Bangladesh J. Bot. **54**(3): 831-840, 2025 (September) Special DOI: https://doi.org/10.3329/bjb.v54i30.85187

SCREENING OF FORMULA FOR GROWING *STROPHARIA RUGOSOANNULATA* USING TOBACCO STALKS AS A SUBSTITUTE AND ITS IMPACT ON SOIL PROPERTIES FOR MUSHROOM CULTIVATION

MENGJIAO DING[1,2,3,4#], HAO SHU[5#], ZIHENG NONG[2,3], MINGHONG LIU, TAIBO LIANG[4], LI ZHANG[6], LI WANG[7], YONG WEI, WENMIN CHEN, BO TAN, YI LI, ZIWEI WANG[5]* AND JUN WAN*

Guizhou Provincial Tobacco Company Zunyi City Branch, Zunyi 563000, China

Key words: *Stropharia rugosoannulata*, Tobacco stalk substitute, Formula screening, Soil nutrients, Enzyme activities, Agronomic traits.

Abstract

To explore the feasibility of substituting sawdust with tobacco stalks for cultivating *Stropharia rugosoannulata* and its impact on soil properties, this study designed cultivation formulas with varying tobacco stalk ratios (0-50%) and analyzed mycelial growth rates, fruiting body agronomic traits, and changes in soil nutrients and enzyme activities. Results showed that the mycelial growth rate first increased and then decreased with a higher tobacco stalk ratio when tobacco stalk ratio was below 50%. T4 (30% tobacco stalks) was optimal with the highest rate (1.69 cm/d), as its tobacco stalk particle size improved mycelium-substrate contact and aeration. D3 (10% tobacco stalks + 90% corn stalks) had the best stipe/cap diameter, individual weight and available K (543.29 mg/kg), while D4 (20% tobacco stalks + 80% corn stalks) showed the highest alkali-hydrolyzed N (118.77 mg/kg) and balanced performance. D6 (100% corn stalks) had the highest available phosphorus content (6.38 mg/kg). Soil enzyme activity analysis indicated that invertase activity peaked in D6 (153.12 mg/g d^{-1}), whereas urease activity was highest in D5 (1.75 μg/g d^{-1}). Correlation analysis showed that the tobacco stalk ratio was extremely positively correlated with the mushroom shape index (r = 0.729**), and urease activity was significantly negatively correlated with fruiting body agronomic traits. In conclusion, T4 promoted mycelial growth, while D3 and D4 aligned soil nutrient improvement with the growth requirements of fruiting bodies, and D4 emerged as the most well-rounded treatment due to its balanced carbon-nitrogen release and favorable fruiting body phenotypic traits.

Introduction

Stropharia rugosoannulata Farl. ex Murrill, an edible fungus with edible and ecological value, has gained attention in recent years for its cultivation technology and resource utilization. Traditional cultivation typically uses materials like sawdust and cottonseed hulls. However, with the accumulation of agricultural waste and the increasing demand for ecological cycles, developing novel alternative growth substrates has become a research hotspot. Tobacco stalks, a major by-product of flue-cured tobacco production, have an annual output of over ten million tons in China. Unfortunately, their utilization rate remains low, and burning or discarding them can cause environmental pollution and resource waste (Hu *et al*. 2024). Studies have shown that tobacco stalks have a suitable carbon - nitrogen ratio and are rich in polysaccharides and proteins, showing potential as a growth substrate for edible fungi (Ye *et al*. 2013, Shen *et al*. 2022). Through the biotransformation of *S. rugosoannulata*, tobacco stalks can be efficiently degraded into fungal proteins and organic fertilizers, aligning with circular economy principles (Fu *et al*. 2015).

*Author for correspondence: <919136656@qq.com>; <346176171@qq.com>. [1]School of Agricultural Sciences, Zhengzhou University, Zhengzhou, 450001, China, [2]College of Tobacco Science of Guizhou University, Guiyang 550025, China. [3]Guizhou Provincial Key Laboratory for Tobacco Quality, College of Tobacco Science, Guizhou University, Guiyang 550025, China. [4]Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou 450001, China. [5]Hubei Xinye Tobacco Sheet Development Co. Ltd., Wuhan 430058, China. [6]China Tobacco Jiangsu Industrial Co. Ltd, Nanjing, 210019, China. [7]China Tobacco Anhui Industrial Co. Ltd, Hefei, 230088, China. Mengjiao Ding and Hao Shu contributed equally to this work.

