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Development of sporeless and low-spored mutants of edible mushroom for alleviating respiratory allergies

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Oyster mushrooms, *Pleurotus* species, are the second largest cultivated mushrooms, accounting for 30% of the global mushroom production. The fruiting bodies of *Pleurotus* are gymnocarpous, i.e. spore discharge begins early and continues until the sporophores are harvested. Estimates have established that a good-sized mushroom can release 100 million spores per hour. The inhalation of these spores induces allergic responses like farmer's lung disease and hay fever. Research was undertaken to solve this problem through the development of sporeless or low-spored strains which would be suitable for commercial production. The UV mutation of the spores of two commercial *Pleurotus* species, *P. florida* (white oyster mushroom) and *P. sajor-caju* (grey oyster mushroom) led to the production of two mutants. The mutant of *P. florida* was totally sporeless with light grey mushrooms, having a white, soft, long central stipe and yielded

40% higher than the parental *P. florida*. The mutant produced from *P. sajor-caju* was low-spored (spore content decreased by 99%) with grey mushrooms having highly wavy margins and a short central stipe. Both these mutants are stable since the last 9 years and more than 40 sub-culture generations. The cultivation technology is similar to the commercial species and the taste and aroma too are similar. These mutants will shortly be released as sporeless and low-spored mutants for commercial cultivation in the country.

Keywords: Mushrooms, mutation, *Pleurotus*, respiratory allergy, sporeless mutants.

ONE of the major problems in cultivating oyster mushrooms, *Pleurotus* species is the abundant production of spores. Many workers involved in the production of mushrooms develop an allergy with symptoms similar to 'extrinsic allergic alveolitis' (EAA)¹. Mushroom worker's lung, a type III IgG-mediated hypersensitivity pneumonitis or EAA is common among mushroom workers². In the mid-seventies, Schulz *et al.*³ described such an illness in Bavarian mushroom workers, caused by spores of the white oyster mushroom, *Pleurotus florida*. The patients showed immediate skin reaction to an extract of *Pleurotus* spores and serum precipitins to *Pleurotus* antigen on analysis with Ouchterlony's gel diffusion. Stewart and Pickering⁴ reported respiratory symptoms in workers cultivating *Agaricus hortensis*. These workers had precipitins to *A. hortensis*, but not to thermophilic actinomycetes. Skin and provocation tests to both these species were negative. In the Netherlands, four workers were reported to have developed EAA after working with oyster mushrooms. After inhalation provocation with the basidiospores of this mushroom, the patients showed the symptoms of the disease⁵. Symptoms of EAA are also commonly seen in workers cultivating shiitake, *Lentinula edodes*. Five mushroom workers involved in the picking of mushrooms were subjected to inhalation provocation with basidiospores of *L. edodes*. All five developed specific symptoms of EAA⁶.

The fruiting bodies of *Pleurotus* are gymnocarpous, i.e. spore discharge begins when the first lamella is formed and continues until the sporophores are harvested. Large amounts of spores are constantly released into the growing atmosphere (Figure 1). Estimates have established that a good-sized mushroom can release 100 million spores per hour⁴. As the mushrooms reach maturity, the level of spores in the atmosphere of the mushroom house is very high. The symptoms associated with *Pleurotus* spore allergy appear 4–6 weeks after first contact with the spores. Severity of allergic response may vary from person to person like general fatigue, mild headache, cough, mild difficulty in breathing, pain in the limbs, etc.^{2,3,7,8}. The spores may also be a source of pollution, which may include new genotypes likely to attack wood or trees. Apart

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from this, during commercialization, the spores settle on fruit bodies, germinate and lead to the formation of a white, velvety film, which gives an unpleasant appearance to the mushroom. Hence, there is a need to produce sporeless or low-spore shedding strains of *Pleurotus* with similar agronomical traits as that of commercial sporulating strain. The mushroom laboratory at the Indian Institute of Horticultural Research, Bangalore, has developed sporeless and low-spore shedding mutants through UV mutation.

