In vitro antifungal activity of lasiodiplodin, isolated from endophytic fungus Lasiodiplodia pseudotheobromae J-10 associated with Sarcandra glabra and optimization of culture conditions for lasiodiplodin production

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Research Article

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Abstract

A macrolide antibiotic, lasiodiplodin was isolated from the endophytic fungus (EF) *Lasiodiplodia pseudotheobromae* J-10 associated with the medicinal plant *Sarcandra glabra*. *In vitro* antifungal assay demonstrated the inhibitory activity of lasiodiplodin against the growth of eight phytopathogenic fungi, with the IC_{50} values ranging between 15.50–249.10 µg/mL. The highest antifungal activities were recorded against *Exserohilum turcicum*, *Colletotrichum capsici*, and *Pestalotiopsis theae*, with IC_{50} values of 15.50, 15.90, and 17.55 µg/mL, respectively. The underlying mechanism of the antifungal activity of lasiodiplodin against *E. turcicum* included the alteration of its colony morphology and disturbance of its cell membrane integrity. In addition, the optimization of *L. pseudotheobromae* J-10 culture conditions increased lasiodiplodin yield to 52.33 mg/L from 0.59 mg/L at pre-optimization. This is the first report on the isolation and identification of antifungal compound from the EF *L. pseudotheobromae* J-10 associated with *S. glabra*, as well as on the optimization of *L. pseudotheobromae* J-10 culture conditions to increase lasiodiplodin yield. The results of this study support that lasiodiplodin is a natural compound with high potential bioactivity against phytopathogens, and provide a basis for further study of the EF associated with *S. glabra*.

1. Introduction

Endophytic fungi (EF) reside in the intercellular or intracellular spaces of the plants without causing any negative effects on the host and are known to play an important role in promoting plant health (Chutulo et al. 2018). Furthermore, EF are recognized as natural biocontrol agents to suppress plant pathogens (Aly et al. 2010; Zhang et al. 2010; Que et al. 2022; Zheng et al. 2021). Currently, studies on the isolation of agriculturally-important bioactive metabolites from EF (especially those associated with medicinal plants) are gaining popularity.

Lasiodiplodia pseudotheobromae, belonging to Botryosphaeriaceae family, is a known pathogenic fungus that induces canker, fruit rot, and dieback in a variety of host plants (Ismail et al. 2012; Nogueira Júnior et al. 2017; Hernandez-Ceja et al. 2021; Zhang et al. 2022). Additionally, it exists as an EF in some plants, producing metabolites with antimicrobial (Wei et al. 2014; Jalil and Ibrahim 2021), herbicidal (Adetunji and Oloke 2013; Adetunji et al. 2017; Adetunji et al. 2018; Adetunji et al. 2020a), enzyme inhibitory (Kumar et al. 2019), and hydrolytic activities (Meshram and Saxena 2016). However, studies on the antifungal effects of its metabolites against phytopathogenic fungi are scarce. Xia ng et al. (2016) found that the culture filtrate of the EF *L. pseudotheobromae* LPS-1 associated with the medicinal plant *Ilex cornuta* controlled *Blumeria graminis* infection on wheat more effectively than the broad-spectrum fungicide triadimefon (10 mg/ml). Furthermore, Que et al. (2022) isolated and identified three antagonistic compounds: indole-3-carbaldehyde (A2-5-6-1), indole-3-carboxylic acid (3-ICA; A2-5-6-2), and jasmonic acid (JA; A2-5-6-3) from the *L. pseudotheobromae* LPS-1 culture filtrate. These studies demonstrate that *L. Pseudotheobromae* species are an underutilized resource with the potential to control agricultural diseases.

In our previous study, the *L. Pseudotheobromae* J-10 strain, isolated from the medicinal plant *Sarcandra glabra*, exhibited a broad-spectrum inhibitory activity against a range of plant pathogenic fungi. Thus, in this study, we focused on isolating and identifying the active component in the *L. Pseudotheobromae* J-10 crude extract and on optimizing the culture conditions to improve the yield and provide a basis for the application of *L. Pseudotheobromae* J-10 metabolites in plant disease control.

