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Biological Characteristics of the Mycelium and Optimization of the Culture Medium for *Phallus dongsun*

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Abstract

This study aimed to elucidate the influence of various culture medium components, including carbon sources, nitrogen sources, inorganic salts, suspension agents, and temperature, on the mycelial growth characteristics of *Phallus dongsun*. Employing single-factor experiments and response surface methodology within glass Petri dishes, the research identified that carrot powder, soybean powder, and $ZnSO_4$ notably enhanced the proliferation of aerial mycelium, significantly augmenting the growth rate of *P. dongsun* mycelium. The resultant mycelium was observed to be dense, robust, and fluffy in texture. In particular, $ZnSO_4$ markedly accelerated the mycelium growth rate. Furthermore, xanthan gum was found to effectively modulate the medium's viscosity, ensuring a stable suspension and facilitating nutrient equilibrium. The optimal cultivation temperature was determined to be 25°C, with mycelial growth ceasing below 5°C and mycelium perishing at temperatures exceeding 35°C. The optimal medium composition was established as follows: wheat starch 5 g/l, carrot powder 5 g/l, soybean powder 7.50 g/l, glucose 10 g/l, $ZnSO_4$ 0.71 g/l, NH_4Cl 0.68 g/l, xanthan gum 0.5 g/l, and agar 20 g/l. Under these optimized conditions, the mycelium of *P. dongsun* exhibited a rapid growth rate (1.04 ± 0.14 mm/day), characterized by a thick, dense, and well-developed structure. This investigation provides a theoretical foundation for the conservation, strain selection, and breeding of *P. dongsun*.

K e y w o r d s: *Phallus dongsun*, nutrient composition, suspension agents, temperature gradient, mycelium growth rate

Introduction

"Dongsun" denotes a folk term for an edible *Phallus* species found in Guizhou Province and southwest China (Li et al. 2015; Li et al. 2020). Its nomenclature derives from its growth period during the cooler seasons of autumn and winter. Additionally, it is classified within the same family as *Dictyophora*. Typically, the "Dongsun" fruiting body, comprising a cap, stalk, and receptacle, is classified under Basidiomycota, Phallomycetidae, Phallales, *Phallaceae*, and *Phallus* (Li et al. 2020). This species is widely distributed (Melanda et al. 2021; Li et al. 2023). As a culinary ingredient, "Dongsun" is flavorful, crunchy, nutrient-rich, and highly nutritious (Kang et al. 2020). It ranks among the most significant traditional edible mushrooms in China, including *P. impudicus*, *P. fragrans*, *P. flavocostatus*, and *P. dongsun*, among others (Li et al. 2020). Research indicates that "Dongsun" yields numerous bioactive compounds, such as crude polysaccharides, proteins, amino acids, flavonoids, phenols, fibrin, ergosterol, and mineral elements (Kikuchi et al. 1984; Wu et al. 2013; Li and Bao 2020; Shou et al. 2020). In traditional Chinese medicine, it is attributed with pharmaco logical properties including stimulating blood circulation, alleviating pain, dispersing wind, and eliminating dampness (Dai et al. 2009; Wu et al. 2019; Li and Bao 2020). Furthermore, it exhibits immunomodulatory (Gupta and Shinde 2016; Buko et al. 2019), anticoagulant, anti-edema, antiseptic, anti-tumor, antioxidant, anti-cholinesterase, anti-inflammatory, and antibacterial properties (Kuznecov et al. 2007; Kuznecova et al. 2007; Yoon and Lee 2019; Shou et al. 2020; Zheng et al. 2022; Qian et al. 2023). Currently, the functionalities of *Phallus* species are garnering increasing attention.

"Dongsun" has been artificially cultivated in Guizhou, China, and represents one of the edible mushroom

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species domesticated in China over the last decade (Li et al. 2023). It is currently cultivated similarly to the *Dictyophora* species, with its production and scale undergoing expansion (Yu et al. 2020). This mushroom has now been recognized as a product with a National Product of Geographical Indication in China. However, the widely cultivated "Dongsun" had been misidentified for a long time as the European material of *P. impudicus*. Fortunately, recent studies have confirmed that it constitutes a new species, distinct from any other *Phallus* taxon, proposed and described as *P. dongsun* (Li et al. 2020; Yang 2020). The results of this study contribute a new species to the roster of cultivated edible mushrooms within the genus *Phallus*.

As production and its scale expand, issues such as slow mycelium growth, low production efficiency, and a high contamination rate have become increasingly apparent in the strain production process (Zhang et al. 2021). In previous studies, glucose, fructose, and brown sugar have been identified as favorable carbon sources for *P. impudicus* or *P. dongsun* mycelium growth. Additionally, mycelium balls tend to form quickly in liquid culture (Zhang et al. 2021; Huang et al. 2022; Meng et al. 2022). Nitrogen sources are critical for mycelial growth; peptone, yeast powder, yeast extract, and beef extract have been recognized as favorable nitrogen sources for mycelial ball formation in liquid culture (Meng et al. 2022; Huang et al. 2022). Findings indicate that K_2PO_4, KCl, $CaCl_2$, VB_6, and 6-BA significantly enhance mycelial growth rate and density, whereas $MgSO_4 \cdot H_2O$ and NaCl deter mycelial growth (Zhang et al. 2021). Potato Dextrose Agar (PDA) is a commonly utilized medium for fungal isolation and purification. In the PDA medium, the *P. impudicus* colony appears white with rhizomorphs. The hyphae are achromatic and transparent, featuring branches, septa, and conspicuous clamps (Lu et al. 2010). However, "Dongsun" tissue blocks easily germinate but fail to germinate or exhibit slow growth in fresh PDA upon transfer to culture. Furthermore, research on the "Dongsun" mother seed medium remains scant. Most practitioners utilize various wood chips as substrates for the "Dongsun" mother seed culture medium, a model characterized by a lengthy production cycle and a high contamination rate, significantly hindering the positive development of the "Dongsun" industry.

In this study, we investigated the effects of carbon sources, nitrogen sources, inorganic salts, suspending agents, and incubation temperature on the biological characteristics of *P. dongson* in Petri dishes utilizing single-factor experiments and response surface methodology. The aim of this research is to develop an optimal mother culture medium for the growth of *P. dongson*, thereby providing scientific support for its conservation, strain selection, and large-scale production.

Experimental

Materials and Methods

Test strain. *P. dongsun* GZCC0351 was isolated and purified from fresh fruiting bodies. The sample was collected from Liulong Town, Dafang County, Bijie City, Guizhou Province, China, and is preserved in the Species Preservation Room at the Guizhou Institute of Biology. Slants were incubated at 25°C on PDA for 7–10 days in glass petri dishes (90 mm in diameter).

Source of materials. All experimental materials were sourced from a consistent supplier and were newly acquired for each use to guarantee substrate stability and uniformity. Brown sugar, wheat bran, carrot powder, potato starch, corn starch, wheat starch, and soybean powder, identified as natural substrates, were procured from Xinghua Lüshuai Food Co., Ltd., (China). Wood powder, a natural substrate, was sourced from Caoxian Luyi Wood Industry Co., Ltd. (China). The following reagents, all of the analytical grade, were acquired from Tianjin Kemiou Chemical Reagent Co., Ltd. (China): KH_2PO_4, $K_2HPO_4 \cdot 3H_2O$, $MgSO_4 \cdot 7H_2O$, $MgCl_2 \cdot 6H_2O$, $ZnSO_4$, $CaCl_2$, $CaSO_4 \cdot 2H_2O$, $Fe_2(SO_4)_3$, $CaCO_3$, NH_4Cl, $(NH_4)_2SO_4$, KNO_3, $NaNO_3$, carbamide, glucose, fructose, sucrose, maltose, lactose, cellulose, xylogen, chitosan, dimethyl sulfoxide, and Tween 80. Additionally, all the analytical grade, sodium carboxymethyl cellulose, sodium alginate, gum arabic, and xanthan gum were purchased from Tianjin Yongda Chemical Reagent Co., Ltd. (China). Furthermore, biochemical reagents such as peptone, yeast powder, yeast extract, beef extract, and soybean powder were procured from Shanghai Bowei Biotechnology Co., Ltd. (China).

Base medium (PDA). Potatoes (200 g) were peeled, sliced thinly, boiled in water until they reached a soft yet firm consistency, and subsequently strained to extract the juice. The extracted juice, along with dextrose (20 g) and agar (20 g), was mixed, and the volume was adjusted to 1000 ml, ensuring the maintenance of the natural pH.

Mycelium culture method. After preparing the medium, it was sterilized at 121°C for 30 minutes, then cooled and aliquoted into 90 mm petri dishes, with a volume of 20 ml/dish. Following solidification, the medium was inoculated with a *P. dongsun* inoculum block (approximately 5 mm in diameter) and incubated at 25 ± 2°C. Cultivation proceeded in darkness for 28 days in a constant-temperature incubator.