Existing research indicates that *S. rugosoannulata* has strong adaptability to culture substrates. Different raw material ratios significantly affect mycelial growth, fruiting body yield, and quality. Studies have shown that mixing corn stover with cottonseed hulls can optimize the nutritional components of fruiting bodies (Tian *et al*. 2024). Research has confirmed that combining bamboo sawdust with corn cobs can enhance the crude protein content (Wang *et al*. 2021). However, research on tobacco stalks as a substitute material is not yet systematic, especially regarding their mechanism of impact on the nutrients and enzyme activity of mushroom-growing soil, which remains to be clarified. Mushroom growing soil, as a key environmental factor for fruiting body development, has its nutrient content and enzyme activity that directly affect mycelial metabolism and fruiting body formation (Wang *et al.* 2016, Xi *et al.* 2023). Studies have shown that incorporating spent mushroom substrate of *S. rugosoannulata* into the soil can significantly enhance soil organic matter and microbial diversity (Liu *et al.* 2021, Peng *et al*. 2023). Nevertheless, the dynamic regulatory effects of raw material ratios during the cultivation process on soil properties require further in-depth investigation.

This study employs gradient formulas with tobacco stalks replacing sawdust to cultivate *S. rugosoannulata*, systematically evaluating impacts on mycelial growth, fruiting body traits, and soil properties. The objectives are to identify optimal substitution ratios, elucidate the feasibility of tobacco stalk valorization, and clarify its synergistic soil-improvement effects, thereby supporting the tobacco industry`s green transition and sustainable mushroom cultivation.

Materials and Methods

The experiment was conducted at the Tobacco College of Guizhou University in Guizhou Province. The experimental soil was yellow soil. The *S. rugosoannulata* strain was provided by the Guizhou Provincial Academy of Agricultural Sciences. Locally sourced mixed-wood sawdust and flue-cured tobacco stalks were used as substrates of was inoculated into sterilized 300 ml glass culture flasks containing varying tobacco stalk ratios (Table 1). Mycelial growth rates were measured weekly until the substrate was fully colonized.

Table 1. Formulations of cultivation medium *Stropharia rugosoannulata*.

Treatment	Wood chips (%)	Tobacco stalks (%)	Wheat grains (%)	Cottonseedhulls (%)	Lime (%)	Gypsum (%)
T1	50	0	30	18	1	1
T2	40	10	30	18	1	1
T3	30	20	30	18	1	1
T4	20	30	30	18	1	1
T5	10	40	30	18	1	1
T6	0	50	30	18	1	1

Table 2. Straw substrate formula test treatments.

Treatment	Rice straw content (%)	Corn straw content (%)	Tobacco straw content (%)
D1	90	-	10
D2	80	-	20
D3	-	90	10
D4	-	80	20
D5	100	-	-
D6	-	100	-

Substrates combining tobacco stalks with rice or corn stalks (Table 2) were adjusted to 65% moisture and 20 cm thickness. All stalks were disinfected with 1% lime water for 24 hrs. Cultivation followed a two-layer substrate with interleaved inoculum. Fruiting bodies were sampled daily to record morphological parameters, including cap diameter, stipe length, and mushroom shape index.

Soil samples were collected at 60 days. The surface soil was moved aside, and soil samples were taken using the five-point method at a depth of 3-5 cm and mixed. After air drying indoors for 14 days, the samples were ground and sieved through 2 mm and 0.25 mm sieves for soil nutrient testing. Uncultivated soil served as the control group (CK).

Soil organic matter (SOM) was determined by potassium dichromate oxidation - colorimetry. Alkali-hydrolyzed nitrogen (AN) was measured by diffusion-absorption method. Available phosphorus (AP) was assayed by sodium bicarbonate extraction-molybdenum-antimony-potassium colorimetry. Available potassium (AK) was analyzed by ammonium acetate extraction-colorimetry (Nannipieri *et al*. 2011).

Sucrase was measured by the 3,5-dinitrosalicylic acid method. Urease was determined by indophenyl blue colorimetry, following reagent kit protocols (Beijing Solarbio Science and Technology Co. Ltd.).

Mycelial growth rate, fruiting body traits, soil nutrient indexes, and enzyme activity data were analyzed and graphed using Microsoft 365 and Origin 2018. Experimental data were subjected to significance tests and Spearman correlation analysis using R software v 4.3.2.

