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PHYSIOLOGICAL DYNAMICS OF SPORE GERMINATION AND INTERSPECIFIC MATING COMPATIBILITY IN OYSTER MUSHROOMS (*PLEUROTUS* SPP.)

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ABSTRACT
Commercial cultivation of oyster mushrooms (*Pleurotus* spp.) is frequently constrained by narrow thermal adaptability and yield plateaus. In this study, seven commercial *Pleurotus* species were evaluated for agronomic traits, sporophore morphology, and basidiospore physiology to facilitate targeted interspecific hybridization. Cumulative yield ranged from 469.3 g in *P. ostreatus* (52.14% biological efficiency [BE]) to 645.5 g in *P. djamor* (71.72% BE). Although *P. ostreatus* yielded the lowest total biomass, it produced the heaviest individual basidiomes (13.40 g), whereas *P. djamor* exhibited the highest flush count (74 basidiomes). Four candidate taxa—*P. sajor-caju*, *P. djamor*, *P. flabellatus*, and *P. ostreatus*—were selected for downstream breeding. Basidiospore germination peaked uniformly at 27°C (62.1%–68.3%) and neutral pH 7.0 (61.0%–68.8%) after 48 h, with severe inhibition at 15°C and pH extremes (4.0 and 10.0). Exogenous sugars exerted osmotic repression compared to sterile water (62.1%–70.1%). Low-temperature preservation (6°C) maintained high spore viability (54.2%–57.0%) up to 150 d versus rapid loss at 27°C (21.0%–22.2%). Single-spore isolates confirmed tetrapolar heterothallism across all four species, displaying slow-growing hyphae devoid of clamp connections and complete basidiome sterility. Interspecific confrontation of 19 verified monokaryons across 135 pairings yielded 27 fertile dikaryotic hybrids (20.0% compatibility) verified by clamp connections. The cross *P. djamor* $\times$ *P. flabellatus* exhibited the highest crossability (36.0%), while *P. djamor* $\times$ *P. ostreatus* demonstrated strong pre-zygotic barriers (8.0%). Selected hybrids manifested prominent heterosis, combining the fleshy basidiome architecture of temperate taxa with the vibrant pigmentation and fecundity of tropical lines.

KEYWORDS: *Pleurotus*; Basidiospore germination; Biological efficiency; Clamp connections; Interspecific hybridization; Monokaryons; Tetrapolar heterothallism

INTRODUCTION

Edible mushrooms are increasingly recognized as vital functional foods and biotechnological agents capable of addressing global protein malnutrition while bioconverting lignocellulosic agro-industrial residues into nutrient-rich biomass. Among commercially cultivated macrofungi, oyster mushrooms (*Pleurotus* spp., Agaricomycetes, Pleurotaceae) hold a preeminent position globally and in India, ranking second only to the white button mushroom (*Agaricus bisporus*) in total commercial output (Barh et al., 2019; Kamal et al., 2022). Their widespread agrarian adoption is driven by advantageous agronomic traits, including the absence of a strict casing requirement, a rapid colonization rate on uncomposted agricultural straws, versatile enzymatic systems for lignin degradation, and exceptional biological efficiency (BE) (Barh et al., 2019). Furthermore, *Pleurotus* basidiomes are rich in high-quality proteins, essential amino acids, dietary fibers, and immunomodulatory polysaccharides (such as ß-glucans), making them an effective tool for nutritional security (Juárez-Hernández et al., 2023).

Despite substantial commercial potential, the oyster mushroom sector in tropical and subtropical regions faces acute operational challenges. Commercial cultivation relies heavily on a limited number of conventional strains that exhibit seasonal constraints and thermal sensitivity. High-temperature regimes during summer months frequently depress basidiome initiation and encourage disease incidence, whereas temperate winter flushes suffer from slow mycelial colonization (Wiafe-Kwagyan et al. 2026**)**. Moreover, commercial cultivars exhibit trade-offs between biological efficiency and organoleptic consumer appeal: tropical taxa such as *Pleurotus djamor* produce high flush counts and visually striking rose-pink basidiomes but often develop thin, brittle pilei with brief post-harvest shelf life; conversely, temperate species such as *Pleurotus ostreatus* and *Pleurotus flabellatus* yield heavier, fleshier, and thicker pilei but produce fewer flushes and exhibit lower overall fecundity under warm cropping regimes (Jamil et al., 2019; Sharma & Lal, 2024). Overcoming this productivity plateau requires the targeted synthesis of elite strains that combine high bioconversion rates and thermal resilience with fleshy, marketable basidiome architecture.