2. Materials And Methods

2.1. Fungal materials

The EF *L. pseudotheobromae* J-10 was isolated from a healthy *S. glabra* stem, which was collected in May 2021 from the Yao Autonomous County, Hezhou City, Guangxi Province of China. Plant pathogenic fungi *Exserohilum turcicum*, *Colletotrichum capsici*, *Pestalotiopsis theae*, *Alternaria oleracea*, and *Ceratocystis paradoxa* were provided by the Laboratory of Phytopathology, College of Agriculture, Guangxi University; *Alternaria citri* was provided by Guangxi Academy of Specialty Crop; and *Fusarium solani* and *Athelia rolfsii* were provided by Key Laboratory of Ecology of Rare and Endangered Species and Environmental Protection, Guangxi Normal University.

2.2. Fermentation culture of the EF *L. pseudotheobromae* J-10 and preparation of its crude extract

The strain J-10 was grown in potato dextrose broth (PDB; supplemented with 200 g/L potato extract and 20 g/L dextrose; Xilong Science Co., Ltd, Sichuan, China). Four agar plugs (0.4 × 0.4 cm) were respectively inoculated into each 1000 mL Erlenmeyer flask containing 400 mL PDB medium. Fermentation was carried out for 45 d at 28 ℃ under stilling culture conditions. The mycelium of the fermented culture was extracted thrice with methanol (Xilong Science Co., Ltd) at 28 ℃, and the extracts were combined and evaporated to dryness under reduced pressure to acquire the crude extract (CE). The CE was further extracted using ethyl acetate (Xilong Science Co., Ltd) and acetone (Xilong Science Co., Ltd), respectively, to obtain ethyl acetate extract, acetone extract, and methanol residue.

2.3. Isolation and purification of the active compound

Based on antifungal activity-directed isolation, the acetone extract was subjected to silica gel open column chromatography and eluted with a linear gradient of petroleum ether/ethyl acetate (2/1 → 1/1 → 1/2 → 1/5 → 0/1) and ethyl acetate/methanol (10/1 → 5/1 → 1/1 → 3/7). Eight fractions (F_1–F_8) were obtained by combining similar fractions using thin layer chromatography (TLC) detection and analysis. A white needle-like crystal was obtained after evaporation of the solvent in the bioactive fraction (F_2), which was then recrystallized to obtain a pure compound (J-10-1A).

2.4. Determination of antifungal activity

The antifungal activity of J-10-1A was determined using a previously described method (Luo et al., 2017; 2019). J-10-1A was dissolved in acetone: H_2O (1:1, v/v) to obtain the desired concentration of 15.50

μg/mL (IC_{50} value of lasiodiplodin against *E. turcicum*). The solution was evenly mixed with molten PDA medium (1:9, v/v) and poured into a Petri dish. The pathogenic fungi were inoculated on the agar medium (diameter 0.4 cm) and incubated at 28 ℃ for 72 h. The diameters of the fungal colonies were measured and the inhibitory rates were calculated. IC_{50} values at 95% confidence intervals (95% CI) were calculated by using the least-squares method.

2.5. Morphological characterization of *Exserohilum turcicum*

The fungal pathogen, *E. turcicum* was cultured on the PDA plates containing lasiodiplodin (test) or acetone: H_2O solution (1:1, v/v) (control) and incubated for 72 h at 28 ℃. Thereafter, the mycelium morphology was observed by macroscopic and microscopic methods. A DM3000 microscope (Leica Microsystems Instrument Incorporated Company, Germany) was used for the microscopic observations.

2.6. Determination of the cell membrane permeability

Cell membrane permeability of *E. turcicum* was determined according to a previously described method with slight modifications (Sun et al. 2017; Luo et al. 2019). The mycelia (1 g) were incubated in a rotary shaker at 120 rpm and 28 ℃ for 7 d and suspended in 10 mL of lasiodiplodin solution at 15.50 μg/mL (the IC_{50} of lasiodiplodin against *E. turcicum*). Acetone: H_2O (1:1, v/v) solution was used as the control. Thereafter, the electrical conductivity (J_0) of each sample was measured. Subsequently, the samples were incubated at 28 ℃ and 120 rpm for 30, 60, 90, 120, 180, and 360 min, and the electrical conductivities (J_1) of the supernatants were measured for each time point. Lastly, the 360 min culture samples were boiled and the final electrical conductivity (J_2) was measured. Each treatment was replicated thrice. The relative permeability of the cell membranes was calculated using the following equation:

$$Relativepermeability = \frac{J_1\ J_0}{J_2\ J_0} \times 100 \text{ Eq. (1)}$$

where J_0 is the initial electrical conductivity, J_1 is the electrical conductivity of lasiodiplodin-treated samples at each time point, and J_2 is the final electrical conductivity of the 360 min lasiodiplodin-treated sample after being boiled.