Results and Discussion

Mycelial growth rates followed an initial increase and subsequent decline with higher tobacco stalk ratios (Table 3). Among all treatments, T4 (30% tobacco stalks) recorded the highest growth rate of 1.69 cm/d, while T6 (50% tobacco stalks) had the lowest rate of 1.20 cm/d. Treatments with added tobacco stalks (T2-T4) showed higher growth rates than T1 (1.36 cm/d), indicating that an appropriate amount (≤ 30%) of tobacco-stalk addition could boost mycelial growth. However, higher tobacco stalk proportions (> 30%, T5-T6) inhibited mycelial expansion. Notably, in the later stages of cultivation, the mycelial density in substrates containing tobacco stalks surpassed that in substrates without them. The small particle size of tobacco stalks increased the contact area between mycelium and substrate and improved aeration, which explained the optimal mycelial density in T4.

Table 3. Mycelial growth rate of *Stropharia rugosoannulata*.

Treatment	Growth rate (cm/d)	Mycelial density
T1	1.36 ± 0.02ab	Sparse
T2	1.49 ± 0.11ab	Moderately dense
T3	1.49 ± 0.27ab	Dense
T4	1.69 ± 0.26a	Dense
T5	1.21 ± 0.04b	Moderately dense
T6	1.20 ± 0.18b	Moderately dense

The data is the mean ± standard error of three experiments, and lowercase english letters indicate differences with $P < 0.05$.

T3 (10% tobacco stalks + 90% corn stalks) exhibited the highest stipe diameter, cap diameter, and individual weight. However, T4 (20% tobacco stalks + 80% corn stalks) demonstrated optimal overall performance, with a mushroom shape index of 0.58 (Fig. 2). T6 (100% corn stalks) showed the highest shape index (0.93) but suppressed fruiting body development, likely due to

metabolic byproduct accumulation. A highly significant positive correlation between tobacco stalk proportion and mushroom shape index (r = 0.729) was found, suggesting that tobacco stalk components could directly impact fruiting body morphogenesis by modulating carbon and nitrogen metabolic enzymes.

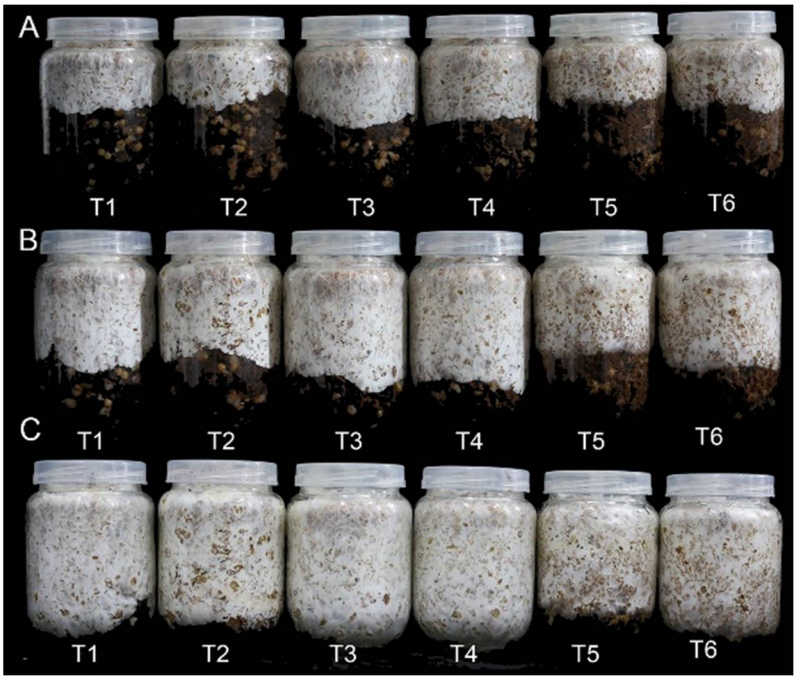

Fig. 1. Growth of hyphae of *Stropharia rugosoannulata* at 7d (A) 35d (B), and 49d (C) after inoculation.