The mutants were produced by UV mutation of the basidiospores of the commercial sporulating species *P. florida* and *P. sajor-caju*. The LD90 was established at 75 and 65 min exposure respectively. The spores that survived were recultured and fructification trial was undertaken on sterilized paddy straw by the conventional mushroom cultivation technique. The sporophores were studied for visual spore production by spore print and microscopically by gill sectioning. The stability of the trait of the mutant strains was studied by repeated subculture and successive tissue culture from each crop for 40 generations. Cultural characterization of the mutant strains was studied in comparison to the parents for germplasm maintenance. The cultural characterization for optimum temperature for mycelial growth was studied by inoculating 5 mm mycelial discs of 7-day-old culture on malt extract agar medium incubated in a BOD incubator at variable temperatures from 10° to 35°C. Radial diameter of the mycelial growth was measured at regular intervals until the medium in the petri plate was completely covered with mycelium. Studies pertaining to requirement of optimal medium for culture maintenance were conducted by inoculating mycelial discs as mentioned earlier, on different solid media in petri plates incubated at $25 \pm 2^\circ\text{C}$. Radial mycelial growth was measured at regular intervals. The density of growth in different broth cultures (liquid media) was studied by inoculating the mycelial discs in 25 ml of the respective liquid media contained in 100 ml conical flasks and incubating the flasks as static cultures at $25 \pm 2^\circ\text{C}$. The optimal pH requirement was studied in malt extract broth (liquid

medium) in a pH range of 3–10. In both the above cases the mycelial mat was filtered through previously weighed Whatman No. 1 filter paper, dried in an oven at 70°C for 48 h to get constant weight. The dried mycelium along with the filter paper was weighed again and the dry weight of mycelium was calculated.

The agronomical traits (spawn running period, yield, sporophore characters and spore count of the parents and mutants) important for commercialization were studied by taking repetitive crops on paddy straw. The environmental condition maintained during spawn running was a temperature of $25 \pm 2^\circ\text{C}$ with ambient humidity. There was no requirement of light or watering at this stage. The environmental conditions maintained for cropping (appearance of mushrooms) was a temperature of $25 \pm 2^\circ\text{C}$, humidity of 80–85% and 8–9 h of fluorescent light using a 40 W bulb. Assessment of spore production was done as follows.

Sporophores showing nil or very faint spore density (visually in spore print compared to commercial sporulating parent) and the commercial parent were used for spore counting by a haemocytometer. The individual fruit bodies from pinhead size to mature harvesting size were collected because in *Pleurotus*, spore shedding starts very early in the pinhead stage itself and all the sporophores in a harvested lot will not be of uniform size. The size of the fruit body was taken at two diameters at the maximum expanded portion of the pileus. The sporophores harvested from the bags of each parent/mutant were grouped as shown in Table 1.

Spores were collected from each fruit body (of all sizes) from one bag. The spore prints were dissolved individually in known volume of sterile distilled water. The spore suspension was counted using the haemocytometer. The spore suspension (10 μl) was placed on the haemocytometer. The spores were counted in all 25 squares. The total number of spores was calculated using the following formula.

Total cell count in 25 squares $\times 10,000 \times$ dilution.

After obtaining the total spores/ml of the spore suspension, the area of the fruit body from which the spore print had been collected was calculated by considering the projected area as an ellipse (*Pleurotus* mushrooms have an irregular, almost ellipsoidal shape) and using the formula $0.7857a, b$, where a and b are the minor and major diameter of the fruit body at its maximum expanded portion.

Table 1. Grouping of harvested sporophores

Pileus width (cm)	Fruit size
6.6–9.5	Big
4.1–6.5	Medium
1.0–4.0	Small

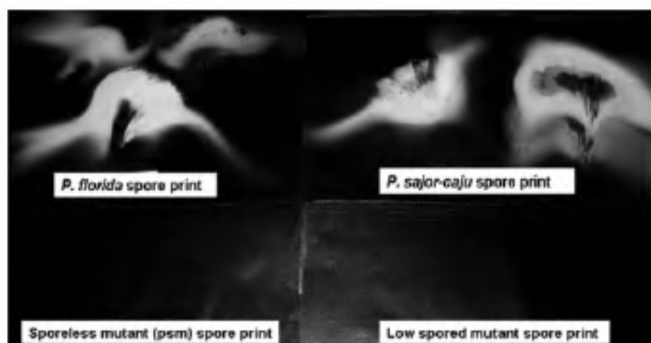


Figure 1. Comparative spore print of commercial sporulating parents and mutants.

Two kilograms of wet paddy straw, which was compacted in a PP bag layered with the spawn was considered as one bag. The number of sporophores per bag was noted. Based on the size (area), the sporophores were categorized into big, medium and small size. The average number of sporophores of the three designated sizes/bag was observed in three flushes. Based on the average number of spores produced by each sporophore size, the total spores produced by one bag over a period of three flushes was calculated. Further, based on the area occupied by one bag and the optimal number of bags which could be accommodated in a room of size $9 \times 14 \times 10$ ft, the total number of spores which could be liberated in the cropping room was calculated.