Screening of single factors for nutrient content. ***Natural substrates.*** This study aimed to investigate the effects of various natural substrates on the biological properties of *P. dongsun* mycelium. Substitutes for the base medium (PDA) included 200 g/l peeled potato,

wheat bran, and 10 g/l of carrot powder, potato starch, corn starch, wheat starch, and wood powder, respectively. A control group was established by using the base medium without any natural substrate.

Suspension agent. Following prior research outcomes, this study examined the impact of various suspension agents on the medium's physical state and the biological properties of *P. dongsun* mycelium. 5 g/l of sodium carboxymethyl cellulose, sodium alginate, gum arabic, xanthan gum, and 5 ml/l of dimethyl sulfoxide, Tween 80 were added to the medium. A control group was employed utilizing the medium without any suspension agent.

Carbon source. In light of prior experimental outcomes, various carbon sources were evaluated for their effects on the biological properties of *P. dongsun* mycelium. Each medium was supplemented with 20 g/l of glucose, fructose, sucrose, maltose, lactose, brown sugar, cellulose, xylogen, and chitosan. A control group was established using a medium devoid of any carbon source.

Nitrogen source. Drawing upon the findings from previous experiments, diverse nitrogen sources were investigated for their influence on the biological properties of *P. dongsun* mycelium. The medium was respectively augmented with 10 g/l of peptone, yeast powder, yeast extract, beef extract, soybean powder, and 1 g/l of carbamide, KNO_3, NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$. A control group was established with the medium lacking any nitrogen source.

Inorganic salt. Following the outcomes of preceding experiments, various inorganic salts were investigated for their influence on the biological characteristics of *P. dongsun* mycelium. Each medium was supplemented with 1 g/l of KH_2PO_4, $K_2HPO_4 \cdot 3H_2O$, $MgSO_4 \cdot \cdot 7H_2O$, $MgCl_2 \cdot 6H_2O$, $ZnSO_4$, $CaCl_2$, $CaSO_4 \cdot 2H_2O$, $Fe_2(SO_4)_3$, $CaCO_3$, NH_4Cl, KNO_3, respectively. A control group was constituted without the addition of any inorganic salt.

Plackett-Burman design. The Plackett-Burman design was employed to identify components that had significant influence on medium optimization. Two natural substrates, one suspension agent, one carbon source, one nitrogen source, and two inorganic salts were investigated for their impact on colony diameter. Utilizing the Plackett-Burman design, a 12-run experiment was conducted to evaluate nine factors, including two virtual variables. Each factor was set at two levels: −1 representing the low level and +1 representing the high level, with the high level being 1.5 times the magnitude of the low level (refer to Table I).

Table I
Range of factors investigated using Plackett-Burman design.

Symbol	Variables	Experimental value	
		Low (−1)	High (+1)
X_1	Glucose (g/l)	10	15
X_2	Wheat starch (g/l)	5	7.5
X_3	Virtual 1	−1	+1
X_4	Carrot powder (g/l)	5	7.5
X_5	Soybean powder (g/l)	10	15
X_6	Virtual 2	−1	+1
X_7	$ZnSO_4$ (g/l)	0.5	0.75
X_8	NH_4Cl (g/l)	0.5	0.75
X_9	Virtual 3	−1	+1

Steepest ascent path. Based on the results of the Plackett-Burman design, the steepest ascent method was applied to $ZnSO_4$ and NH_4Cl to determine the centroid of the response surface design. The main effects, significance, step size, and direction of change of the factors were analyzed through the regression equation of the Plackett-Burman design model.

Response surface optimization experiment. Based on the single-factor design, Plackett-Burman design, and steepest ascent path design, a response surface methodology (central composite design, CCD) encompassing 20 runs was employed. The variables tested (soybean powder, $ZnSO_4$, NH_4Cl) were designated as A, B, and C, respectively, and were evaluated at five distinct levels, incorporating factorial points (−1, +1), axial points (−1.6818, +1.6818), and a central point (0), as depicted in Table II.

Testing the temperature gradient. Building upon the outcomes of preceding experiments, the impact of varying temperatures on the biological characteristics of *P. dongsun* mycelium was investigated. Subsequently, medium plates were inoculated and incubated within a climate chamber at temperatures of $5 \pm 2°C$, $10 \pm 2°C$, $15 \pm 2°C$, $20 \pm 2°C$, $25 \pm 2°C$, $30 \pm 2°C$, and $35 \pm 2°C$, respectively.

Table II
Factors and levels of response surface central composite design.

Symbol	Variables	Code level				
		−1.6828	−1	0	1	1.6818
A	Soybean powder (g/l)	5.80	7.50	10	12.50	14.20
B	$ZnSO_4$ (g/l)	0.45	0.63	0.88	1.13	1.30
C	NH_4Cl (g/l)	0.47	0.57	0.72	0.86	0.98

Statistical Analysis. Each experiment was conducted with five replicates. Upon completion of the cultivation period, the colony diameter resulting from mycelial elongation was measured using the criss-cross method. For colonies exhibiting incomplete diameters, measurements were recorded at their widest points (Zhang et al. 2021). Furthermore, the mycelial growth rate was calculated employing the subsequent mathematical equation (1).

$$\text{Mycelial growth rate}\,\frac{(mm)}{d} = \\ = \frac{\text{Colony diameter}\,(mm) - \text{Inoculum block diameter}\,(mm)}{2 \times \text{culture time}\,(d)} \quad (1)$$

Colony diameter and mycelial growth rate were presented as mean values ± standard deviation (SD). An analysis of variance (ANOVA), followed by Tukey's post hoc test, was utilized to conduct multiple comparisons for significant differences at $p < 0.05$. The Design-Expert® software, version 13.0.1.0 (StatEase Inc., USA, www.statease.com), was employed to design experiments, regression, and graphical analysis of the data obtained. Bar and line graphs were generated using the OriginPro® 2021 software package, version 9.8.0.200 (OriginLab Corporation, USA).

Results

Effect of natural substrates on the biological properties. The effect of natural substrates on the biological properties was investigated. Adding wood powder to the medium resulted in the fastest mycelial growth and largest colony diameter (0.85 ± 0.13 mm/d), followed by wheat bran, wheat starch, and carrot powder. However, the differences among these substrates were not statistically significant. Upon adding carrot powder, mycelial growth was observed to be both dense and vigorous, in contrast to the other test groups, which exhibited either dense or moderately dense growth. Adding carrot powder, wood powder, and wheat bran to the medium facilitated the most vigorous mycelial growth. The poorest mycelial growth was observed with the addition of potato powder. Considering colony diameter, growth density, and overall mycelial development, the incorporation of wheat bran, wood powder, wheat starch, and carrot powder into the medium proved to be beneficial for the growth of *P. dongsun* mycelium (Table III, Fig. 1). The study aimed to investigate the effects of composite natural substrates on the biological characteristics of *P. dongsun* mycelium, with wheat starch, carrot powder, and wood powder being added individually or in combination to the culture media (Table IV). Results indicated that the FA4 treatment exhibited the fastest mycelial growth rate and the largest colony diameter (67.07 ± 4.79 mm). FA4 and FA6 treatments demonstrated the most vigorous mycelial growth, albeit with sparse colony density. The FA5 treatment resulted in the densest mycelial density and superior growth. In conclusion, the FA5 treatment was identified as the optimal natural substrate combination, comprising 10 g/l wheat starch and 10 g/l carrot powder (Table IV, Fig. 2).

Effect of suspension agent on the biological properties. When suspension agents were added to the medium, *P. dongsu* mycelium grew adherent to the surface of the medium with less aerial mycelium, resulting in faster mycelial growth, denser colonies, and vigorous growth. However, there were differences in the rate of mycelial growth and overall growth (Table V, Fig. 3). A medium suspension can be formed when sodium carboxymethyl cellulose, sodium alginate, gum arabic, and xanthan gum are added. However, only with the addition of xanthan gum do the medium become more viscous and the suspension stabilize. Adding

Table III
Effects of natural substances on the growth of *Phallum dongsun*.