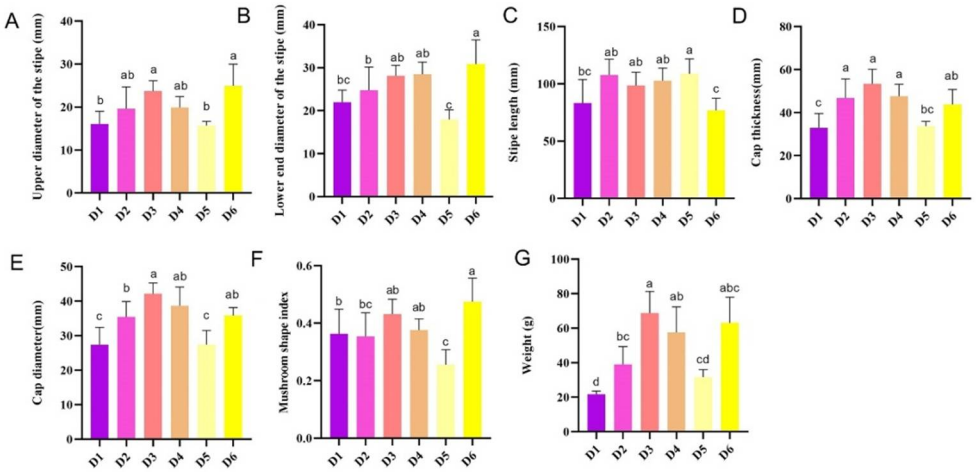

Fig. 2. Agronomic traits of fruiting bodies of *Stropharia rugosoannulata* in various treatments. A: Upper diameter of the stipe, B: Lower diameter of the stipe, C: Stipe length, D: Cap thickness, E: Cap diameter, F: Mushroom shape index, and G: Weight.

All treatments showed significantly higher contents of alkali-hydrolyzed nitrogen, available phosphorus, and available potassium than (Table 4). D3 achieved the highest available potassium (543.29 mg/kg). D6 had the highest available phosphorus (6.38 mg/kg), alongside an unusually elevated organic matter content (29.01 g/kg), likely due to increased soil carbon stocks from undecomposed tobacco stalk residues. D4 recorded the highest alkali-hydrolyzed nitrogen (118.77 mg/kg), indicating that the combination of tobacco stalks and corn stalks could balance carbon and nitrogen release. This probably promoted the secretion of extracellular enzymes by the mycelium to break down the substrate and release nitrogen. Overall, when the tobacco-stalk proportion was ≤ 20% (D1 - D4), the improvement in soil nutrients matched the growth requirements of fruiting bodies better.

Table 4. Effects of planting *Stropharia rugosoannulata* on soil nutrients.

Treatment	Alkali-hydrolyzed nitrogen （mg/kg）	Available phosphorus (mg/kg)	Available potassium (mg/kg)	Organic matter (g/kg)
CK	108.07 ± 0.00c	3.82 ± 0.27e	171.59 ± 2.97f	25.63 ± 1.47b
D1	117.21 ± 2.20a	5.82 ± 0.25b	367.42 ± 2.26d	26.40 ± 0.50ab
D2	111.98 ± 0.86bc	5.52 ± 0.15bc	400.06 ± 5.19c	25.83 ± 0.22b
D3	115.38 ± 0.41ab	6.37 ± 0.17a	543.29 ± 7.33a	24.95 ± 0.35b
D4	118.77 ± 2.10a	4.23 ± 0.08d	363.00 ± 2.26d	24.49 ± 0.54b
D5	115.86 ± 0.34ab	5.32 ± 0.09c	348.08 ±7.38e	24.64 ± 1.56b
D6	117.96 ± 3.50a	6.38 ± 0.05a	444.61 ± 3.63b	29.01 ± 1.45a

As shown in Fig. 3, invertase activity was highest in D6 (153.12 mg/g ·d^{-1}). This suggests that D6 could significantly enhance soil invertase activity using corn stalks as the sole substrate. This indicates that certain components in corn stalks might be more conducive to microbial growth and metabolism, thereby promoting invertase production. Urease activity was highest in D5 (1.75 μg/g ·d^{-1}) and lowest in D6 (0.43 μg/g ·d^{-1}), which shows that rice straw can effectively enhance soil urease activity while maize straw's unbalanced C/N ratio inhibits urease production.