Conventional breeding via confrontation pairing of sexually compatible single-spore isolates (SSIs) offers a reliable and legally unencumbered pathway to exploit heterosis in edible basidiomycetes (Barh et al., 2019). The mating system of *Pleurotus* species is governed by bifactorial (tetrapolar) heterothallism controlled by two unlinked, multiallelic mating-type loci (*A* and *B*) (Singh&Kamal, 2017). In this system, homokaryotic basidiospores give rise to self-sterile monokaryons that lack clamp connections; mutual fertilization and subsequent dikaryotization occur only when compatible monokaryons carrying distinct alleles at both mating loci encounter each other, giving rise to clamp-bearing, fertile dikaryons (Larraya, 2000; Barh et al., 2019). While intraspecific crosses yield high compatibility frequencies, interspecific hybridization presents severe pre- and post-zygotic barriers, including allorecognition antagonism, barrage formation, and vegetative incompatibility (Kues et al., 2009). Systematic exploration of interspecific compatibility across diverse commercial *Pleurotus* taxa remains critical for introgressing divergent gene pools and exploiting dominant gene complementation (Bahukhandi & Sharma, 2002).

A fundamental prerequisite for establishing an efficient SSI-based hybrid breeding platform is a precise understanding of basidiospore physiology and viability dynamics. Basidiospores serve as the haploid genetic bridge for monokaryon generation. However, basidiospore germination and longevity are heavily governed by micro-environmental parameters, including temperature, hydrogen ion concentration (pH), and exogenous nutritional osmolarities (Feofilova et al, 2012). Inadequate germination conditions impede the recovery of representative single-spore progenies, while improper spore storage leads to rapid desiccation, loss of cellular integrity, and diminished viability (Corrales et al,2023). Despite empirical cultivation of *Pleurotus*, comprehensive comparative investigations detailing the physiological thresholds of basidiospore germination across distinct subtropical and temperate *Pleurotus* taxa remain sparse.

MATERIALS AND METHODS

1. Germplasm Collection and Parental Strain Maintenance

Seven commercially relevant and morphologically distinct species of oyster mushroom (*Pleurotus* spp.)—*Pleurotus sajor-caju*, *Pleurotus djamor*, *Pleurotus florida*, *Pleurotus sapidus*, *Pleurotus flabellatus*, *Pleurotus citrinopileatus*, and *Pleurotus ostreatus*—were procured from the Directorate of Mushroom Research (DMR), Solan, and institute culture repositories, or collected directly from natural habitats and commercial production facilities. Fresh sporocarps collected from natural habitats were packed aseptically in sterile polyethylene bags, labeled with ecological metadata, and transported to the laboratory at 4°C within 4–6 hours.

Baseline pure cultures were obtained by excising sterile internal tissue blocks (0.5-1.0 cm^2) from the stipe–pileus junction under aseptic conditions in a laminar airflow cabinet. Excised explants were inoculated onto Potato Dextrose Agar (PDA) amended with streptomycin sulfate (50 mg/L) to suppress bacterial proliferation and incubated at 25 ± 2°Cin darkness. Verified dikaryotic mycelia—confirmed by the presence of true septal clamp connections under light microscopy—were maintained on PDA slants at 4°C and cryogenic glycerol suspensions (15-20%v/v) at 20°C with routine subculturing every 45–60 days to prevent strain senescence. Based on baseline performance, four core species—*P. sajor-caju*, *P. djamor*, *P. flabellatus*, and *P. ostreatus*—were advanced to comprehensive spore physiology and hybridization pipelines.

2. Spore Print Collection and Inoculum Preparation

Spore prints were harvested from healthy, fully expanded basidiomes of the four selected parental species. The stipe was severed aseptically, and the pileus was placed lamellae-downward onto sterile glass slides or blackened paper discs positioned inside sterile Petri dishes under ambient room conditions (25 ± 2°C) for 8–12 hours to allow dense basidiospore deposition.

Deposited spores were scraped under aseptic conditions and resuspended in sterile distilled water containing 0.1% (v/v)Tween-80 to prevent clump formation. The stock suspension was homogenized via vortexing and serially diluted (10^{-1} to 10^{-4}) to achieve a uniform spore density yielding approximately 2 to 5 spores per low-power microscopic field (100×).