The cultural characterization results showed that malt extract agar medium (solid), malt extract broth (liquid) and temperature of $25 \pm 2^\circ\text{C}$ were the optimal conditions for the mycelial growth of the parental species and the

mutants (Figures 2–4). Thus mutation did not bring about any change in the nutritional and temperature requirements, which remained the same for the parental species and the mutants. The optimum pH range for *P. florida*, *P. sajor-caju* and the low-spored mutants was between 5.5 and 6.5, as against that of sporeless mutant which was between 7 and 7.5 (Figure 5).

As seen in Table 2, the spawn running period of the low-spored mutant was longer compared to the totally sporeless mutant and the commercial species. In the present study, the total cropping cycle of the low-spored mutant was 50 days compared to its parent *P. sajor-caju* (44 days). The sporeless mutant showed early fructification (2–3 days) compared to the low-spored mutant and the commercial species. Its cropping cycle was the shortest (38 days) compared to its parent *P. florida* (40 days). This shows its greater ability to colonize straw. The yield and biological efficiency of the sporeless mutant was

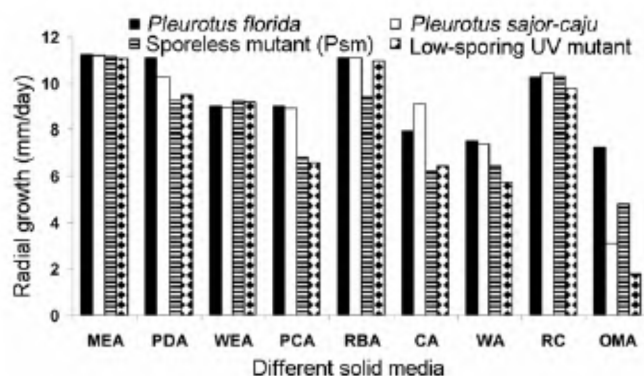


Figure 2. Radial growth of mycelium of mutants and parents on different media. MEA, Malt extract agar; PDA, Potato dextrose agar; WEA, Wheat extract agar; PCA, Potato carrot agar; CA, Carrot agar; WA, Water agar; OMA, Oatmeal agar; RC, Raper's complete medium and RBA, Rice bran agar. CD at 0.1%: *Pleurotus florida* = 0.54; *Pleurotus sajor-caju* = 0.26; Sporeless = 1.68 and Low-spored = 0.46.

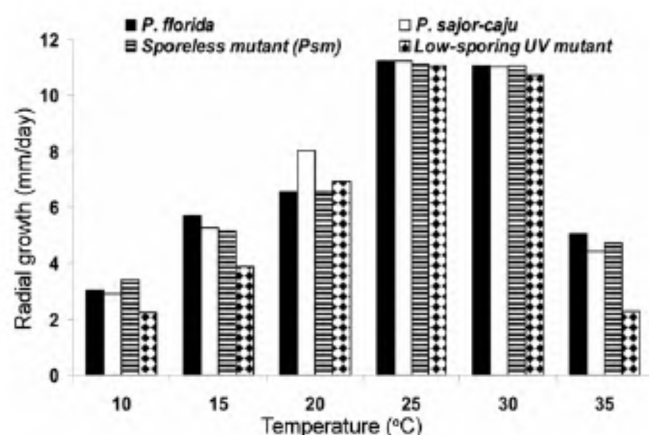


Figure 3. Radial growth of mycelium of mutants and parents at different temperatures. CD at 1%: *P. florida* = 0.38; *P. sajor-caju* = 0.35; Sporeless = 0.71 and Low spored = 0.35.

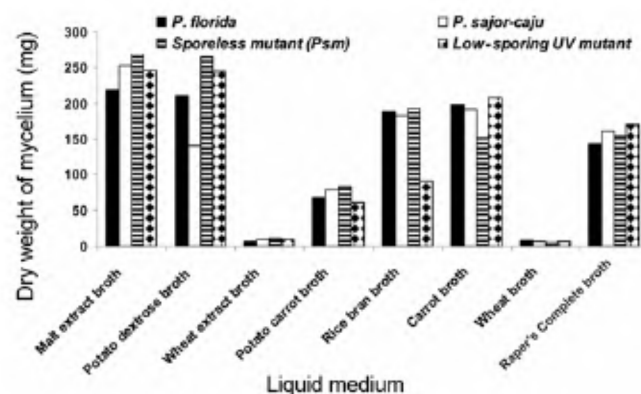


Figure 4. Mycelial growth of mutants and parents in different liquid media. CD at 1%: *P. florida* = 25.92; *P. sajor-caju* = 46.38; Sporeless = 46.09; Low spored = 29.54.