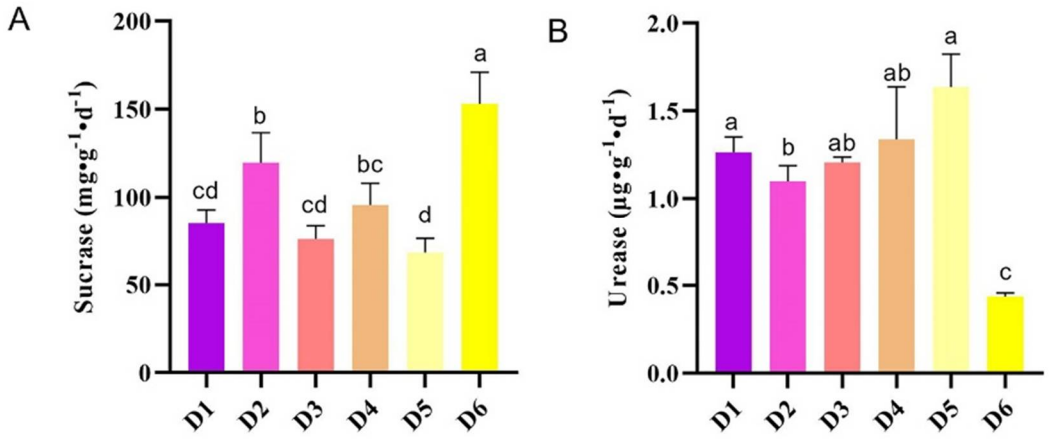

Fig. 3. Trends in sucrase (A) and urease (B) changes in mushroom planting soil under different treatment conditions.

Table 5 reveals a significant negative correlation between urease activity and fruiting body agronomic traits such as overall height and cap diameter (r = -0.643 to -0.529), indicating that high urease activity may accelerate nitrogen mineralization, thereby causing local nitrogen excess and suppressing fruiting body development. Alkaline-hydrolyzed nitrogen also showed a highly significant negative correlation with stipe diameter (r = -0.599), possibly related to nitrogen-induced stipe elongation. The highly significant positive correlation between tobacco stalk proportion and mushroom shape index (r = 0.729) further supports the hypothesis that tobacco stalk components optimize marketable traits by regulating morphogenesis-related gene expression (Li *et al*. 2022).

Table 5. Spearman correlation between nutrient indicators and agronomic traits of fruiting bodies.

Correlation Coefficient	Tobacco stalk content	AN	AP	AK	SOM	Sucrase	Urease
Total length	-0.184	-0.353	-0.286	0.078	-0.615*	0.440	-0.643**
Cap diameter	-0.046	-0.246	-0.297	0.074	-0.531	0.478	-0.596*
Cap thickness	0.00	-0.320	-0.269	0.047	-0.699*	0.407	-0.529*
Diameter of the stipe	-0.009	-0.599*	0.088	0.372	-0.252	0.143	-0.343
Stipe length	-0.355	-0.416	-0.198	0.169	-0.462	0.423	-0.593*
Weight	-0.189	-0.623**	-0.027	0.272	-0.42	0.269	-0.343
Mushroom shape index	0.729**	0.299	0.22	0.002	0.301	-0.319	0.232

** Significant correlation at 0.01 level * Significant correlation at 0.05 level.

In the mycelial growth experiment, the mycelium cultivated with tobacco stalks grew faster than that without tobacco stalks. This aligns with Qi *et al*. (2018), who found a similar trend in *Pleurotus ostreatus* cultivation. Gu *et al*. (2024) reported that in *Pleurotus eryngii* cultivation, the mycelial growth rate only increased with rising tobacco stalk content in the substrate. This likely stems from differing tolerance to tobacco-stalk components among fungal species. In this study, the growth of *S. rugosoannulata* showed good tolerance to increasing tobacco stalk content. Sawdust generally has a higher C/N ratio than tobacco stalks (Wan *et al.* 2014, Huang *et al.* 2023, Yang *et al.* 2024). For *S. rugosoannulata*, mycelial growth is faster at higher C/N ratios and slows down as the C/N ratio decreases, with the mycelium becoming denser (Yang *et al.* 2021). As the tobacco stalk content in the substrate increased, the mycelium tended to become denser. When the tobacco stalk content reached 40 and 50%, the mycelial growth rate declined, and the high concentration of tobacco stalk components (e.g., phenolic compounds) might exert toxic effects (Hu *et al*. 2024). Zhang *et al*. (2024) reported that as the nitrogen content in the culture environment increased from 0 to 1.4%, the mycelial growth rate of *S. rugosoannulata* first rose and then fell, consistent with this study. At the end of cultivation, the mycelium density of the treatment without tobacco stalks was the sparsest, while other treatments were relatively denser. The small particle size of tobacco stalks provided a larger contact area between the mycelium and substrate and better aeration than sawdust (Yang *et al.* 2022). Tobacco stalks contain more small-molecule compounds like sugars, organic acids, and proteins than sawdust, which can promote mycelial growth (Liu *et al*. 2008). Thus, tobacco stalks have the potential to replace some culture materials for *S. rugosoannulata* cultivation and hold broad application prospects. However, the proportion of tobacco stalk substrate must be strictly controlled to balance nutrient supply and metabolic inhibition.