2.7. Orthogonal designed experiment

On the basis of a previous orthogonal designed experiment using five factors, four levels were adopted to optimize the culture conditions to improve the yield of lasiodiplodin in *L. pseudotheobromae* J-10. L_{16} (4^5) orthogonal table design was adopted for each factor level designed, and the settings are shown in Table 1.

Table 1

L_{16} (4^5) orthogonal test factors and levels

Factor	Symbol code	Level			
		1	2	3	4
Initial pH	A	5	6	7	8
Temperature (°C)	B	25	28	31	34
Liquid volume[a] (mL)	C	150	200	250	300
Carbon:nitrogen ratio (C:N, g:g)[b]	D	1/0	2/1	1/1	1/2
Culture duration (d)	E	5	10	15	20

Note: a: each flask was 500 mL and b: sucrose and yeast extract were supplemented to adjust C: N ratio (based on a previous orthogonal designed experiment).

2.8. Analytical procedures

An equal amount of methanol was added into the fermentation flask (containing broth and mycelia) standing for 24 h, and the solid and liquid components were separated by filtration. The solid component was extracted twice using methanol and filtered. Subsequently, all the filtrates were combined, evaporated to dryness, and then dissolved in 1 mL of methanol. The lasiodiplodin content in the solution was analyzed by high performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan). A reversed-phase Inertsil ODS-HL C_{18} column (250 mm × 4.6 mm, 5 µm, Shimadzu, Inc., Kyoto, Japan) was used for separation by using methanol: H_2O (60:40, v/v) as a mobile phase at 1 mL/min flow rate. The temperature was maintained at 40°C, and the UV detection wavelength was set at 200 nm. The sample injection volume was 5 µL. The LC-solution multi-PDA workstation was employed to acquire and process chromatographic data. Lasiodiplodin was detected and quantified using the HPLC standards. The linear equation of lasiodiplodin analysis was as follows:

$$Y = 2.84998 \times 10^6 X + 5554.6 \ (R^2=0.9965) \quad \text{Eq. (2)}$$

where Y was the peak area, X was the quality (µg) of the sample injected each time, and R was the correlation coefficient.

2.9. Statistical analysis

All the tests were replicated thrice, and the results were represented as mean ± standard deviation (SD). Analysis of variance (one-way ANOVA; PROC ANOVA; SAS version 8.2) was used to detect significant differences in the data. p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. The active compound from the *L. pseudotheobromae* J-10 crude extract

An antifungal compound, J-10-1A was isolated from the EF *L. pseudotheobromae* J-10 by using bioassay-guided fractionation. The purified compound was a white needle-like crystal, which was soluble in methanol and acetone. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.67 (s, 1H), 6.25 (d, J = 2.1 Hz, 1H), 6.20 (d, J = 2.1 Hz, 1H), 5.04 (m, 1H), 3.67 (s, 3H), 2.48 (m, 2H), 2.39 (m, 1H), 1.82 (m, 1H), 1.66–1.51 (m, 4H), 1.43–1.29 (m, 4H), 1.21 (d, J = 6.8 Hz, 3H), and 1.18–1.09 (m, 2H). ^{13}C NMR (100 MHz, acetone-d_6) δ 167.83, 159.07, 157.98, 142.31, 117.17, 107.92, 96.83, 71.07, 55.20, 32.32, 30.05, 29.69, 26.56, 24.98, 24.40, 20.78, and 18.95. ESI: m/z $[M-H]^-$ = 291.1599. Comparative analysis of the spectroscopic data with those in the literature, identified compound J-10-1A as lasiodiplodin (Yang et al. 2007; Wang et al. 2009), a macrolide antibiotic (Yang et al. 2006a). Its chemical structure is given in Fig. 1.

