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Evaluation of the Antifungal Bio-Efficacy of Shiitake Spent Mushroom Substrate Against *Sclerotinia sclerotiorum*: Influence of Concentration and Storage Period Under *in vitro* Conditions

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Abstract

Shiitake (*Lentinula edodes*) cultivation produces substantial Spent Mushroom Substrate (SMS), a potential resource for sustainable disease management. This study evaluated the antifungal activity of crude SMS extract against *Sclerotinia sclerotiorum*, a major soil-borne pathogen causing white mould in tomato. The SMS was tested at concentrations ranging from 10 to 60 per cent across three storage durations, i.e., fresh, 3 and 6 months. Results showed that minimal mycelial inhibition was observed at 10 per cent concentration, ranging from 0 to 1.48 per cent, while the maximum inhibition occurred at 60 per cent, ranging from 27.77 to 66.66 per cent, with significant differences across treatments. Both the concentrations and storage critically influenced the antifungal efficacy of SMS. These findings highlight the potential of shiitake SMS as an eco-friendly, value-added by-product for managing soil-borne pathogens and aligning with sustainable agricultural practices.

Keywords: Antifungal Activity, Crude Extract, Spent Mushroom Substrate

Mushrooms serve as an important dietary component, known for their health-promoting properties and rich nutritional value (Nidhi *et al.* 2023; Thakur *et al.* 2023). The shiitake mushroom (*Lentinula edodes* [Berkeley] Pegler), an edible fungus native to East Asia, is widely cultivated and valued for its nutritional and therapeutic properties (Sharma *et al.* 2024; Sud *et al.* 2024). Shiitake mushrooms are known for their therapeutic potential, which is mainly linked to bio-active compounds such as ergosterol, glucans, lectins and polysaccharides. These bioactive compounds contribute to a broad spectrum of beneficial effects, including antimicrobial, antioxidant, anti-inflammatory, antiviral and antitumor properties (Baptista *et al.* 2023). Shiitake is among the most widely cultivated and consumed mushroom species across the globe (Ahmad *et al.* 2023) and contributes about 26 per cent of the total mushroom production worldwide (Singh *et al.* 2020). With the rise in mushroom production, substantial quantities of SMS are generated, i.e. typically around 5kg of SMS for every 1kg of fresh mushrooms harvested (Kumar *et al.* 2022).

SMS, often referred to as Spent Mushroom Substrate, is the leftover growing material that remains after the mushroom crop has been fully harvested, and it is the primary byproduct of the mushroom industry (Martín *et al.* 2023). It is naturally rich in organic matter and retains a considerable amount of key nutrients, including

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nitrogen, phosphorus and potassium, along with a varied population of beneficial microorganisms (Aiduang *et al* 2025). SMS is widely utilised as a nutrient-rich bio-fertiliser and soil amendment as its high organic matter content helps to improve soil structure, enhance water retention and support better crop yields. Its diverse microbial and enzymatic profile further adds value, enabling the breakdown of environmental contaminants such as pesticides and heavy metals. Importantly, SMS also contributes to plant health by reducing the incidence of soilborne diseases when applied to soil, and it fosters beneficial microbial communities that can suppress soilborne pathogens. Due to its rich nutrient content and useful physical properties, SMS is viewed not as waste but as a valuable resource that can be re-purposed for agriculture and environmental restoration (Martín *et al.* 2023).

MATERIALS AND METHODS

All the experiments were conducted at the Centre for Mushroom Research and Training Unit (CMRT), Department of Plant Pathology, CSKHPKV, Palampur.

Collection of Diseased Plant Material

An infected tomato (*Solanum lycopersicum*) plant showing characteristic symptoms of white mould, including water-soaked lesions on the leaves, growth of white cottony mycelium and formation of black sclerotia in the stem, was collected in paper bags from the infected fields of Plant Pathology and was further investigated under laboratory conditions.

Isolation and Purification of the Pathogen

Plant tissues containing both diseased and adjoining healthy areas were cut into small sections, surface sterilised with 0.2 per cent sodium hypochlorite and rinsed two to three times with sterile distilled water. The cut plant tissue pieces were transferred onto 9 cm Petri dishes containing 25-30 ml of Potato Dextrose Agar (PDA) and incubated in the dark at 25 ± 1 °C for 3-4 days to encourage fungal growth. Similarly, sclerotia collected from infected plants were surface sterilised with 0.2% sodium hypochlorite, rinsed with sterile distilled water and placed on PDA plates, which were incubated at 25 ± 1 °C for 3 days to allow germination. Fungal colonies emerging from both tissue pieces and sclerotia were purified by transferring single hyphal tips onto fresh PDA plates to obtain pure cultures. These pure isolates were then multiplied under controlled conditions for further studies, with cultures monitored regularly and sub-cultured as needed to maintain viability of the pathogen (Faruk & Rahman 2022).