Natural substance	Colony diameter (mm)	Mycelium growth rate (mm/d)	Colony density	Hyphal growth
Control group	29.64 ± 7.07^{de}	0.44 ± 0.13^{de}	#	++
Peeled potato	20.75 ± 0.79^{e}	0.28 ± 0.01^{e}	##	+
Carrot powder	42.17 ± 3.23^{bcd}	0.66 ± 0.06^{bcd}	###	+++
Wood powder	54.75 ± 5.1^{a}	0.88 ± 0.09^{a}	##	+++
Wheat bran	52.71 ± 7.22^{ab}	0.85 ± 0.13^{ab}	##	++++
Potato starch	37.63 ± 3.63^{bcd}	0.58 ± 0.06^{bcd}	#	+
Corn starch	37.58 ± 5.35^{bcd}	0.58 ± 0.10^{bcd}	#	+
Wheat starch	48.12 ± 9.48^{abc}	0.77 ± 0.17^{abc}	##	++

Different small letters in the same column indicate significance at the 5% level
– sparse colony density, ## – dense colony density, ### – densest colony density,
+ – weak mycelial growth, ++ – moderate mycelial growth, +++ – favorable mycelial growth,
++++ – strong mycelial growth

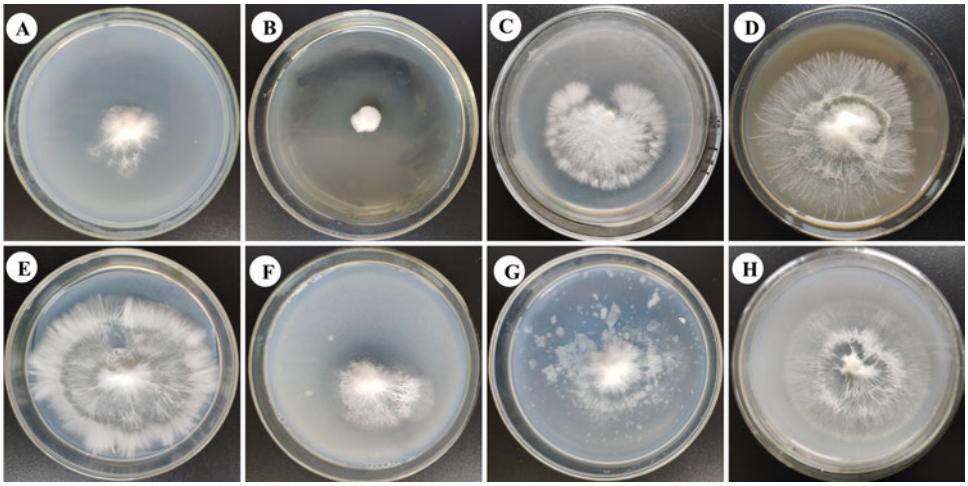


Fig. 1. Effects of natural substances on the growth of *Phallus dongsun*.
A – control group, B – peeled potato, C – carrot powder, D – wood powder, E – wheat bran, F – potato powder, G – corn starch, H – wheat starch

Table IV
Effects of composite natural substances on the growth of *Phallus dongsun*.

Test group	Natural substance (g/l)				Colony diameter (mm)	Mycelium growth rate (mm/d)	Colony density	Hyphal growth
	Peeled potato	Wheat starch	Carrot powder	Wood powder				
FA0	200	0	0	0	26.78 ± 4.76^{c}	0.38 ± 0.09^{c}	###	++
FA1	0	20	0	0	56.19 ± 6.44^{b}	0.91 ± 0.12^{b}	##	+++
FA2	0	0	20	0	51.57 ± 7.10^{b}	0.83 ± 0.13^{b}	##	+++
FA3	0	0	0	20	34.34 ± 4.57^{c}	0.52 ± 0.08^{c}	#	++
FA4	0	10	0	10	67.07 ± 4.79^{a}	1.10 ± 0.09^{a}	#	+++
FA5	0	10	10	0	52.35 ± 5.21^{b}	0.84 ± 0.09^{b}	###	++++
FA6	0	0	10	10	56.19 ± 8.14^{b}	0.91 ± 0.15^{b}	#	++++
FA7	0	10	10	10	50.66 ± 9.46^{b}	0.81 ± 0.17^{b}	##	+++

Different small letters in the same column indicate significance at the 5% level
– sparse colony density, ## – dense colony density, ### – densest colony density, + – weak mycelial growth, ++ – moderate mycelial growth, +++ – favorable mycelial growth, ++++ – strong mycelial growth

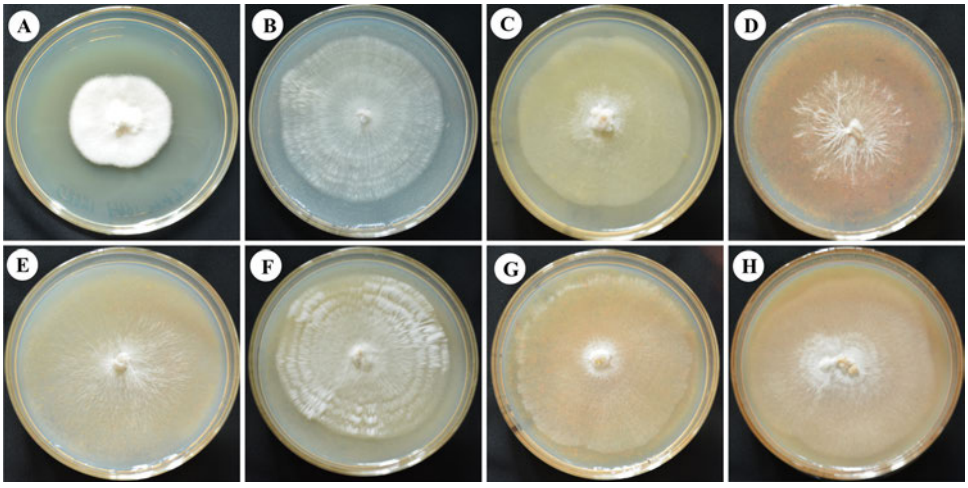


Fig. 2. Effects of composite natural substances on the growth of *Phallus dongsun*.
A – FA0, B – FA1, C – FA2, D – FA3, E – FA4, F – FA5, G – FA6, H – FA7

Table V
Effects of suspension agents on the physical state of the medium and the growth of *Phallus dongsun*.

Suspension agent	Medium physical state	Colony diameter (mm)	Mycelium growth rate (mm/d)	Colony density	Hyphal growth
Control group	Sediment	44.45 ± 6.1^{abc}	0.70 ± 0.11^{abc}	##	+++
Sodium carboxylmethyl cellulose (g/l)	Unstable suspension	45.05 ± 8.48^{ab}	0.72 ± 0.15^{ab}	##	++++
Sodium alginate (g/l)	Unstable suspension	44.10 ± 8.64^{ab}	0.70 ± 0.15^{ab}	###	++++
Dimethyl sulfoxide (ml/l)	Sediment	33.11 ± 8.35^{c}	0.50 ± 0.15^{c}	##	++++
Tween 80 (ml/l)	Sediment	38.95 ± 6.89^{bc}	0.61 ± 0.12^{bc}	#	++++
Gum arabic (g/l)	Unstable suspension	50.73 ± 5.4^{a}	0.82 ± 0.1^{a}	##	++++
Xanthan gum (g/l)	Stable suspension, high viscosity	48.03 ± 6.06^{ab}	0.77 ± 0.11^{ab}	###	++++

Different small letters in the same column indicate significance at the 5% level
– sparse colony density, ## – dense colony density, ### – densest colony density, + – weak mycelial growth, ++ – moderate mycelial growth, +++ – favorable mycelial growth, ++++ – strong mycelial growth

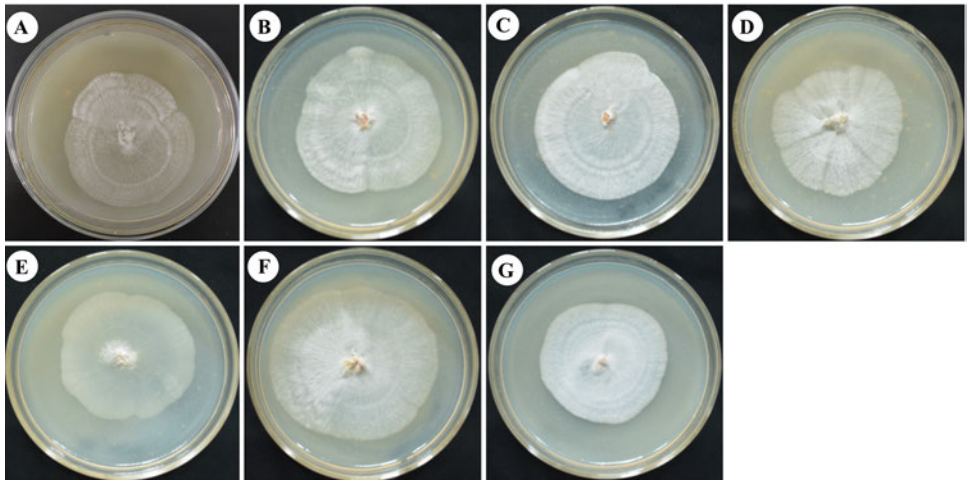


Fig. 3. Effects of suspension agents on the physical state of the medium and the growth of *Phallus dongsun*.
A – control group, B – sodium carboxylmethyl cellulose, C – sodium alginate, D – dimethyl sulfoxide, E – Tween 80, F – gum arabic, G – xanthan gum

dimethyl sulfoxide and Tween 80 to the medium resulted in no suspension formation and led to medium precipitation. The results indicated that xanthan gum is the best-suspending agent. A higher concentration of xanthan gum significantly affected the viscosity of the medium. Varying concentrations of xanthan gum had negligible effects on the growth of *P. dongsu* mycelium, which all exhibited average, vigorous growth and high colony density (Table VI, Fig. 4). However, considering the physical state of the medium, increased concentrations of xanthan gum made it more difficult to disperse and increased the viscosity, adversely affecting the medium's partitioning. In summary, an optimal concentration of 0.5 g/l xanthan gum was determined for the culture medium.