3.2. Lasiodiplodin toxicity towards phytopathogenic fungi

The results of lasiodiplodin toxicity analysis towards eight plant pathogenic fungi are shown in Table 2. Lasiodiplodin showed varying degrees of inhibition against the tested phytopathogens with the IC_{50} values ranging between 15.50–249.10 µg/mL. It showed the most effective toxicity against *E. turcicum*, *C. capsici*, and *P. theae*, with IC_{50} values of 15.50, 15.90, and 17.55 µg/mL, respectively (< 20 µg/mL), followed by *C. paradoxa*, *A. citri*, *A. rolfsii*, and *A. oleracea*, with the IC_{50} values of 27.45, 40.35, 46.15, and 52.30 µg/mL. However, lasiodiplodin demonstrated relatively low toxicity toward *F. solani*, with an IC_{50} value of 249.10 µg/mL.

Table 2
Lasiodiplodin toxicity against eight tested phytopathogenic fungi

Plant pathogenic fungi	IC_{50} (µg/mL; 95% Confidence interval)
Exserohilum turcicum	15.50 (11.30–19.70)
Ceratocystis paradoxa	27.45 (19.41–35.49)
Pestalotiopsis theae	17.55 (11.01–24.09)
Alternaria oleracea	52.30 (37.70–66.90)
Alternaria citri	40.35 (30.01–50.69)
Colletotrichum capsici	15.90 (9.89–21.91)
Fusarium solani	249.10 (170.68–327.52)
Athelia rolfsii	46.15 (31.91–60.39)

3.3. Effects of lasiodiplodin on Exserohilum turcicum colony and mycelial morphologies

E. turcicum was inoculated on plates containing lasiodiplodin (test) or acetone: H_2O solution (1:1, v/v; control), and after incubating for 72 h at 28 ℃, the colony and mycelial morphologies were determined. There were significant differences in the colony and mycelial morphologies between the lasiodiplodin-treated (test) group and the control group. The lasiodiplodin-treated colonies (Fig. 2b) were shriveled, and their diameters were smaller than those of the control group (Fig. 2a). Light microscopic analysis revealed that compared with the control (Fig. 2c), lasiodiplodin-treated mycelia had several skinny, shriveled, and distorted aberrations; moreover, no obvious mycelial separation was observed (Fig. 2d).

3.4. Effects of lasiodiplodin on *Exserohilum turcicum* cell membrane permeability

The changes in the relative permeability of *E. turcicum* mycelia cytomembrane are shown in Fig. 3. The relative permeability was positively correlated with the treatment time. During the 30–360 min intervals, the relative permeability of the lasiodiplodin-treated group increased by 0.39–2.74 times, significantly higher than that of the control group (0–1.21 times). The sharp increase in the relative permeability in the test group indicated that lasiodiplodin could induce destruction of the cytoplasmic membranes and cause efflux of the intercellular content, ultimately leading to fungal death.

3.5. Optimization of cultural conditions

Results of the orthogonal test are shown in Table 3. A comparative analysis of the *R* values of the orthogonal test revealed that the most important factor for the optimal production of lasiodiplodin in *E. turcicum* was the C: N ratio, followed by the fermentation time; the effects of initial pH value, temperature, and liquid volume were comparatively low. In addition, the optimal fermentation conditions were obtained as follows: $A_3B_2C_4D_1E_4$, *i.e.*, initial pH, 7; temperature, 28 ℃; liquid volume, 300/500 mL; C: N (sucrose:yeast extract) ratio, 1:0; and fermentation time, 28 d. Following these fermentation conditions, lasiodiplodin yield increased to 52.33 mg/L, much higher than that at pre-optimization (0.59 mg/L). It is worth noting that $A_4B_3C_2D_1E_2$ could be more suitable for practical application, considering the shorter fermentation period and low fermentation cost, *i.e.*, initial pH, 8; temperature, 31 ℃; liquid volume, 200/500 mL; C: N ratio, 1:0; fermentation time, 7 d, with the lasiodiplodin yield reaching 45.104 mg/L.