3. Spore Germination Assays

All physiological germination trials were conducted using cavity slides containing an equal spore load or on 2%Water Agar (WA: 15g/Lagar in distilled water) supplemented with 50mg/L streptomycin sulfate. Spore germination was evaluated microscopically at 24 and 48 hours post-inoculation. A spore was scored as germinated when germ tube emergence exceeded the diameter of the basidiospore. All treatments were performed in triplicate with at least 100 spores scored per replicate:

- Thermal Regimes: Spore suspensions prepared in sterile tap water were incubated at 15°C, 20°C, 24°C, 27°C, and 30°C in precision BOD incubators under dark conditions.
- pH Gradients: The influence of hydrogen ion concentration was tested by resuspending basidiospores in sterile buffer solutions adjusted across a wide gradient (pH 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, and 10.0). Suspensions were loaded into cavity slides and incubated at the physiological optimum of 27°C.
- Carbon and Osmotic Sources: The effect of exogenous carbohydrates was assessed by suspending spores in 1% (w/v) solutions of glucose, fructose, sucrose, maltose, and soluble starch prepared in sterile tap water with the pH buffered to 6.5–7.0, using sterile tap water as the reference control. Inoculated cavity slides were maintained at 27°C.

- Storage Viability and Shelf-Life: Basidiospores were stored under two distinct thermal conditions: refrigeration at 6°C and ambient incubation at 27°C. Germination capacity in sterile water was periodically monitored at intervals of 1, 7, 30, 90, 120, and 150 days of storage.

4. Single-Spore Isolation (SSI) and Monokaryon Validation

To isolate haploid progeny, 100muL of the diluted basidiospore suspension was spread across 2% WA plates using a sterile glass spreader and incubated at 27 ± 1°Cfor 24–48 hours. Individually germinating spores with distinct germ tubes were identified under a compound stereomicroscope and transferred to individual PDA slants using a micro-manipulating glass needle.

A panel of 50–80 single-spore isolates (SSIs) per parental species was cataloged and incubated at 25°C for 5–7 days. The haploid (monokaryotic) status of each isolate was verified by two distinct criteria:

1. Microscopic Verification: Mycelial tips stained with lactophenol cotton blue were examined at 400×magnification across 20–30 microscopic fields per isolate to confirm the complete absence of septal clamp connections.
2. Physiological Screening: Colony morphology was observed for slower radial expansion rates, low aerial mycelium, and lack of fruiting body formation in mock cultivation beds (sterility check).

Verified homokaryons were coded systematically: *P. sajor-caju* (SSIs 1–5, excluding 3), *P. djamor* (SSIs 6–10), *P. flabellatus* (SSIs 11–15), and *P. ostreatus* (SSIs 16–20).

5. Interspecific Confrontation Pairing and Hybrid Confirmation

Controlled interspecific mating was conducted using diallel confrontation pairing grids on 90 mm PDA plates. Active mycelial plugs (3–5 mm diameter) excised from the advancing margins of 10-day-old monokaryotic colonies were placed 2–3 cm apart at opposite ends of the Petri dish. Plates were sealed with Parafilm and incubated in the dark at 25-28°C for 7–14 days.

A total of 135 cross combinations were executed across all parental pairs:

- *P. sajor-caju*×*P. djamor* (4×5 = 20 pairings)
- *P. sajor-caju*×*P. flabellatus* (4×5 = 20 pairings)
- *P. sajor-caju*×*P. ostreatus* (4×5 = 20 pairings)
- *P. djamor*×*P. flabellatus* (5×5 = 25pairings)
- *P. djamor*×*P. ostreatus* (5×5 = 25 pairings)
- *P. flabellatus*×*P. ostreatus* (5×5 = 25 pairings)

Confrontation zones were monitored visually for compatibility indicators: compatible crosses merged seamlessly with dense, vegetative barrage lines at the interaction zone, whereas incompatible pairings displayed antagonistic demarcation lines or contact inhibition zones. Dikaryotization and hybrid status were confirmed cytologically by excising mycelial plugs from the junction zone, staining with lactophenol cotton blue, and verifying the presence of true clamp connections at the hyphal septa under light microscopy at 400× to 1000× magnification.

6. Statistical Analysis

All spore germination assays and compatibility frequencies were carried out in triplicate. Germination percentage values were subjected to angular (arcsine) transformation prior to analysis to normalize variance. Data were analyzed using analysis of variance (ANOVA) in a Completely Randomized Design (CRD), with standard error of the mean (SEm) and Critical Difference (CD) calculated at a 5% level of significance to evaluate treatment differences.