Effect of carbon source on the biological properties. When glucose, maltose, and cellulose were utilized as carbon sources in the culture, *P. dongsun* mycelium exhibited rapid growth, high colony density, and an optimal growth rate, notably with glucose, which demonstrated a mycelial growth rate of 0.77 ± 0.06 mm/d. When fructose, sucrose, lactose, and brown sugar served as carbon sources, the mycelium grew and maintained a high density; however, its performance was comparable to the control group, showing no significant difference. Utilizing chitosan as a carbon source resulted in suboptimal and slower mycelium growth, which was inferior to the control; conversely, when lignin served as the carbon source, the mycelium failed to germinate and grow (Table VII, Fig. 5). In conclusion, glucose was determined to be the optimal carbon source.

Effect of nitrogen source on the biological properties. Nitrogen source screening experiments demonstrated that the $NaNO_3$ exhibited the fastest mycelial growth rate (0.74 ± 0.09 mm/d), with vigorous growth, yet the colony density was notably sparse. In contrast, the soybean powder demonstrated a faster mycelial growth rate (0.69 ± 0.09 mm/d), experiencing vigorous growth and dense colonies; however, the difference

Table VI
Effects of xanthan gum concentration on the physical state of the medium and the growth of *Phallus dongsun*.

Xanthan gum (g/l)	Medium physical state	Colony diameter (mm)	Mycelium growth rate (mm/d)	Colony density	Hyphal growth
Control group	Sediment	60.41 ± 7.95^{a}	0.98 ± 0.14^{a}	##	+++
0.5	Stable suspension, Suitable viscosity	65.34 ± 6.52^{a}	1.08 ± 0.12^{a}	##	+++
1.0	Stable suspension, Suitable viscosity	66.04 ± 5.46^{a}	1.09 ± 0.10^{a}	##	+++
1.5	Stable suspension, Higher viscosity	62.63 ± 7.24^{a}	1.03 ± 0.13^{a}	##	+++
2.0	Stable suspension, Higher viscosity	61.06 ± 5.46^{a}	1.00 ± 0.10^{a}	##	+++
2.5	Stable suspension, high viscosity	56.23 ± 6.40^{a}	0.91 ± 0.11^{a}	##	+++
3.0	Stable suspension, high viscosity	60.68 ± 4.23^{a}	0.99 ± 0.08^{a}	#	+++

Small letter in the same column indicate significance at the 5% level
– sparse colony density, ## – dense colony density, ### – densest colony density, + – weak mycelial growth,
++ – moderate mycelial growth, +++ – favorable mycelial growth, ++++ – strong mycelial growth

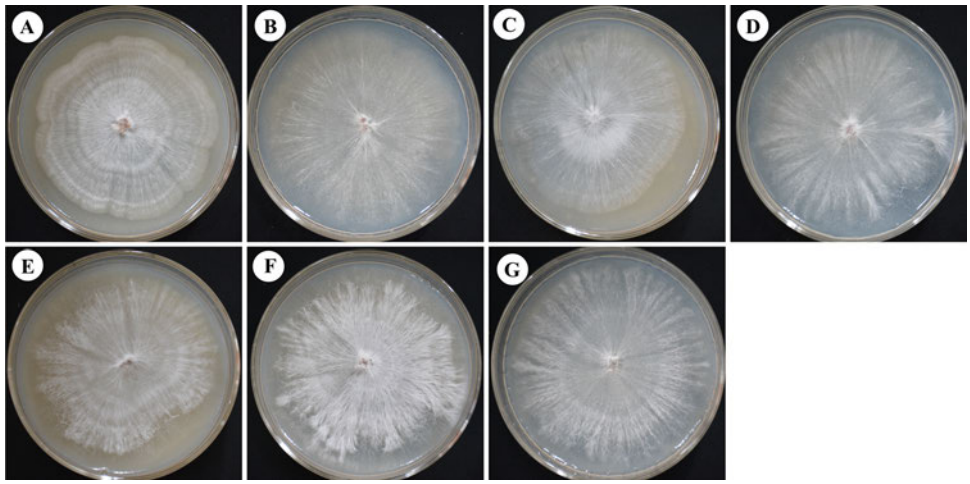


Fig. 4. Effects of xanthan gum concentration on the physical state of the medium and the growth of *Phallus dongsun*.
A – Control group, B – 0.5 g/l, C – 1.0 g/l, D – 1.5 g/l, E – 2.0 g/l, F – 2.5 g/l, G – 3.0 g/l

Table VII
Effects of carbon sources on the growth of *Phallus dongsun*.

Carbon source (g/l)	Colony diameter (mm)	Mycelium growth rate (mm/d)	Colony density	Hyphal growth
Control group	36.84 ± 1.29^{bcd}	0.56 ± 0.02^{bcd}	##	++
Glucose	48.32 ± 3.63^{a}	0.77 ± 0.06^{a}	###	+++
Fructose	37.92 ± 6.61^{bcd}	0.58 ± 0.12^{bcd}	##	++
Sucrose	32.43 ± 3.27^{d}	0.49 ± 0.06^{d}	##	+++
Maltose	48.18 ± 2.97^{ab}	0.77 ± 0.05^{ab}	###	+++
Lactose	37.13 ± 6.36^{bcd}	0.57 ± 0.11^{bcd}	###	++
Brown sugar	36.57 ± 4.02^{cd}	0.56 ± 0.07^{cd}	##	+++
Cellulose	47.89 ± 5.17^{abc}	0.76 ± 0.09^{abc}	##	+++
Xylogen	0.00 ± 0.00^{f}	0.00 ± 0.00^{f}	n.d.	n.d.
Chitosan	18.07 ± 6.54^{e}	0.23 ± 0.12^{e}	##	+

Different small letters in the same column indicate significance at the 5% level
– sparse colony density, ## – dense colony density, ### – densest colony density,
+ – weak mycelial growth, ++ – moderate mycelial growth,
+++ – favorable mycelial growth, ++++ – strong mycelial growth,
n.d. – no data for the experimental group

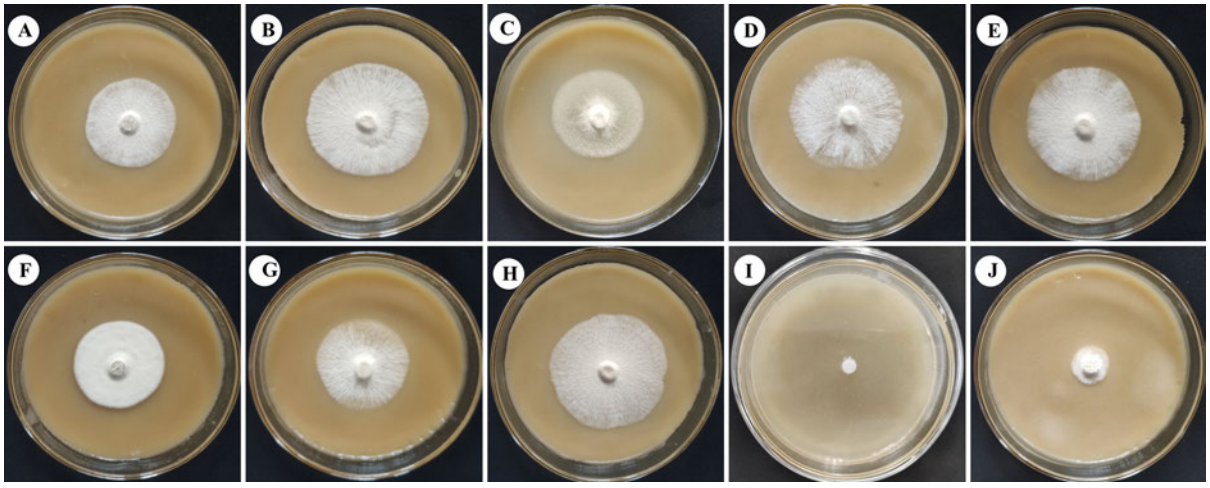


Fig. 5. Effects of carbon sources on the growth of *Phallus dongsun*.
A – control group, B – glucose, C – fructose, D – sucrose, E – maltose, F – lactose, G – brown sugar, H – cellulose, I – xylogen, J – chitosan

from the $NaNO_3$ was minimal. Adding peptone, yeast powder, yeast paste, and carbamide to the culture medium significantly inhibited the growth of *P. dongsun* mycelium, resulting in a slow growth rate and suboptimal growth (Table VIII, Fig. 6). In conclusion, soybean powder was determined to be the best nitrogen source.