The analysis of fruiting body agronomic traits showed that T3 recorded the highest values in most indicators except the mushroom shape index. This might be due to the added tobacco stalks

shortening the growth cycle, accelerating aging and cap opening. The mushroom shape index is also vital for evaluating mushroom shape and marketability (Kora 2020). The shape index increased with rising tobacco - stalk content, indicating that substrate composition affects fruiting body formation (Song *et al.* 2009). D4 showed moderate values in all indicators, with a shape index of 0.58 (higher than D1). A larger shape index implies a bigger cap diameter and shorter stipe, meeting market demands (Sakshi *et al.* 2025).

The nutrient content of cultivated soil was significantly higher than that of CK. During growth, *S. rugosoannulata* secretes extracellular enzymes like cellulase and hemicellulase, breaking down substrates and releasing nutrients into the soil (Jiang *et al.* 2020). This study found that soil cultivated with *S. rugosoannulata* had significantly higher levels of alkali-hydrolyzed nitrogen, available potassium, and available phosphorus than CK. Similar results were reported by Chen *et al.* (2022). During cultivation, the lower soil layer was relatively oxygen-deficient, yet the mycelium grew towards the oxygen-rich upper layer. This indicates that the improvement in soil nutrients from incorporating spent mushroom substrate is not only due to nutrient release from mycelium and substrate decomposition but also due to the effects of different cultivation formulas on mycelium, which in turn influence the surrounding environment during fruiting body cultivation (Tang *et al.* 2022). In the cultivated soil, the changes in alkaline-hydrolyzed nitrogen and organic matter were relatively small. This is because most of the nitrogen from the straw was used for mycelial growth. The increase in alkaline - hydrolyzed nitrogen in the upper soil layer originated from a small portion of the substrate's nitrogen and the improved nutrient transformation capacity of the soil due to mycelial growth. Moreover, the cultivated soil received little external organic matter supplementation during the cultivation process.

Soil enzymes are crucial for various chemical and biochemical processes and are often used to evaluate soil nutrient levels. Invertase and urease enhance soluble nutrients in the soil and are closely related to organic matter transformation and respiration intensity (Nannipieri *et al.* 2011). They directly depend on soil microbial numbers and respiration intensity (Richard *et al.* 2013). In this experiment, D6 (100% corn stalks) significantly increased soil invertase activity. During cultivation, the substrate was decomposed by *S. rugosoannulata*, providing easily - utilizable small - molecule substances for soil microorganisms and boosting their activity and invertase levels. Zhou *et al.* (2010) found that the application of *Pleurotus ostreatus* spent mushroom substrate could increase soil invertase activity. It was also found that D5 (100% rice straw) significantly increased soil invertase activity.This might be related to the different nutritional compositions, structures, and impacts on soil microbial communities among various straws. In practical applications, the types and ratios of straws can be selected and adjusted based on soil enzyme activity improvement goals and straw availability to optimize soil enzyme activities and promote the growth of *S. rugosoannulata* and soil ecosystem health. Studies have shown that soils amended with *S. rugosoannulata* spent substrate had significantly increased bacterial diversity, with nitrogen - fixing and deep - soil - carbon - decomposing bacteria becoming dominant. *S. rugosoannulata* cultivation can alter soil microbial communities and enhance soil urease and invertase activities (Yang *et al.* 2024), which is similar to the results of this experiment.