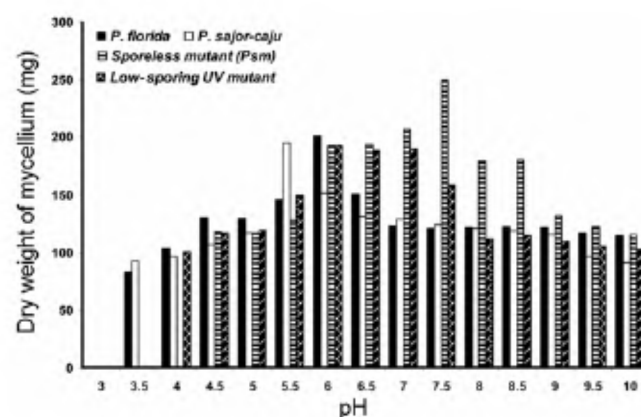


Figure 5. Mycelial growth of mutants and parents at different pH values. CD at 1%: *P. florida* = 18.4694; *P. sajor-caju* = 15.9966; Sporeless = 30.7727 and Low spored = 33.2008.

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Table 2. Comparative agronomical traits of parents, sporeless and low-spored UV mutants

Traits	<i>Pleurotus florida</i>	<i>Pleurotus sajor-caju</i>	Sporeless mutant (Psm)	Low-spored UV mutant
Number of days for spawn run on paddy straw	17–20	18–20	17–20	21–25
Number of days for first flush	5–7	6–8	3–5	7–8
Total yield (g/kg wet straw)	189.83	188.77	221.45	162.76
Biological efficiency (%)	61.09	62.02	70.93	52.97
Spores/m ³ (room volume = 33.89 m ³)	94.18 × 10 ⁸	45.4 × 10 ⁸	No spore	0.084 × 10 ⁸
Stipe length (mm)	33.4–43.2	30.3–48.5	34.2–43.3	24.7–30.7
Stipe thickness (mm)	7.9–11.0	6.30–10.30	9.0–12.20	5.30–8.60
Pileus length (mm)	31.1–74.8	28.7–70.0	37.7–72.1	28.7–56.4
Pileus width (mm)	48.2–90.1	49.7–76.8	43.5–77.9	36.2–65.4
Pileus thickness (mm)	3.60–4.80	3.30–4.80	2.80–3.40	3.80–5.40
Number of gills (per cm ²)	12.83	17.10	9.60	13.36
Average weight of single fresh sporophore (g)	2.27–12.23	2.0–15.86	2.61–12.63	1.8–10.47

CD at 1% for yield = 33.92.