Table 3
Results of $L_{16}(4^5)$orthogonal test

Test group	A	B	C	D	E	Lasiodiplodin (mg/L)
1	1	1	1	1	1	2.60
2	2	2	2	2	1	4.15
3	3	3	3	3	1	1.19
4	4	4	4	4	1	0.37
5	4	3	2	1	2	45.10
6	3	4	1	2	2	24.20
7	2	1	4	3	2	9.83
8	1	2	3	4	2	3.87
9	2	4	3	1	3	45.03
10	1	3	4	2	3	22.12
11	4	2	1	3	3	9.68
12	3	1	2	4	3	6.07
13	3	2	4	1	4	52.33
14	4	1	3	2	4	26.04
15	1	4	2	3	4	6.06
16	2	3	1	4	4	20.15
K_1	8.66	11.13	14.16	36.27	2.08	
K_2	19.79	17.51	15.35	19.13	20.75	
K_3	20.95	22.14	19.03	6.69	20.72	
K_4	20.30	18.91	21.16	7.52	26.14	
R	12.28	11.01	7.01	29.58	24.07	

Note: *K* represents the total yield of lasiodiplodin for every factor at each level and *R* stands for range (max-min).

4. Discussion

This is the first report on the isolation of lasiodiplodin from the EF *L. Pseudotheobromae* J-10 associated with the medicinal plant *S. glabra*. Lasiodiplodin is a known macrolide antibiotic (Yang et al. 2006a) that

is ubiquitous in plants and microorganisms and demonstrates important biological activities, including antileukemic (Leet et al. 1982), cytotoxic (Cao et al. 2011; Sobreira et al. 2016), and antimicrobial activities (Yang et al. 2006b; Li et al. 2016). In this study, we demonstrated the broad-spectrum antifungal activity of lasiodiplodin against eight tested plant pathogenic fungi, with the highest activity against *E. turcicum*, *C. capsici*, and *P. theae*. The antifungal activity of lasiodiplodin was mediated via the alteration in the colony morphology and disruption of the cell membrane integrity of the tested fungal pathogens. Yang et al. (2006b) first reported the antimicrobial activity of lasiodiplodin and its antifungal activity against phytopathogen, *F. oxysporum* f. sp. cubense. Additionally, Li et al. (2016) found that lasiodiplodin demonstrates inhibition against phytopathogen, *Ralstonia solanacearum*. These results support that lasiodiplodin is a natural compound with high potential bioactivity against phytopathogens.

Efficient production of lasiodiplodin is critical for its application. Based on previous single factor tests, the optimal combination of the five influencing factors, temperature, pH, fermentation time, C/N ratio, and liquid volume, to improve lasiodiplodin yield in *L. Pseudotheobromae* J-10 was obtained by orthogonal experiment. To the best of our knowledge, there is no report on the optimization of culture conditions for lasiodiplodin production, although it is commonly used in other fungi (He et al 2012; Wang et al. 2022; Peng et al. 2022). The results of this study indicate that C: N ratio had the greatest effect on lasiodiplodin yield in *L. pseudotheobromae* J-10. Moreover, Adetunji et al. (2020b) found that C: N ratio significantly affected the herbicidal activity of *L. Pseudotheobromae* C1136 metabolite, and the phytotoxic metabolite amended with C: N at 10:1 exhibited maximum disease severity on *Chromolaena odorata* and *Echinochloa crus-galli* weeds. C and N are popularly used as the basic components for the optimization of media to permit mass production of spores or vegetative propagules (Gao et al. 2007). There are several reports regarding the influence of the C: N ratio on the development, sporulation, and mycelia biomass of fungi with biopesticidal potential (Tapia et al. 2012; Bushra and Narzish 2016). Furthermore, the addition of other factors, such as metal ions, host extracts, precursors, epigenetic modifiers, and enzyme inhibitors, to the fermentation media has been widely reported to enhance secondary metabolite production in fungi (Pan et al. 2019). Jalil et al. (2021) found that the addition of the host extract in the culture medium could enhance the anti-MRSA activity of the EF *L. pseudotheobromae* IBRL OS-64 against MRSA ATCC 33591. Therefore, several factors are yet to be screened to enhance the production of lasiodiplodin and other active metabolites in *L. pseudotheobromae* J-10 of *S. glabra*.