RESULTS

1. Baseline Screening and Parental Germplasm Selection

Evaluation of seven commercial *Pleurotus* species revealed substantial variation in yield parameters and sporophore morphology (Fig 1) (Table 1). Cumulative sporophore yield per 3 kg wet substrate ranged from 469.3 g in *P. ostreatus* (52.14% BE) to 645.5 g in *P. djamor* (71.72% BE). *Pleurotus djamor* produced the highest sporophore count (74) and total yield. *Pleurotus sajor-caju* (598.4 g; 66.48% BE) and *P. florida* (595.2 g; 66.13% BE) were statistically at par. While *P. ostreatus* exhibited the lowest total biomass, it produced the heaviest individual basidiomes (13.40 g/fruit body), followed by *P. flabellatus* (11.60 g/fruit body). Based on biological efficiency, thermotolerance, pileus fleshiness, and distinct sporophore pigmentation, four parent taxa—*P. sajor-caju*, *P. djamor*, *P. flabellatus*, and *P. ostreatus*—were selected for downstream spore physiology and hybridization assays.

Figure 1. Basidiome morphology of the four selected parental *Pleurotus* species cultivated on wheat straw: (a) *P. sajor-caju*, (b) *P. djamor*, (c) *P. flabellatus*, and (d) *P. ostreatus*.



Pleurotus sajor-caju



Pleurotus dijamor



Pleurotus flabellatuse



Pleurotus ostreatus

Table 1. Baseline agronomic performance and sporophore morphology of seven *Pleurotus* species.

Species	Sporophore Count (No.)	Total Yield (g/3 kg substrate)	Individual Weight (g)	BiologicalEfficiency (%)	Sporophore Morphology	Cropping Season
P. sajor-caju	65	598.4	9.20	66.48	Dull brown/buff, lobed, fleshy-leathery	March–April
P. djamor	74	645.5	8.70	71.72	Rose-pink, wavy margin, thin-fleshed	March–April
P. florida	56	595.2	10.62	66.13	Creamy white, fan-shaped	March–April
P. flabellatus	44	510.5	11.60	56.72	White, fan-shaped, decurrent	Sept–March

					lamellae	
P. citrinopileatus	45	515.3	11.45	57.25	Bright golden yellow, delicate pileus	Oct–March
P. sapidus	47	520.1	11.07	57.78	Dull white, imbricate clusters	March–April
P. ostreatus	35	469.3	13.40	52.14	Grayish-brown/purplish, thick, fleshy	March–April
CD (P=0.05)	1.75	2.49	—	—	—	—
SEm (pm)	0.58	0.82	—	—	—	—

2. Physiological Dynamics of Basidiospore Germination and Viability

Basidiospore germination across all four parent taxa was optimized at 27°C and pH 7.0 after 48 h of incubation (Table 2). Germination was severely inhibited under extreme chilling (15°C; 5.2%–7.1% at 48 h) and pH extremes (pH 4.0 and 10.0; ≤ 3.5%). Exogenous carbohydrates repressed germination compared to sterile tap water (62.1%–70.1%), with simple hexoses (glucose: 29.2%–36.3%; fructose: 28.7%–39.4%) exerting stronger osmotic repression than disaccharides or polysaccharides (maltose: 47.5%–56.3%; starch: 36.5%–44.0%). Basidiospore viability declined with storage age, but low-temperature preservation (6°C) preserved 54.2%–57.0% viability through 150 days, compared to a steep reduction to 21.0%–22.2% under ambient storage (27°C).

Table 2. Basidiospore germination and viability dynamics across cardinal physiological parameters (48 h incubation; storage up to 150 d).