Effect of inorganic salt on the biological properties. Inorganic salt screening experiments indicated that the $ZnSO_4$ exhibited the fastest mycelial growth rate (0.80 ± 0.16 mm/d), characterized by vigorous growth, and was followed by NH_4Cl (0.76 ± 0.13 mm/d) and $Fe_2(SO_4)_3$ (0.65 ± 0.18 mm/d). Incorporating $MgSO_4 \cdot 7H_2O$ and $CaCO_3$ into the culture medium significantly inhibited the growth of *P. dongsun* mycelium (Table IX, Fig. 7). To explore the effects of composite inorganic salts on the biological characteristics of *P. dongsun* mycelium, $ZnSO_4$, NH_4Cl, and $Fe_2(SO_4)_3$ were each added either individually or in combination to the culture media (Table X). The findings revealed that FM-F demonstrated the fastest mycelial growth

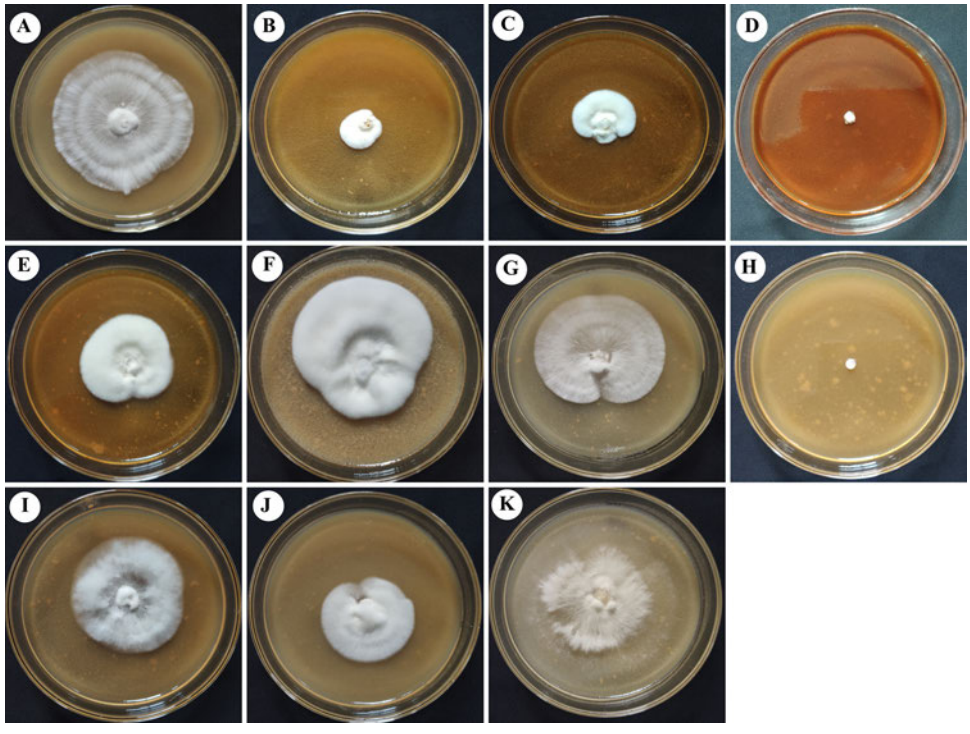


Fig. 6. Effects of nitrogen sources on the growth of *Phallus dongsun*.
A – control group, B – peptone, C – yeast powder, D – yeast extract, E – beef extract, F – soybean powder, G – KNO_3, G – carbamide, I – NH_4Cl, J – $(NH_4)_2SO_4$, K – $NaNO_3$

Table VIII
Effects of nitrogen sources on the growth of *Phallus dongsun*.

Nitrogen source (g/l)	Colony diameter (mm)	Mycelium growth rate (mm/d)	Colony density	Hyphal growth
Control group	36.01 ± 5.30^{c}	0.55 ± 0.09^{c}	##	+++
Peptone	19.16 ± 4.26^{cd}	0.25 ± 0.08^{cd}	##	+
Yeast powder	9.16 ± 2.35^{cd}	0.07 ± 0.04^{cd}	##	+
Yeast extract	5.72 ± 0.63^{d}	0.01 ± 0.01^{d}	#	+
Beef extract	39.86 ± 5.18^{ab}	0.62 ± 0.09^{ab}	##	++
Soybean powder	44.18 ± 5.17^{ab}	0.69 ± 0.09^{ab}	###	+++
KNO_3	40.62 ± 4.33^{ab}	0.63 ± 0.08^{ab}	##	+++
Carbamide	5.70 ± 0.16^{d}	0.01 ± 0.00^{d}	#	+
NH_4Cl	37.73 ± 5.98^{ab}	0.58 ± 0.11^{ab}	##	++
$(NH_4)_2SO_4$	35.66 ± 3.32^{b}	0.54 ± 0.06^{b}	##	++
$NaNO_3$	46.49 ± 5.31^{a}	0.74 ± 0.09^{a}	#	+++

Different small letters in the same column indicate significance at the 5% level
– sparse colony density, ## – dense colony density, ### – densest colony density,
+ – weak mycelial growth, ++ – moderate mycelial growth, +++ – favorable mycelial growth,
++++ – strong mycelial growth

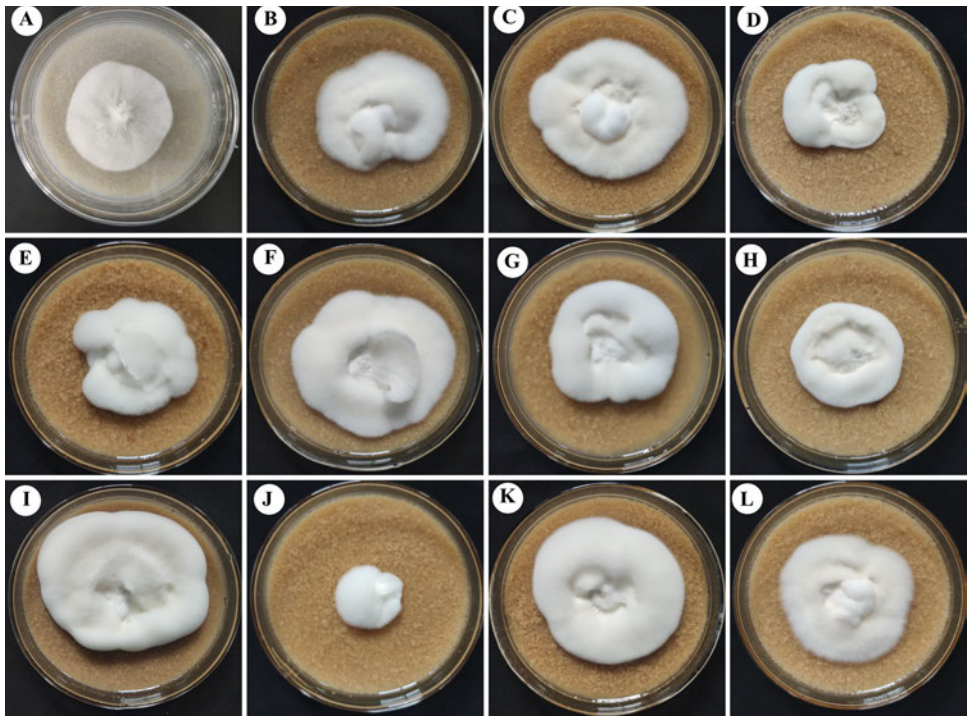


Fig. 7. Effects of inorganic salts on the growth of *Phallus dongsun*.
A – control group, B – KH_2PO_4, C – $K_2HPO_4 \cdot 3H_2O$, D – $MgSO_4 \cdot 7H_2O$, E – $MgCl_2 \cdot 6H_2O$, F – $ZnSO_4$, G – $CaCl_2$, H – $CaSO_4 \cdot 2H_2O$, I – $Fe_2(SO_4)_3$, J – $CaCO_3$, K – NH_4Cl, L – KNO_3

rate (1.00 ± 0.13 mm/d). In conclusion, FM-F emerged as the optimal inorganic salt combination, comprising $ZnSO_4$ 0.5 g/l and NH_4Cl 0.5 g/l (Table X, Fig. 8).

Screening of important variables using Plackett-Burman design. The data indicated that process optimization plays a crucial role in enhancing the efficiency of colony diameter (Table XI). Analysis of the regression coefficients for 9 factors (Table XII) revealed that X_1, X_7, and X_8 positively affected colony diameter. X_2, X_4, and X_5 demonstrated negative impacts. Variables affecting a confidence level above 95% are deemed significant factors. Based on these results (Table XII), two factors, X_7 ($ZnSO_4$) and X_8 (NH_4Cl), were identified as significant for the colony diameter of *P. dongsun* mycelium.

Steepest ascent path. Based on the coefficients from the colony diameter regression equation derived

Table IX
Effects of inorganic salts on the growth of *Phallus dongsun*.

