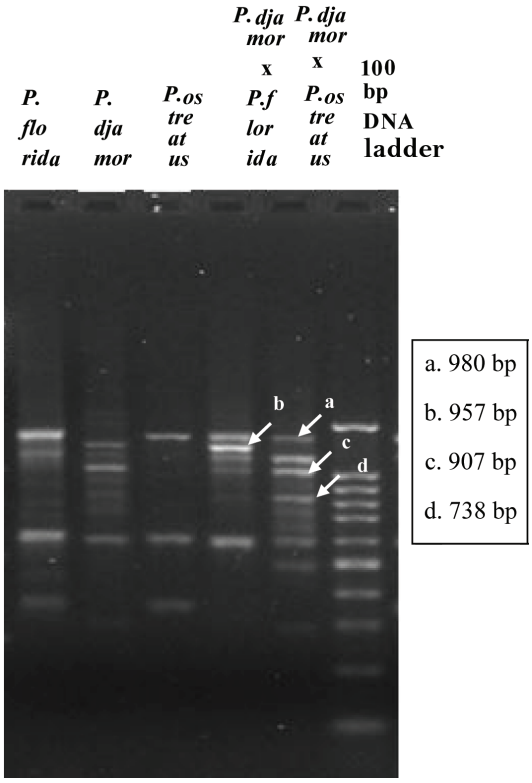
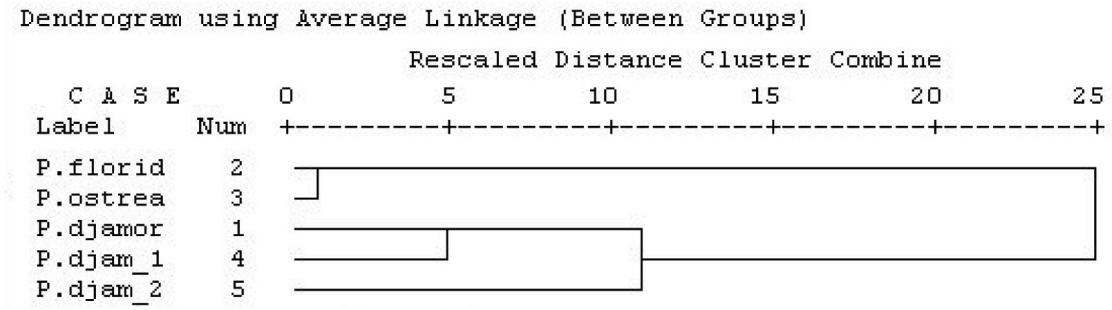


Plate 10. RAPD profile of species of *Pleurotus* and their hybrids amplified by primer OPT 5 (GGGTTTGGCA); *P. djamor* x *P. florida* share 980 bp of *P. florida* and 957 bp and 907 bp of *P. djamor P. djamor* x *P. ostreatus* share 938 bp, 907 bp and 738 bp of *P. djamor* and 980 bp of *P. ostreatus*

all RAPD bands from both parents were found in RAPD profiles of the hybrids. This indicated the high penetrance and dominant nature of RAPD markers.

A typical RAPD reaction produced multiple amplification products and each band was assumed to represent a unique genetic locus (Mishra et al., 2012). Based on the presence or absence of amplicons, bands were scored (Table 4). The bivariate data was analysed to generate Jaccard's similarity coefficient (Table 5). The pair wise coefficient values ranged from 0.143 to 0.750. The hybrids, *P. djamor* x *P. florida*and *P. djamor* x *P. ostreatus* had 66.7 and 57.1 per cent similarity to *P. djamor*. The hybrids formed a single cluster with *P. djamor*, when subjected to unweighed pair group method analysis using SPSS software (Figure 1). Abdulgani et al. (2017) carried out hybridisation between *P. pulmonarius* and *P. citrinopileatus*; and further analysis with RAPD confirmed that the five hybrids developed showed high genetic homology with *P. pulmonarius*.

Among the two hybrids produced by interspecific hybridization of *P. djamor* with *P. florida* and *P. ostreatus, P. djamor* x *P. ostreatus* was found to be promising with regard to improved BE, reduced



The scale is based on Jaccard's co-efficient of similarity

Figure 1. Dendrogram of species of *Pleurotus* and their hybrids constructed by the UPGMA of binary matrix obtained from RAPD

Table 4.RAPD scoring pattern of species of *Pleurotus* and their hybrids

Amplicon size	*P. florida*	*P. djamor*	*P. ostreatus*	*P. djamor* x *P. florida*	*P. djamor* x*P . ostreatus*
980 bp	1	0	1	1	1
957 bp	0	1	0	1	0
938 bp	1	1	0	1	1
907 bp	0	1	0	1	1
738 bp	0	1	0	0	1
500 bp	1	1	1	1	1
397 bp	0	0	0	0	1
271 bp	1	0	1	0	0

Table 5. Similarity matrix for Jaccard's coefficient of species of *Pleurotus* and their hybrids

	P. djamor	*P. florida*	*P. ostreatus*	*P. djamor* x *P. florida*	*P. djamor* x *P. ostreatus*
P. djamor	1	0.286	0.143	0.667	0.571
P. florida		1	0.750	0.500	0.429
P. ostreatus			1	0.333	0.286
P. djamor x *P. florida*				1	0.571
P. djamor x *P. ostreatus*					1

crop period, better texture and increased nutrient composition. Molecular characterization of hybrids was done with RAPD and it revealed that the hybrids were more genetically related tothe parent, *P. djamor*. The hybrid of *P. djamor* and *P. ostreatus* having superior characteristics with improved pileus size and slightly delicate texture favouredpackaging, safe transit and storage.Hence this hybrid having high consumer preference could be suggested for commercial cultivation which in turn could also increase farmers' revenue in short period.

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