Previously, *S. glabra*, an important medicinal plant, was reported to demonstrate significant antifungal activity against phytopathogens (Tong et al. 2022). Notably, the EF *L. pseudotheobromae* J-10 (used in this study), and the other EF from *S. glabra* (Song et al. 2022), also displayed broad-spectrum antifungal activity against phytopathogenic fungi. These results underpin that EF could possess activities similar to the host, and also provide a basis for further study of EF in *S. glabra*.

5. Conclusion

It is the first report on the isolation of lasiodiplodin from the EF *L. Pseudotheobromae* J-10 associated with the medicinal plant *S. glabra*, as well as the optimization of *L. Pseudotheobromae* J-10 culture

conditions to increase lasiodiplodin yield. Lasiodiplodin demonstrated broad-spectrum antifungal activity against eight phytopathogenic fungi, with the highest efficacy against *E. turcicum*, *C. capsici*, and *P. theae*. Its mode of action includes alteration of the colony morphology and disruption of the cell membrane integrity of the fungus. The optimal culture conditions to increase lasiodiplodin yield were as follows: initial pH, 7; temperature, 28 ℃; liquid volume, 300/500 mL; C: N ratio (sucrose: yeast extract), 1:0; fermentation time, 28 d, following which lasiodiplodin yield increased to 52.33 mg/L. The results of this study support that lasiodiplodin is a natural compound with high potential bioactivity against phytopathogens, and provided a basis for further study of EF in *S. glabra*.

Declarations

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Declaration of competing interest

The authors state that they have no competing financial interests or personal relationships that could have appeFared to influence the work reported in this paper.

Authors' contributions

Siyu Meng designed the study and performed the experiments. Zhiyong Deng and Huilu Shi were responsible for data validation. Haiyu Luo wrote the manuscript. Yecheng Deng supervised the study and critically revised the manuscript. All of the authors have read and approved the final manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

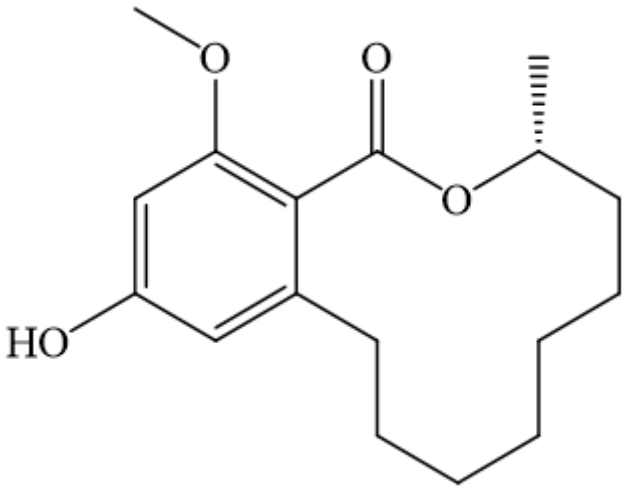

Figure 1

Structure of lasiodiplodin

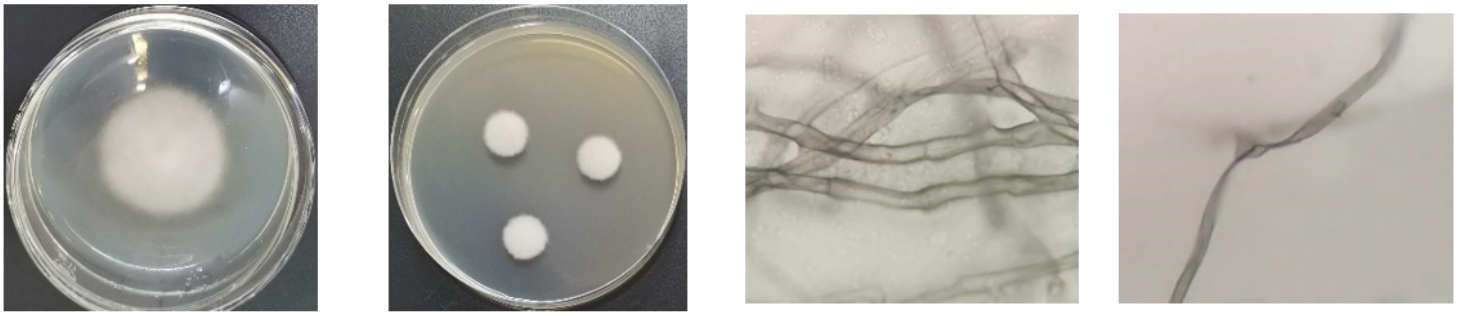

Figure 2

The effect of lasiodiplodin on the colony growth a Fnd mycelial morphology of Exserohilum