Physiological Factor	P. sajor-caju	P. djamor	P. flabellatus	P. ostreatus	CD (P=0.05)	SEm (±)
Temperature (°C)						
15	5.2 (13.20)	6.2 (14.40)	7.1 (15.40)	6.2 (14.40)	1.69–2.15	0.54–0.68
20	23.3 (28.90)	20.3 (26.80)	21.0 (27.30)	18.0 (25.10)	—	—
24	60.1 (50.80)	58.2 (49.70)	55.2 (47.90)	58.3 (49.80)	—	—
27 (Optimum)	68.3 (55.70)	63.0 (52.50)	65.3 (53.90)	62.1 (52.00)	—	—
30	65.2 (53.80)	58.2 (49.70)	56.0 (48.40)	55.0 (47.90)	—	—
Media pH						
4.0	2.1 (8.30)	0.0 (0.00)	0.0 (0.00)	2.2 (8.50)	1.51–1.62	0.51–0.55
6.0	55.7 (51.80)	52.3 (46.30)	55.1 (47.90)	56.8 (48.90)	—	—
6.5	62.3 (52.10)	60.7 (45.20)	60.3 (50.90)	60.2 (50.90)	—	—
7.0 (Optimum)	68.8 (56.04)	63.0 (52.50)	61.5 (51.60)	61.0 (51.80)	—	—
7.5	60.0 (50.80)	59.1 (50.20)	58.7 (44.40)	60.0 (50.80)	—	—
8.0	28.1 (31.01)	28.2 (32.07)	27.2 (31.40)	30.3 (33.40)	—	—
10.0	3.5 (0.00)	0.0 (0.00)	0.0 (0.00)	3.2 (10.30)	—	—
Carbon Source (1% w/v)						
Tap Water (Check)	68.2 (55.60)	65.2 (53.80)	62.1 (52.00)	70.1 (56.90)	1.68–2.51	0.55–0.82
Glucose	35.0 (36.20)	29.2 (32.70)	29.2 (32.70)	36.3 (37.04)	—	—
Fructose	39.4 (38.90)	28.7 (32.40)	29.0 (32.60)	38.4 (38.20)	—	—

Sucrose	42.5 (40.50)	41.0 (39.80)	38.5 (38.30)	42.0 (40.40)	—	—
Maltose	55.1 (47.90)	47.5 (43.60)	48.6 (44.20)	56.3 (48.60)	—	—
Starch	39.2 (38.70)	41.2 (39.90)	36.5 (37.20)	44.0 (41.50)	—	—
Viability at 150 Days						
Refrigerated (6°C)	57.0 (49.02)	57.0 (49.02)	55.2 (47.90)	54.2 (47.60)	1.96–2.05	0.64–0.67
Ambient (27°C)	22.1 (28.04)	21.0 (27.30)	22.2 (28.10)	21.6 (27.70)	2.05–2.62	0.67–0.85

Values in parentheses indicate angular (arcsine) transformed values.

3. Monokaryon Recovery and Interspecific Hybridization

Nineteen confirmed monokaryotic single-spore isolates (SSIs devoid of clamp connections; SSIs 1, 2, 4, 5 for *P. sajor-caju*; 6–10 for *P. djamor*; 11–15 for *P. flabellatus*; 16–20 for *P. ostreatus*) were paired across 135 confrontation crosses (Fig 2) (Table 3). Overall compatibility was 20.0% (27/135), marked by prominent hyphal clamp connections at the interaction zones. The cross *P. djamor*× *P. flabellatus* displayed the highest mating frequency (36.0%, 9/25), with high receptivity centered around SSI 11. *Pleurotus sajor-caju*× *P. djamor* yielded 30.0% compatibility (6/20), driven primarily by SSI 2 and SSI 7. In contrast, *P. djamor*×*P. ostreatus* showed strong reproductive barriers with only 8.0% compatibility (2/25). Inter-crosses involving *P. ostreatus* with *P. sajor-caju* and *P. flabellatus* yielded 15.0% (3/20) and 16.0% (4/25) compatible dikaryons, respectively.

Figure 2.Macro- and micro-morphological indicators of interspecific hybridization in *Pleurotus*: (a) Dual-culture confrontation pairing showing a compatible vegetative hyphal junction and an incompatible barrage standoff on PDA; (b) Light micrograph of monokaryotic hypha displaying simple septa devoid of clamp connections; (c) Dikaryotic hybrid mycelium (*Psc*×*Pd-5*) displaying a characteristic clamp connection at the hyphal septum (1000× magnification)