Morphological and Cultural Characterisation of the Pathogen

A 5mm mycelial disc was taken from the actively growing region of a 4-day-old culture and transferred onto a fresh 90mm Petri plate containing PDA. The inoculated plates were incubated at 25 ± 1 °C. Linear mycelial growth was measured at 48 and 72 hours, while observations of colony colour, texture and growth pattern were recorded after 72 hours. These assessments allowed for the evaluation of cultural and morphological characteristics of the pathogen.

Microscopic Examination

The pathogen's morphological characteristics were studied while cultured on PDA medium. Petri plates were prepared and inoculated at the centre with a 5-day-old culture of the pathogen using a uniform disc. The plates were incubated at 25 ± 1 °C and observations like growth patterns and structural features were recorded. Detailed studies were conducted using a microscope to examine slides of the pathogen. Characteristics of the mycelium, including branching patterns, width and colour, as well as features of the sclerotia, such as shape and colour, were recorded.

Molecular Characterisation

Molecular identification of *Sclerotinia* sp. was carried out using genomic DNA extracted through the Cetyl Trimethyl Amonium Bromide (CTAB) method described by Murray and Thomson (1980). For confirmation of pathogen identity, the cultured plate was directly sent to Eurofins Genomics Pvt. Ltd., Bangalore, India, where the amplified products were sequenced.

Sample Preparation

The SMS sample of *Lentinula edodes* was collected from CMRT of the Department of Plant Pathology at various storage stages of spent mushroom substrate, i.e. fresh, 3- and 6-month storage periods.

Preparation of Spent Mushroom Substrate Crude Extract

The extract was prepared by taking fresh, 3- and 6-month-old powdered SMS(s). The SMS and distilled water were mixed in a 1:3 ratio, i.e. 100g of SMS in 300 ml of distilled water. The prepared extract was boiled to concentrate the volume to 100ml. After boiling, the mixture was subsequently cooled at room temperature. At last, the antifungal potent SMS extract was derived by filtering the concentrated SMS extract through muslin cloth, followed by Whatman No. 1 filter paper to remove particulate matter. The filtrate obtained was considered the crude extract of SMS, which was collected in sterile containers and maintained at 4 °C for subsequent antimicrobial evaluation and bioassay studies.

Antifungal Efficacy of SMS Extracts Evaluated through the Poisoned Food Technique

The extract at different concentrations of 10%, 20%, 30%, 40%, 50% and 60% was prepared and incorporated into PDA, after which the mixture was autoclaved at 121 °C at 15 psi for 15 minutes before being poured into sterilised Petri plates. A disc of actively growing fungal mycelium, 5 mm in diameter, was taken from a 6-7-day-old culture and carefully placed at the centre of each Petri plate. Plates without the SMS extract were served as a control. All plates were incubated at 25 ± 1 °C. Each treatment and control was replicated three times to ensure consistency, precision and statistical analysis of the experiment. After seven days of incubation, the radial growth of the fungus was measured and compared against the control to assess the antagonistic effect of the extracts. The efficacy of each extract was assessed by measuring the radial growth of fungal colonies after incubation and calculating the percentage inhibition compared to untreated controls.

Per cent Inhibition (%)

$$\text{Percentage inhibiton \%} = \frac{C - T}{C} \times 100$$

Where:

C = colony diameter in control

T = colony diameter in treatment

RESULTS AND DISCUSSIONS

Morpho-Cultural and Microscopic Characterisation of *Sclerotinia sclerotiorum*

After a seven-day incubation period on PDA, *S. sclerotiorum* showed changes in mycelial colour, sclerotia production and growth pattern. The colonies of *S. sclerotiorum* were fast-growing with white, fluffy, and aerial mycelia. Sclerotia developed in the growing culture at the margins of the fungal colonies, forming concentric rings and radiating patterns. Sclerotia exhibited variability in shape, mostly globose to cylindrical, and were characterised by a dark black rind enclosing a white inner cortex. Each sclerotium measured about 2-7 mm in length and 2-4 mm in width. Each PDA plate yields approximately 15-20 sclerotia. During the development of sclerotia, three macroscopically distinct stages were observed: an initial phase characterised by a white appearance, a developmental phase in which they turned beige, and a maturation phase marked by the acquisition of a typical black pigmentation. Under the microscope, the hyphae were hyaline, branched and multinucleate (Plate 1). No conidia or conidiophores were produced (Prova *et al.* 2018).