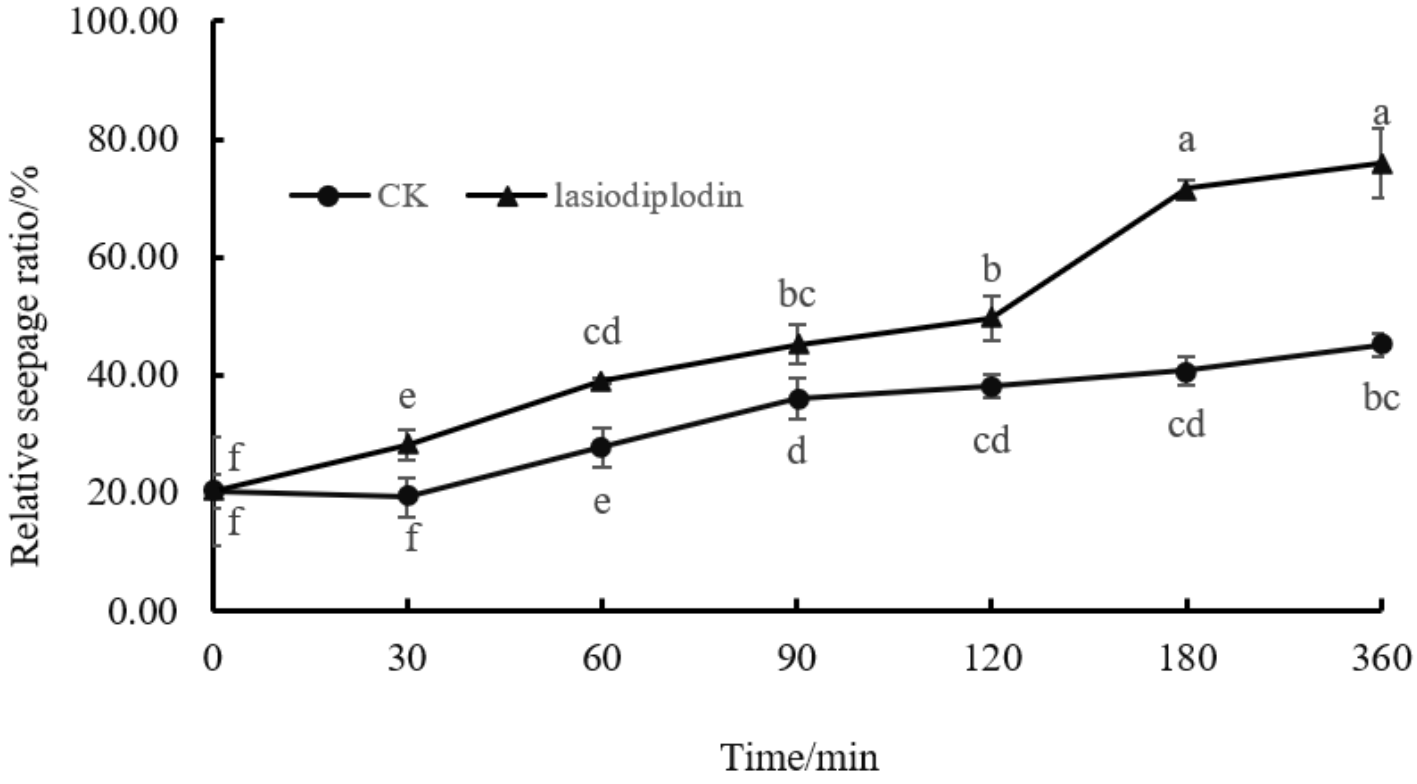

Figure 3

Effects of lasiodiplodin on *Exserohilum turcicum* cell membrane permeability. The test group was cultured on PDA supplemented with 15.50 μg/mL lasiodiplodin (IC_{50} value of lasiodiplodin against *E. turcicum*). “Control” is the control group cultured on PDA supplemented with acetone: H_2O (1:1; v/v). Small case alphabets indicate significant differences among the treatments (p = 0.05).