Inorganic salt (g/l)	Colony diameter (mm)	Mycelium growth rate (mm/d)	Colony density	Hyphal growth
Control group	40.00 ± 7.53^{abc}	0.62 ± 0.13^{abc}	##	+++
KH_2PO_4	33.66 ± 8.64^{bcd}	0.51 ± 0.15^{bcd}	##	+++
$K_2HPO_4 \cdot 3H_2O$	36.14 ± 5.64^{bcd}	0.55 ± 0.10^{bcd}	###	++
$MgSO_4 \cdot 7H_2O$	29.51 ± 4.16^{cd}	0.43 ± 0.07^{cd}	##	++
$MgCl_2 \cdot 6H_2O$	39.37 ± 4.56^{abc}	0.61 ± 0.08^{abc}	##	++
$ZnSO_4$	50.14 ± 9.16^{a}	0.80 ± 0.16^{a}	###	++++
$CaCl_2$	37.11 ± 8.19^{abc}	0.57 ± 0.15^{abc}	###	+++
$CaSO_4 \cdot 2H_2O$	37.09 ± 4.72^{abcd}	0.57 ± 0.08^{abcd}	#	++
$Fe_2(SO_4)_3$	41.85 ± 9.97^{abc}	0.65 ± 0.18^{abc}	###	++++
$CaCO_3$	20.79 ± 2.32^{d}	0.28 ± 0.04^{d}	#	+
NH_4Cl	48.00 ± 7.14^{ab}	0.76 ± 0.13^{ab}	###	+++
KNO_3	40.40 ± 6.27^{abc}	0.63 ± 0.11^{abc}	###	++

Different small letters in the same column indicate significance at the 5% level
– sparse colony density, ## – dense colony density, ### – densest colony density,
+ – weak mycelial growth, ++ – moderate mycelial growth,
+++ – favorable mycelial growth, ++++ – strong mycelial growth

Table X
Effects of composite inorganic salt on the growth of *Phallus dongsun*.

Test group	Inorganic salt (g/l)			Colony diameter (mm)	Mycelium growth rate (mm/d)	Colony density	Hyphal growth
	$ZnSO_4$	$Fe_2(SO_4)_3$	NH_4Cl				
FM-A	0	0	0	42.23 ± 8.15^{b}	0.66 ± 0.15^{b}	##	++
FM-B	1	0	0	50.04 ± 4.62^{ab}	0.80 ± 0.08^{ab}	###	+++
FM-C	0	1	0	39.01 ± 2.45^{b}	0.61 ± 0.04^{b}	##	++
FM-D	0	0	1	39.07 ± 0.24^{ab}	0.61 ± 0.00^{ab}	##	++
FM-E	0.5	0.5	0	49.51 ± 6.03^{ab}	0.79 ± 0.11^{ab}	###	++
FM-F	0.5	0	0.5	61.10 ± 7.26^{a}	1.00 ± 0.13^{a}	###	++++
FM-G	0	0.5	0.5	50.05 ± 8.99^{ab}	0.80 ± 0.16^{ab}	###	++
FM-H	0.5	0.5	0.5	44.52 ± 5.92^{b}	0.71 ± 0.11^{b}	###	+++

Different small letters in the same column indicate significance at the 5% level
– sparse colony density, ## – dense colony density, ### – densest colony density, + – weak mycelial growth,
++ – moderate mycelial growth, +++ – favorable mycelial growth, ++++ – strong mycelial growth

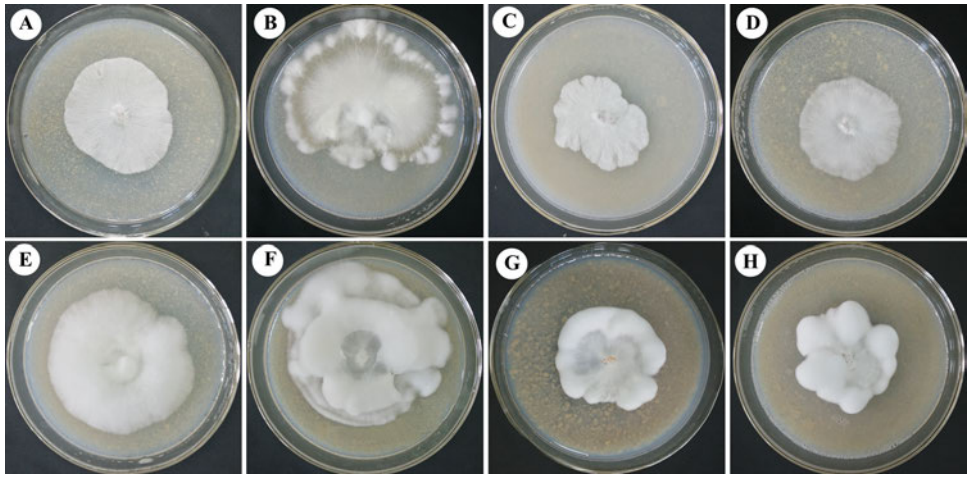


Fig. 8. Effects of composite inorganic salts on the growth of *Phallus dongsun*.
A – FM-A, B – FM-B, C – FM-C, D – FM-D, E – FM-E, F – FM-F, G – FM-G, H – FM-H

Table XI
Plackett-Burman design and response values.

Run	Variables								Y Colony diameter (mm)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	
1	1	1	−1	1	1	1	−1	−1	49.92 ± 3.47
2	−1	1	1	−1	1	1	1	−1	59.97 ± 6.54
3	1	−1	1	1	−1	1	1	1	74.45 ± 3.25
4	−1	1	−1	1	1	−1	1	1	60.35 ± 2.28
5	−1	−1	1	−1	1	1	−1	1	59.05 ± 4.68
6	−1	−1	−1	1	−1	1	1	−1	61.95 ± 5.93
7	1	−1	−1	−1	1	−1	1	1	63.52 ± 4.59
8	1	1	−1	−1	−1	1	−1	1	58.35 ± 4.82
9	1	1	1	−1	−1	−1	1	−1	62.39 ± 4.49
10	−1	1	1	1	−1	−1	−1	1	53.93 ± 6.15
11	1	−1	1	1	1	−1	−1	−1	50.62 ± 4.74
12	−1	−1	−1	−1	−1	−1	−1	−1	48.70 ± 2.69

X_1–X_8 code values are shown in Table I

Table XII
Results of the regression analysis of the Plackett–Burman design.

Source	Coefficient estimate	df	Sum of squares	Means quares	*F*–value	*p*–value (Prob > *F*)
Model	58.60	6	486.42	81.07	5.14	0.0464*
X_1	1.28	1	19.53	19.53	1.24	0.3162
X_2	−1.11	1	14.90	14.90	0.9453	0.3756
X_4	−0.0640	1	0.0491	0.0491	0.0031	0.9576
X_5	−1.36	1	22.24	22.24	1.41	0.2882
X_7	5.17	1	321.04	321.04	20.37	0.0063**
X_8	3.01	1	108.66	108.66	6.90	0.0468*
Residual		5	78.79			
Cor Total		11	565.20			

* – significant at 0.05 level, ** – significant at 0.01 level

from the Plackett-Burman design model, the steepest ascent path for optimizing colony diameter involved primarily adjusting the levels of $ZnSO_4$ and NH_4Cl. The magnitude and direction of changes were dictated by the regression equation from the Plackett-Burman design model, as illustrated in Table XIII. Colony diameter reached its peak at the "$O+3\Delta$" factor level before decreasing. This "$O+3\Delta$" factor level, marking the maximum response, was subsequently chosen as the central point for the central composite design.

Response surface optimization experiment. To assess the impact of medium components on colony diameter, an evaluation involving soybean powder, $ZnSO_4$, and NH_4Cl was conducted using a response surface optimization experiment. Detailed experimental designs and results are presented in Table XIV. A regression analysis was conducted to align the response function with the experimental data. Based on the variables presented in Table XV, the model, denoted as equation (2), articulates the colony diameter (Y) in relation to the concentrations of soybean powder (A), $ZnSO_4$ (B), and NH_4Cl (C):

$$Y = 62.04 - 3.24 \times A - 1.15 \times B - 0.23 \times C + 0.16 \times AB + 1.14 \times \times AC - 0.81 \times BC - 1.40 \times A^2 - 0.32 \times B^2 - 0.96 \times C^2 \quad (2)$$

The F-test analysis of variance (ANOVA) results demonstrated that the regression achieved statistical significance at the 99% confidence level (Table XV), with a high goodness of fit value ($R^2 = 0.9242$), signifying the model's accurate representation of the relationship between the investigated factors and response values. The "F-value" for "Lack of Fit" was found to be insignificant, indicating the model's retention of fit. Furthermore, the linear terms of A and B, the interaction term of AC, and the quadratic terms of A^2 and C^2 were identified as significant model terms for colony diam-

Table XIII
Experimental design and results of the steepest ascent path.