Molecular Characterisation

The sequencing results obtained from Eurofins Genomics India Pvt. Ltd. were analysed by the Basic Local Alignment Search Tool (BLAST) tool developed by the National Centre for Biotechnology Information (NCBI), which revealed a high degree of sequence homology of the pathogens with respective reference sequences available at GenBank. Thus, confirming the identity of *Sclerotinia* sp. as *S. sclerotiorum*. The sequences were submitted to the GenBank database through the NCBI submission portal, and accession numbers were obtained. The accession number obtained for *S. sclerotiorum* (PV271190) showed 100 per cent identity with *S. sclerotiorum* isolate (MG516658) (Figure 1). Further, a phylogenetic tree was developed for the pathogens, which were identified on a molecular basis using the MEGA 12 software to establish the evolutionary relationship among them. The obtained nucleotide sequences were analysed, and a phylogenetic tree was prepared using the neighbour-joining method.

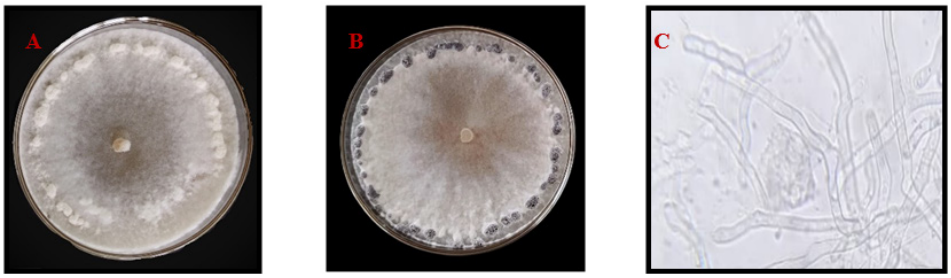


Plate 1. **(A)** Initial stage of sclerotia appearance **(B)** Pure culture of the *Sclerotinia sclerotiorum* showing white fluffy mycelium and black sclerotia **(C)** Microscopic view of transparent (hyaline) mycelial structures of *S. sclerotiorum*.

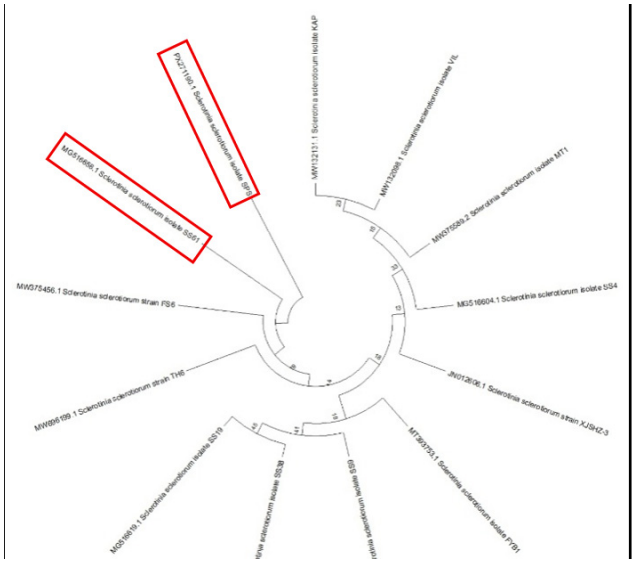


Figure 1. Phylogenetic tree of *Sclerotinia sclerotiorum* (PV271190) based on ITS 1 and ITS 4 gene sequences constructed using the neighbour-joining method.

The phylogenetic tree clearly represents the clustering of isolates with their respective reference sequence from NCBI, which helped in confirming their identity at the species level.

Evaluation of Antifungal Activity of SMS Crude Extracts Against *Sclerotinia sclerotiorum*

The crude extract from shiitake mushroom spent substrate SMS effectively inhibited the mycelial growth of *S. sclerotiorum*, demonstrating a clear antagonistic effect. The degree of inhibition was directly correlated with the concentration of the extract and its age. At the lowest concentration (10 per cent), mycelial growth inhibition was negligible, ranging from 0.0 per cent at fresh to 1.48 per cent after 6 months. Similarly, the 20 per cent extract showed weak and inconsistent effects, showing mycelial inhibition between 0.0 per cent at fresh and 8.14 per cent after 6 months of storage.