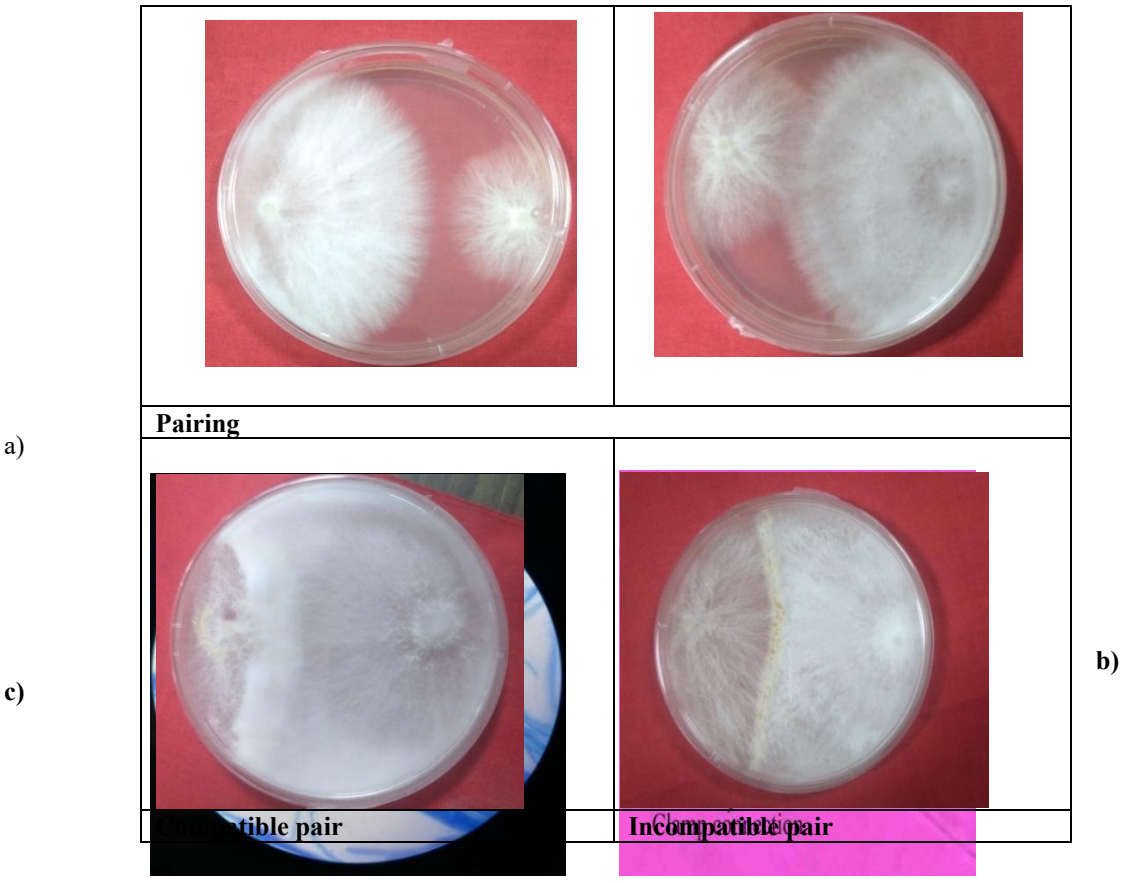


Table 3. Confrontation pairing matrix and cross-compatibility among monokaryotic single-spore isolates (SSIs) of four *Pleurotus* species.

Interspecific Cross	Total Pairings	Compatible Pairs (No.)	Compatibility (%)	Compatible Monokaryon Combinations (Dikaryon Designation)
P. sajor-caju*× *P. djamor	20	6	30.0	1×7 (Pd-1), 2×10 (Pd-2), 4×7 (Pd-3), 2×7 (Pd-4), 2×8 (Pd-5), 2×9 (Pd-6)
P. sajor-caju*× *P. flabellatus	20	3	15.0	2×12 (Pf-1), 2×14 (Pf-2), 4×14 (Pf-3)
P. sajor-caju*× *P. ostreatus	20	3	15.0	1×17 (Pos-1), 2×17 (Pos-2), 2×19 (Pos-3)
P. djamor*× *P. flabellatus	25	9	36.0	10×13 (Pf-1), 8×13 (Pf-2), 7×14 (Pf-3), 6×11 (Pf-4), 7×11 (Pf-5), 7×12(Pf-6), 8×11 (Pf-7), 10×14 (Pf-8), 8×11 reciprocal (Pf-9)
P. djamor*×*P. ostreatus	25	2	8.0	7×19 (Pos-1), 9×17 (Pos-2)
P. flabellatus*× *P. ostreatus	25	4	16.0	13×18 (Pos-1), 12×18 (Pos-2), 15×18 (Pos-3), Secondary sweep (Pos-4)
Total / Overall	135	27	20.0	—

4. Basidiome Morphology and Agronomic Heterosis of Hybrids

Cultivation of confirmed dikaryotic hybrids on pasteurized wheat straw revealed marked phenotypic diversity and transgressive heterosis in sporophore architecture and biomass productivity (Fig. 4). All 27 fertile dikaryons successfully formed basidiomes, manifesting intermediate to superior fleshiness and coloration compared to their parental lines. Among the generated crosses, *Psc ×Pd-5* produced the highest cumulative yield (688.8 g/3 kg wet substrate; 76.44% BE), combining the dense cluster architecture of *P. sajor-caju* with the vibrant pigmentation of *P. djamor*. Furthermore, hybrids derived from *P. flabellatus*×*P. ostreatus* (e.g., *Pf* × *Pos-2*) exhibited enhanced pileus thickness and individual sporophore weight (13.10 g), significantly surpassing both parental baselines in cumulative biological efficiency.