Factor level	Inorganic salt (g/l)		Colony diameter (mm)	Mycelium growth rate (mm/d)	Colony density	Hyphal growth
	$ZnSO_4$	NH_4Cl				
Step size (Δ)	0.125	0.073	n.d.	n.d.	n.d.	n.d.
Origin (O)	0.5	0.5	48.13 ± 3.00	0.77 ± 0.05	###	+++
$O+1\Delta$	0.625	0.573	52.99 ± 5.85	0.85 ± 0.10	###	+++
$O+2\Delta$	0.750	0.646	54.20 ± 7.01	0.87 ± 0.13	###	+++
$O+3\Delta$	0.875	0.719	61.07 ± 2.20	1.00 ± 0.04	###	++++
$O+4\Delta$	1.000	0.792	60.43 ± 10.18	0.99 ± 0.18	###	++++
$O+5\Delta$	1.125	0.865	59.56 ± 4.15	0.88 ± 0.30	###	++++
$O+6\Delta$	1.250	0.938	48.82 ± 4.68	078 ± 0.08	###	++++
$O+7\Delta$	1.375	1.011	51.52 ± 9.49	0.83 ± 0.17	###	+++
$O+8\Delta$	1.500	1.084	32.24 ± 6.24	0.48 ± 0.11	###	++
$O+9\Delta$	1.625	1.157	35.39 ± 12.32	0.54 ± 0.22	###	+++
$O+10\Delta$	1.75	1.230	29.47 ± 15.78	0.43 ± 0.28	##	+
$O+11\Delta$	1.875	1.303	26.49 ± 8.02	0.38 ± 0.14	##	+
$O+12\Delta$	2.00	1.376	23.38 ± 7.40	0.32 ± 0.13	##	+

– sparse colony density, ## – dense colony density, ### – densest colony density, + – weak mycelial growth, ++ – moderate mycelial growth, +++ – favorable mycelial growth, ++++ – strong mycelial growth, n.d. – no data for the experimental group

Table XIV
Response surface central composite design and matching results.

Run	Variables			Y Colony diameter (mm)
	A	B	C	
1	−1	−1	−1	64.56 ± 5.88
2	1	−1	−1	54.69 ± 4.98
3	−1	1	−1	62.58 ± 7.79
4	1	1	−1	54.35 ± 7.41
5	−1	−1	1	63.78 ± 5.37
6	1	−1	1	59.50 ± 4.29
7	−1	1	1	59.59 ± 9.21
8	1	1	1	54.91 ± 3.34
9	−1.6818	0	0	63.33 ± 3.93
10	1.6818	0	0	53.13 ± 4.86
11	0	−1.6818	0	62.65 ± 4.13
12	0	1.6818	0	59.95 ± 4.14
13	0	0	−1.6818	60.88 ± 1.57
14	0	0	1.6818	58.10 ± 4.80
15	0	0	0	62.62 ± 2.36
16	0	0	0	61.43 ± 1.60
17	0	0	0	61.72 ± 7.81
18	0	0	0	64.65 ± 4.31
19	0	0	0	59.99 ± 2.18
20	0	0	0	61.76 ± 1.83

A, B and C code values are shown in Table II

eter. The findings reveal that the maximum values for colony diameter are attained through the regression equation and two-dimensional contour plots (Fig. 9). The model forecasted a maximum colony diameter of 64.75 mm at the optimized medium component levels of soybean powder 7.50 g/l, $ZnSO_4$ 0.71 g/l, and NH_4Cl

Table XV
ANOVA for response surface quadratic polynomial model.

Source	df	Sum of squares	Means quares	*F*-value	*p*-value (Prob > *F*)
Modle	215.88	9	23.99	13.54	0.0002**
A – Soybean powder	143.14	1	143.14	80.81	< 0.0001**
B – $ZnSO_4$	17.91	1	17.91	10.11	0.0098**
C – NH_4Cl	0.6925	1	0.6925	0.3910	0.5458
AB	0.1922	1	0.1922	0.1085	0.7487
AC	10.44	1	10.44	5.89	0.0356*
BC	5.22	1	5.22	2.94	0.1169
A^2	28.37	1	28.37	16.02	0.0025**
B^2	1.46	1	1.46	0.8214	0.3861
C^2	13.22	1	13.22	7.46	0.0211*
Residual	17.71	10	1.77		
Lack of Fit	5.81	5	1.16	0.4882	0.7750
Pure Error	11.90	5	2.38		
Cor Total	233.60	19			
R^2	0.9242				
Adjusted R^2	0.8559				

* – significant at 0.05 level, ** – significant at 0.01 level

0.68 g/l. Validation testing demonstrated the rapid growth of *P. dongsun* mycelium, with the colony diameter and mycelial growth rate achieving 63.00 ± 3.89 mm and 1.04 ± 0.14 mm/d at 28 days, respectively. These outcomes were closely aligned with the model's predicted values and can facilitate the evaluation of medium components and mycelial growth in *P. dongsun*.

The impact of various temperatures on the biological characteristics. The impact of various temperatures on the biological characteristics of *P. dongsun* mycelium was significant, as illustrated in Fig. 10 and 11. The findings indicated that under culture conditions at 20 and 25°C, the colonies were dense, vigorous, and robust, significantly surpassing those of other treatments. Mycelium growth was slow and weak under culture conditions at 10, 15, and 30°C. Furthermore, at 35°C, the mycelium failed to germinate, and the mycelium on the inoculum block subsided, indicating death. In conclusion, the optimal temperature range for the growth of *P. dongsun* mycelium is 20–25°C, with growth ceasing below 5°C and death occurring above 35°C.

Discussion

This investigation focused on how medium nutrients, the physical state of the medium, and temperature gradients influence the biological characteristics of *P. dongsun* mycelium. In the carbon source screening phase, adding natural substrates (wheat starch and carrot powder) and a single carbon source (glucose) to the culture medium resulted in the dense, thick, and rapid growth of *P. dongsun* mycelium. This suggests that *P. dongsun* can utilize monosaccharides, disaccharides, and polysaccharides derived from natural ingredients. This outcome slightly diverges from prior research, which identified brown sugar as the optimal carbon source due to its content of vitamins, amino acids, trace elements, and other nutrients conducive to mycelial growth (Zhang et al. 2021). The study concluded that *P. dongsun* mycelium extensively utilizes various carbon sources. Incorporating wheat starch, carrot powder, and glucose into the medium, which collectively encompasses mono-, di-, and polysaccharide forms, ensures a continuous and efficient carbon supply, significantly enhancing the growth of *P. dongsun* mycelium.

According to previous studies, organic nitrogen sources, particularly yeast paste, and peptone, foster denser and more rapidly growing mycelium than inorganic nitrogen sources (Zhang et al. 2021; Huang et al. 2022). This research found $NaNO_3$ to accelerate mycelial growth, while soya flour contributed to denser and thicker mycelium. Conversely, peptone, yeast powder, yeast paste, and urea were found to inhibit mycelial growth. Inorganic salt screening indicated that $ZnSO_4$, $Fe_2(SO_4)_3$, and NH_4Cl enhanced *P. dongsun* mycelial growth, aligning with previous findings (Zhang et al. 2021), whereas magnesium sulfate was inhibitory. This nuanced understanding of how different nitrogen sources and inorganic salts affect mycelial growth can guide future cultivation strategies for *P. dongsun.* It was also observed that $NaNO_3$, KNO_3, NH_4Cl, $(NH_4)_2SO_4$,