The mycelial inhibitory effect became more evident from 30 per cent concentration onwards. At this concentration, mycelial inhibition increased from 4.81 per cent at fresh to 28.51 per cent at 6 months of storage. The 40 per cent extract showed greater suppression, rising from 15.92 per cent initially to 32.59 per cent after 6 months. At 50 per cent, mycelial inhibition ranged from 23.33 per cent at fresh, 25.18 per cent at 3 months, followed by 6 months of storage, which showed 37.40 per cent mycelial inhibition, confirming progressive enhancement of activity with storage. The strongest effect was recorded at 60 per cent concentration, where inhibition recorded a substantial rise from 27.77 per cent at fresh to 66.66 per cent at 6 months of storage (Table 1 and Figure 2). This demonstrated that both higher concentrations and longer storage periods significantly improve the antifungal activity of SMS extracts. Overall, the best treatment was a 60 per cent concentration stored for six months, which achieved 66.66 per cent mycelial inhibition, suggesting that antimicrobial compounds become more effective with time.

CONCLUSION

This study demonstrated that crude extracts from shiitake Spent Mushroom Substrate (SMS) possess significant antifungal activity against *S. sclerotiorum*. The inhibitory effect was strongly influenced by both extract concentration and storage duration, with higher concentrations and longer storage periods showing progressively stronger inhibition of mycelial growth. The SMS stored for six months proved most effective and achieved nearly 66.66 per cent mycelial growth

Table 1. Effect of crude extracts from fresh, three- and six-month-old shiitake spent mushroom substrate on growth and inhibition of *Sclerotinia sclerotiorum*

Concentrations (%)	Mycelial Growth (mm)			Mycelial Growth Inhibition (%)		
	Month					
	0	3	6	0	3	6
10	90.00 (9.53)	90.00 (9.53)	88.66 (9.46)	0.00 (1.00)	0.00 (1.00)	1.48 (1.53)
20	90.00 (9.53)	87.66 (9.41)	82.66 (9.14)	0.00 (1.00)	2.59 (1.89)	8.14 (3.00)
30	85.66 (9.30)	78.00 (8.88)	64.33 (8.08)	4.81 (2.40)	13.33 (3.78)	28.51 (5.43)
40	75.66 (8.75)	73.66 (8.64)	60.66 (7.85)	15.92 (4.11)	18.14 (4.37)	32.59 (5.79)
50	69.00 (8.36)	67.33 (8.26)	56.33 (7.57)	23.33 (4.93)	25.18 (5.11)	37.40 (6.19)
60	65.00 (8.12)	60.00 (7.81)	30.00 (5.56)	27.77 (5.36)	33.33 (5.85)	66.66 (8.22)
Control	90.00 (9.53)	90.00 (9.53)	90.00 (9.53)	-	-	-
CD (p=0.05)	(0.41)	(0.22)	(0.36)			
SE(d)	0.19	0.10	0.16			

*Mean of three replications. Values in parentheses are transformed values

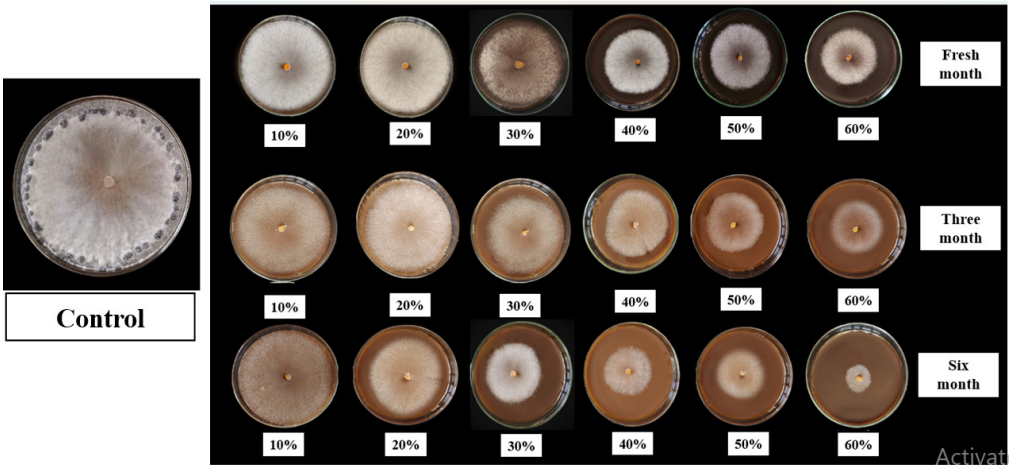


Figure 2. *In vitro* evaluation of *Lentinula edodes* spent mushroom substrate at different concentrations and storage periods against *Sclerotinia sclerotiorum*.

inhibition at 60 per cent concentration. These findings suggest that shiitake SMS contains antimicrobial compounds with antifungal potential, and the use of aged SMS may provide a low-cost and environmentally friendly approach to suppressing *S. sclerotiorum*.

Conflict of Interest: The authors declare that they have no competing interests.

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