Figure 4. Comparative basidiome morphology of parental lines (*Pleurotus sajor-caju* and *P. djamor*) and their interspecific dikaryotic hybrids (*Psc* × *Pd-5* and *Psc* ×*Pd-1*).



P.sajor-cajuP. djamor



Hybrid (Psc x Pd -5)Hybrid (Psc x Pd-1)

DISCUSSION

Oyster mushrooms (*Pleurotus* spp.) occupy a premier position in global and Indian commercial mushroom production owing to their wide substrate adaptability, excellent nutritional profile, and high biological efficiency. In India, *Pleurotus* species account for approximately 21% of total mushroom production, serving as a critical dietary and commercial bioresource (Kamal et al., 2022). However, broader market expansion has been constrained by narrow seasonal adaptability and the lack of high-yielding cultivars combining desirable organoleptic traits (such as fleshy pilei and appealing color morphs) with resilience to fluctuating ambient temperatures. Interspecific hybridization offers a robust genetic pathway to combine non-overlapping agronomic traits and break existing yield plateaus(Dwivedi et al., 2019).

The baseline evaluation of seven commercial *Pleurotus* species demonstrated marked phenotypic and agronomic variation under prevailing ambient conditions. *Pleurotus djamor* exhibited the highest sporophore count and cumulative yield (645.5 g/3 kg wet substrate; 71.72% BE) characterized by distinct rose-pink basidiomes. This pronounced fecundity and flush density in *P. djamor* relative to *P. sajor-caju* aligns with previous comparative evaluations where pink oyster lines consistently yielded higher numbers of primordial aggregations and basidiomes (Jamil et al., 2019; Sharma & Lal, 2024). Conversely, *P. ostreatus* and *P. flabellatus* yielded fewer basidiomes but produced significantly heavier, fleshier pilei (13.40 g and 11.60 g per fruiting body, respectively), corresponding to morphological records described for temperate and subtropical *Pleurotus* accessions (Stamets, 2011). These divergent morpho-agronomic traits provided an ideal phenotypic basis for selecting *P. sajor-caju*, *P. djamor*, *P. flabellatus*, and *P. ostreatus* as parental stocks for downstream hybridization.

Successful recovery of viable haploid germplasm requires precise calibration of spore physiology. In the present study, basidiospore germination across all four test species was optimal at 27°C and neutral pH (7.0), achieving 61.0%–68.8% germination after 48 h. The precipitous reduction in germination at 15°C and complete arrest at pH extremes (4.0 and 10.0) indicate that germ tube emergence is strictly dependent on membrane fluidity and the active proton electrochemical gradient across the fungal plasmalemma. Similar thermal and hydrogen ion optima have been reported for basidiospore germination across the *P. ostreatus–sapidus* complex (Poyedinok et al. 2015). Notably, exogenous hexoses (glucose and fructose) exerted an osmotic repression on germ tube initiation compared to sterile tap water. This reflects a well-documented physiological response in higher basidiomycetes where exogenous simple sugars trigger catabolite repression or hypertonic delay, whereas metabolically inert or dilute aqueous conditions trigger prompt endogenous reserve utilization (trehalose and glycogen stores) to drive germ tube emergence (Walker & White, 2017).

Storage kinetics highlighted an operational threshold for spore bank maintenance. Low-temperature storage (6°C) preserved high viability (>54%) for up to 150 days, while ambient room temperature (27°C) led to severe viability loss within 90 days, dropping to ~21% by Day 150. Feofilova (2003) similarly demonstrated that chilled basidiospores of *P. ostreatus* maintain near-complete germinability up to one year, whereas ambient exposure leads to rapid desiccation and lipid peroxidation of unencapsulated spore walls. Thus, refrigerated storage of spore prints provides a stable foundation for phased monokaryon collection programs.

Single-spore isolates (SSIs) across all four species displayed classic monokaryotic characteristics: slow-growing, thin, appressed vegetative hyphae devoid of clamp connections, and an inability to form basidiomes on wheat straw substrates. This confirms that these *Pleurotus* species operate under a bifactorial (tetrapolar) heterothallic incompatibility system, governed by two independent unlinked multiallelic mating loci (*A* and *B*) that prevent self-fertilization in homokaryons (Lee et al., 2026).