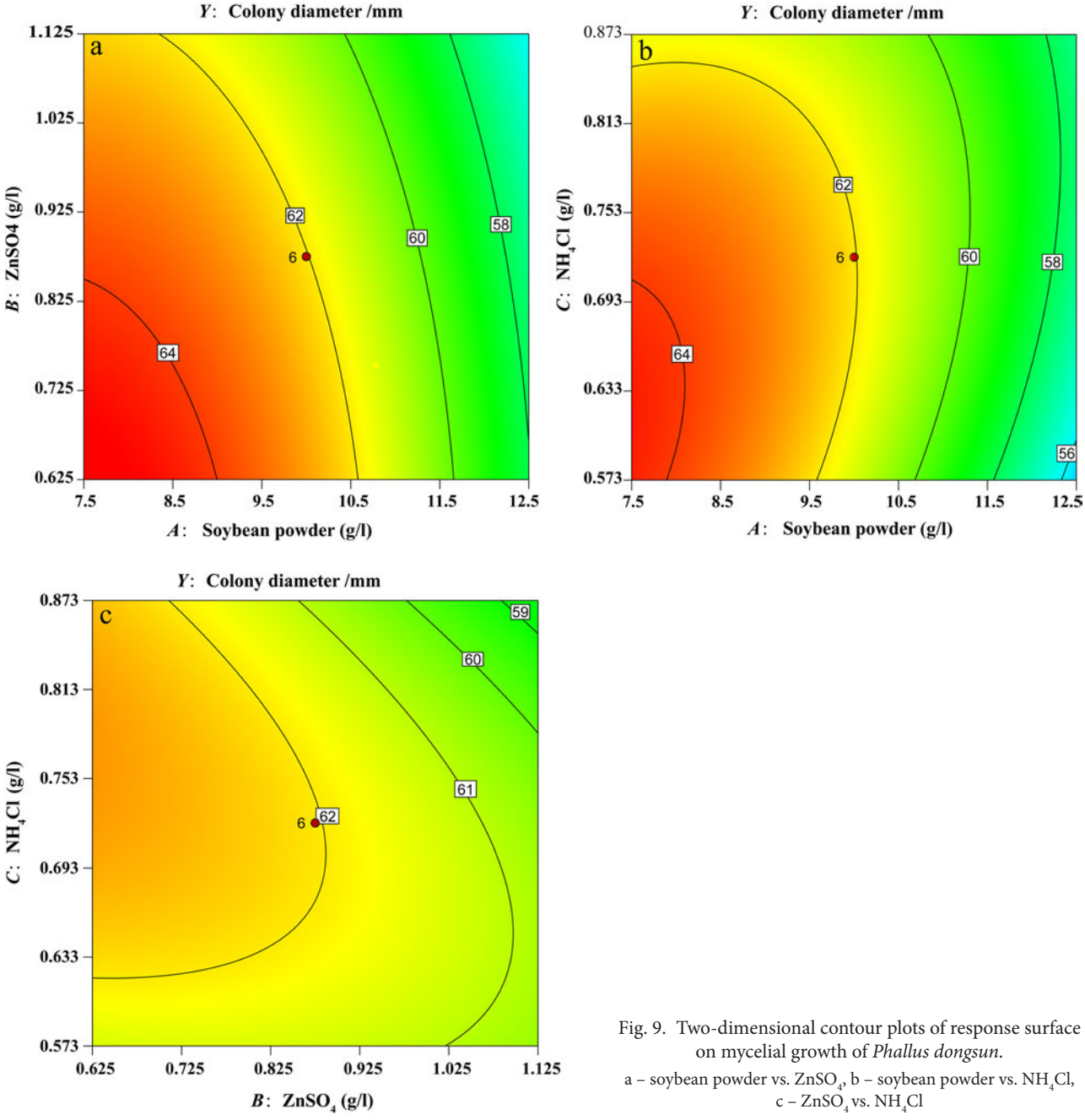


Fig. 9. Two-dimensional contour plots of response surface on mycelial growth of *Phallus dongsun*.
a – soybean powder vs. $ZnSO_4$, b – soybean powder vs. NH_4Cl, c – $ZnSO_4$ vs. NH_4Cl

$ZnSO_4$, and $Fe_2(SO_4)_3$ enhanced mycelial growth, with the compositions containing NO_3^-, NH_4^+, and SO_4^{2-} either individually or in combination. Nitrogen is a critical factor in plant growth and development, with NO_3^- and NH_4^+ serving as plants' primary sources of inorganic nitrogen. NH_4^+ can be directly utilized in nitrogen assimilation following its uptake by plants. NO_3^- must undergo an energy-intensive reduction process to be assimilated by plant cells (Xiao et al. 2023). Elemental sulfur, a vital component of cells, exists in diverse forms within living organisms and the environment, playing crucial roles in signaling, redox balance, gene expression promotion, and the maintenance of fundamental metabolic processes (Wasmund et al. 2017). Microorganisms play pivotal roles in all significant sulfur metabolic pathways and are key drivers of the sulfur biocycle (Wang et al. 2022). Consequently, NO_3^-, NH_4^+, and SO_4^{2-} are involved in nitrogen and sulfur metabolism during *P. dongsun* growth and their underlying biological mechanisms warrant further investigation

Upon incorporating sodium carboxymethyl cellulose and sodium alginate into the liquid medium, *Morchella sextelata* mycelium balls exhibited homogeneity and high biomass (Zheng et al. 2020). This study revealed that adding carrot, wheat starch, and soya flour to the medium led to precipitation and nutrient imbalance. Including xanthan gum in the medium resulted in a low concentration, stable suspension, reduced propensity for precipitation, and favorable mycelium growth. Suspension aids, or thickeners, can significantly

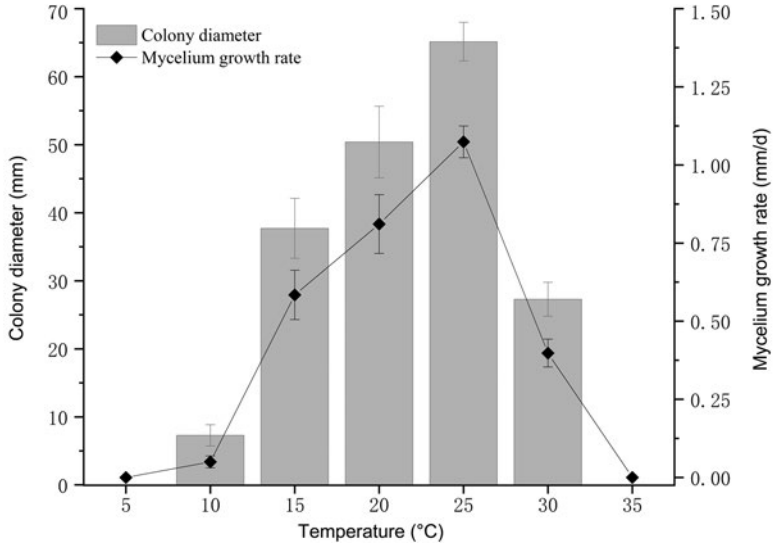


Fig. 10. Effects of different temperature on colony diameter and mycelial growth rate of *Phallus dongsun*.

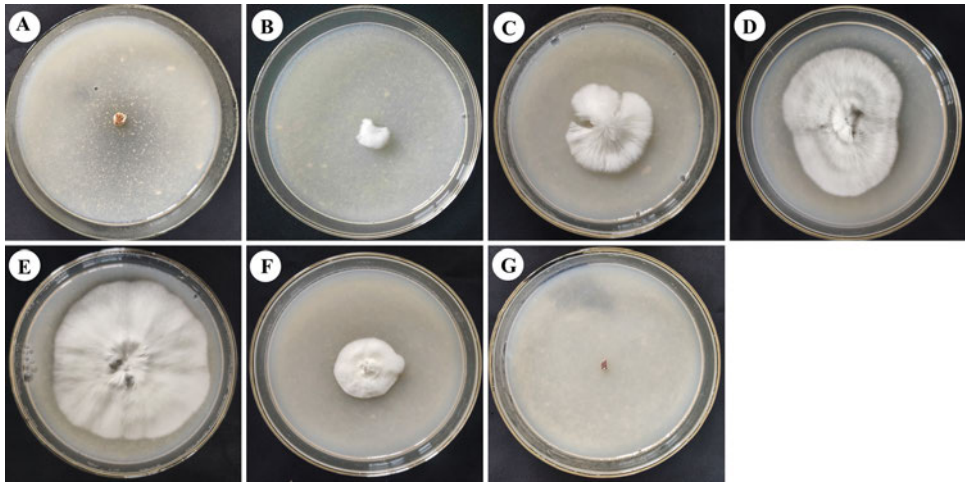


Fig. 11. Effects of different temperature on mycelial growth of *Phallus dongsun*.
A – 5°C, B – 10°C, C – 15°C, D – 20°C, E – 25°C, F – 30°C, G – 35°C

alter the viscosity of liquids and also modify the morphology of fungal hyphae (Dahlstrom et al. 2000).

In the one-factor temperature test, mycelial growth rate and momentum initially increased and decreased as the temperature rose. At the optimal temperature of 25°C, the mycelium exhibited its fastest growth; at temperatures below 5°C, growth ceased, and at temperatures above 35°C, the mycelium perished, aligning with the findings of Luo and Chen (2016). The study demonstrated that temperature stress altered the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in macrofungal mycelium. This imbalance in intracellular reactive oxygen species (ROS) metabolism and subsequent accumulation of free radicals inhibited mycelial growth, as Liu et al. (2010) observed.

This study evaluated the composition, physical state, and temperature gradient of the *P. dongsun* medium using single-factor experiments, Plackett-Burman design, and response surface methodology. The results indicated that an optimized medium composition comprising 5 g/l wheat starch, 5 g/l carrot powder, 7.50 g/l soybean powder, 10 g/l glucose, 0.71 g/l $ZnSO_4$, 0.68 g/l NH_4Cl, 0.5 g/l xanthan gum, 20 g/l agar, and an incubation temperature of 25°C resulted in vigorous, uniform, thick, and dense growth of *P. dongsun* mycelium, with a growth rate of 1.04 ± 0.14 mm/d. These findings offer a theoretical foundation for the conservation, selection, and breeding of *P. dongsun* strains, and scientific support for its large-scale and industrial production.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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