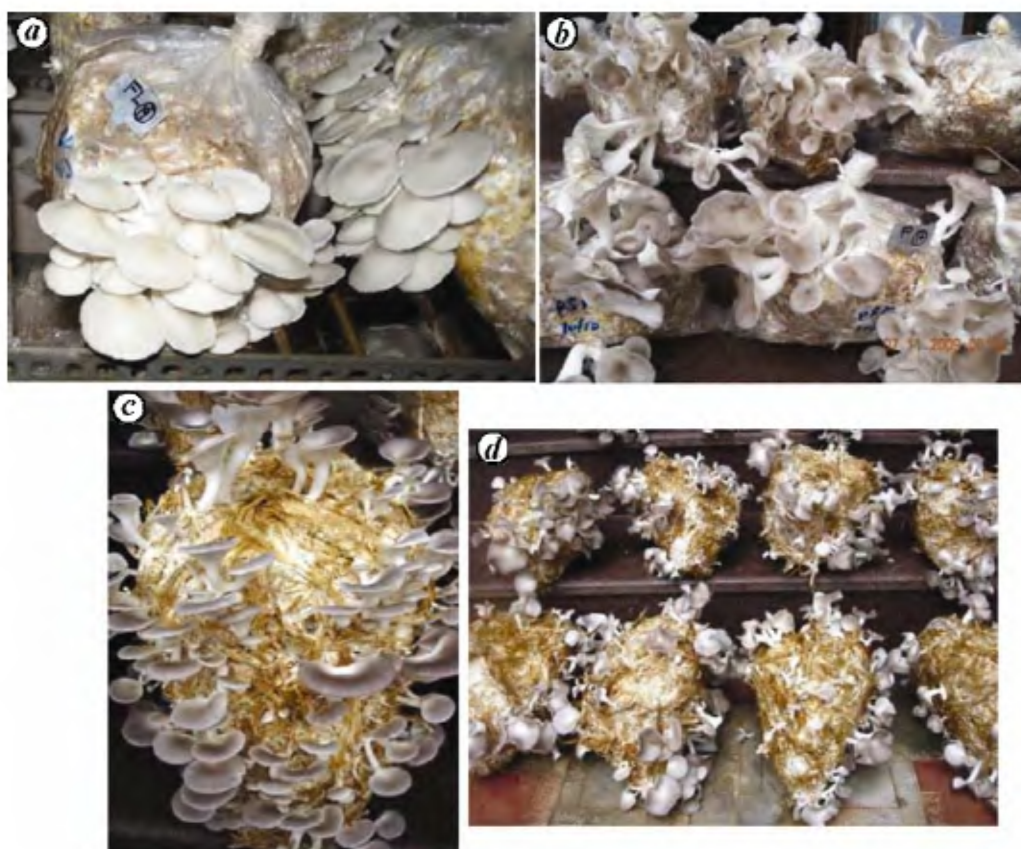


Figure 6. *a*, Commercial spore producing *P. florida*. *b*, Sporeless mutant of *P. florida*. *c*, Commercial-spore producing *P. sajor-caju*. *d*, Low-spored mutant of *P. sajor-caju*.

higher compared to the low-spored mutant and the commercial species. This is an interesting observation as the yield of such sporeless or low-spored mutants has been reported to be low by earlier workers^{9,10}.

The most important agronomical difference was observed in the number of spores produced by the mutants compared to commercial species. The sporeless mutant was completely devoid of spores (Figure 1) and this trait

was found to be stable over more than 48 subcultures carried out during the last 9 years. The spore content of the low-spored mutants was reduced by 98.49% compared to its parent *P. sajor-caju* and by 99.14% compared to *P. florida*. There was no visible spore print in low-spored mutant (Figure 1). However, few spores were seen microscopically in the gill section, and hence it was termed as low-spored. This trait has also been stable over

48 subculture generations done till date. Mutation brought about a prominent change in the shape of the sporophores. The two commercial species showed expanded and wide pileus with an eccentric stipe (Figure 6a and c). The sporeless mutant showed a long, funnel-shaped pileus with a central stipe (Figure 6b). The low-spored mutant showed a highly lobed flower-like pileus with a central stipe (Figure 6d). Another characteristic change brought about due to mutation was the drastic reduction in the number of gills/cm² in the sporeless mutants (Table 2).

The sporeless and low-spored mutants developed during this study have been stable for this character. These mutants will help in mitigating the respiratory allergies among mushroom growers. They can also be used as excellent tools for the genetic understanding of basidio-spore formation in mushrooms. The mutants can be further utilized for the commercial production of sporeless strains through genetic manipulation.

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A unique example of a wooden boat of *Bridelia retusa* in ancient India entrapped in the Holocene sediments at Derde, Ratnagiri Coast, Maharashtra

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Derde is a coastal village in Dapoli taluka, Ratnagiri District, Maharashtra, India. It is located on a small stream 1 km north of and 6 km inland from the mouth of the Vashishti River. A small, completely preserved wooden boat was found in a trench 2 m below the surface. The timber of the boat has been identified as *Bridelia retusa* Spreng. (trade name – kassi) and belongs to the family Euphorbiaceae. ¹⁴C dating showed that the boat belongs to AD 784 ± 102. A study of the geomorphological features of the area combined with aerial photographs revealed that an impulsive hurricane might have resulted in sudden toppling of the boat, sometime around the 8th century. This is a solitary evidence on the use of *B. retusa* for boat-building in ancient India.

Keywords: Boat-building, Holocene, hurricane, timber.

DERDE (17°36'10"N, 73°12'55"E) is a coastal village in Dapoli taluka, Ratnagiri District, Maharashtra, India (Figure 1). It is located on a small stream (nala) about 1 km north of the Vashishti River and 6 km inland from the coast. The boat was discovered during the excavation of a well in June 2007. The circular trench (Figure 2) had a diameter 4.3 m. The boat was found buried at a depth of 1.9 m from the surface, in an upturned position within a stratum of sand. The boat was 2.6 m high and the exposed bow portion of the boat was about 1.6 m. The stern portion of the boat was engulfed inside the stratum. There were a number of properly preserved wooden planks, 10–13 cm in thickness. It was observed that wooden pegs were used in the construction of the boat; metal nails were absent. About 15 wood samples of the planks were collected. In a preliminary study it was found that all the wood samples were identical. Therefore, one sample was sent for anatomical analysis and for ¹⁴C dating.

The study area is surrounded by small hills on three sides and the Vashishti River flows on the southern side. In this area the gradient is very low. In the surrounding area along the small stream there are very thin patches of gravelly deposit.

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