Research indicates that the porosity, water-holding capacity, and composition of covering soil materials significantly impact fruiting body development and yield and quality. In this study, high correlations were observed between urease activity and nitrogen-nutrient indexes with fruiting body agronomic traits, as well as between tobacco stalk content and mushroom shape index (He *et al.* 2023). The strong positive correlation between tobacco stalk proportion and mushroom shape index ($r = 0.729$) suggests two potential mechanisms: (1) Specific components in tobacco stalks (e.g., polysaccharides or phenolic compounds) regulate the activity of carbon- and nitrogen-metabolism enzymes (such as glutamine synthetase), thereby influencing mycelial differentiation

(Li *et al.* 2022); (2) A higher proportion of tobacco stalks may shorten the fruiting body growth cycle, accelerating cap expansion and stipe shortening (Kora 2020). However, T6 (100% corn stalks) exhibited the highest shape index (0.93), yet overall development was suppressed, likely due to an excessively high C/N ratio in the single substrate or the accumulation of metabolic by-products (Zhang *et al.* 2024).

Soil enzyme activity analysis revealed that D6 (100% corn stalks) had the highest invertase activity (153.12 mg/g $\cdot d^{-1}$), whereas D5 (100% rice straw) had the highest urease activity (1.75 μg/g $\cdot d^{-1}$). The elevated invertase activity might be linked to the high content of soluble sugars in corn stalks, which provide a rapid energy source for microbes, thereby stimulating enzyme synthesis (Zhou *et al.* 2010). In contrast, D6 showed the lowest urease activity (0.43 μg/g $\cdot d^{-1}$), indicating that the imbalanced C/N ratio (> 60) in the sole corn-stalk substrate inhibited urease production (Nannipieri *et al.* 2011). Further correlation analysis revealed significant negative associations between urease activity and fruiting body agronomic traits (e.g., total height and cap diameter) (r = -0.643** to -0.529*), suggesting that high urease activity accelerates nitrogen mineralization, leading to local nitrogen excess and suppressing fruiting body development (He *et al.* 2023). Moreover, the strong positive correlation between tobacco-stalk proportion and mushroom shape index (r = 0.729**) indicates that tobacco stalk components may optimize fruiting body marketability by regulating the expression of morphogenesis-related genes (such as auxin-encoding genes) (Li *et al.* 2022).

Previous studies have found that carbon and nitrogen metabolism-related enzymes (e.g., glutamine synthetase, glutamate synthase, acidic xylanase, and neutral xylanase) serve pivotal roles in fruiting body development, consistent with our findings. Excessive nitrogen availability in the substrate severely inhibits both mycelial proliferation and fruiting body formation (Li *et al.* 2022). Furthermore, tobacco stalk amendment levels exhibited a strongly significant positive correlation with mushroom shape index (r = 0.729), likely attributable to the specific regulatory effects of tobacco stalk components on fruiting body development, thereby directly regulating morphogenesis processes. Therefore, future research should combine transcriptomics to explore the molecular regulatory mechanisms of tobacco stalk components on mycelial differentiation and continuously refine cultivation formulas for large-scale *S. rugosoannulata* production.

Among all treatments, T4 (30% tobacco stalk substitution for sawdust) represents the optimal formulation for mycelial biomass accumulation. Conversely, D4 (20% tobacco stalks + 80% corn stalks) emerged as the superior formulation for both fruiting body quality and soil nutrient enhancement. Future studies should integrate multi-omics approaches to decode the molecular mechanisms by which tobacco-stalk bioactive compounds regulate mycelial metabolism, and establish standardized cultivation protocols for industrial-scale *S. rugosoannulata*.

Acknowledgements

The work was supported by the National Natural Science Foundation of China (42467007), Guizhou Science and Technology Partnership Initiative, Basic Research Grant [2024] Youth 177, the Science and Technology Project of Zunyi Tobacco Company (2022XM06), the Technical Service Project of Anhui Tobacco Base Unit (2024340000340003). Jiangsu Tobacco Industry Co., Ltd. Technology Project (H202226), the Key Program for Science and Technology of CNTC (No.110202402016), the Science and Technology Program of Guizhou Provincial Branch of the CNTC (No. 2024520000240022, 2023520000240241), the Program of Hubei Xinye Tobacco Slice Development Co. Ltd. (No. N2024420114360067). Guizhou Provincial Key Laboratory Construction Project for Tobacco Quality Improvement and Efficiency Enhancement Guizhou Science and Technology Innovation Platform ZSYS (2025) 028.

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(Manuscript received on 16 May 2025; revised on 14 October 2025)