Confrontation pairings between sexually compatible monokaryons yielded 27 fertile dikaryons out of 135 crosses (20.0% overall compatibility). Successful dikaryotization was characterized by the induction of persistent clamp connections at every septal junction along the interaction zone, signaling reciprocal nuclear exchange and regular conjugate nuclear division (Abdulgani et al., 2017; Jyothi &Thara, 2020). Marked variations in crossability were recorded among the species pairs:

- The interspecific cross *P. djamor*×*P. flabellatus* showed the highest hybridization rate (36.0%), suggesting a close phylogenetic affinity and lower interspecific barrier between these two tropical/subtropical taxa.
- *Pleurotus sajor-caju*× *P. djamor* yielded a 30.0% compatibility rate, heavily anchored by the broad mating receptivity of SSI-2 and SSI-7.
- In contrast, pairings between *P. djamor* and *P. ostreatus* displayed strong pre-zygotic barriers, yielding only 8.0% compatibility (2/25). This vegetative antagonism (barrage formation and zone lines) reflects significant genetic divergence, mitochondrial incompatibilities, or allorecognition barriers commonly observed between distinct species complexes in *Pleurotus* (Kües and Navarro-González, 2009).

Vegetative growth on agar media demonstrated that the generated dikaryotic hybrids exhibited distinct thermal plasticity, growing vigorously at 27°C and 30°C. When cultivated on wheat straw substrate, all 27 interspecific hybrids formed fertile basidiomes with improved textural firming and visual pigmentation over their parents. Most hybrid families exhibited varying degrees of heterosis:

- Hybrids of *P. sajor-caju*×*P. djamor* and *P. djamor*×*P. flabellatus* outperformed their lower-yielding parents (*P. sajor-caju* and *P. flabellatus*, respectively) and incorporated the desirable fleshiness and thick stipes of the white-spored taxa alongside the pink pigmentation of *P. djamor*.

- Hybrids from the cross *P. flabellatus*× *P. ostreatus* displayed transgressive heterosis, producing higher cumulative biological yields and fleshier caps than both respective parental lines.

These findings corroborate the observations of Singh and Kamal (2017), who demonstrated that interstock and interspecific *Pleurotus* dikaryons often display superior vegetative vigor and yield over parental monokaryons through dominant gene complementation. Similarly, Bahukhandi and Sharma (2002) documented up to 23.3% yield increases in interspecific *Pleurotus* hybrids. The selected hybrids identified here provide a viable germplasm reservoir combining high bio-conversion efficiency, dense basidiome architecture, and broader climatic adaptability for sustainable commercial cultivation.

CONCLUSION

The present study establishes a streamlined conventional breeding and physiological protocol for developing superior, climate-resilient *Pleurotus* cultivars through targeted interspecific hybridization. Baseline evaluation of seven commercial species identified *P. sajor-caju*, *P. djamor*, *P. flabellatus*, and *P. ostreatus* as optimal parental lines possessing complementary traits in yield, fleshiness, and sporophore pigmentation. Spore physiological dynamics confirmed strict optima for basidiospore germination at 27°C and pH 7.0 across all species, whereas exogenous sugars caused osmotic repression and low temperatures severely restricted germination. Cold storage at 6°C preserved spore viability above 54% through 150 days, establishing practical guidelines for short- to medium-term germplasm conservation.

Microscopic and cultural screening of single-spore isolates verified bifactorial (tetrapolar) heterothallism and vegetative sterility in homokaryons. Dual confrontation pairing among 19 monokaryons yielded 27 clamp-bearing dikaryotic hybrids out of 135 crosses (20.0% compatibility). Mating frequencies revealed differential cross-compatibility, peaking in *P. djamor*×*P. flabellatus* (36.0%) while remaining constrained by pre-zygotic barriers in *P. djamor*×*P. ostreatus* (8.0%). Subsequent substrate cultivation demonstrated marked heterosis, with hybrid **Psc ×Pd-5** achieving the highest cumulative yield (688.8 g/3 kg substrate; 76.44% biological efficiency) and hybrid **Pf ×Pos-2** displaying enhanced pileus fleshiness. Overall, this breeding framework provides a reliable approach for breaking yield plateaus and introgressing elite morphological traits in commercial oyster mushroom cultivation.

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