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# Evaluation of ergosterol composition and esterification rate in fungi isolated from mangrove soil, long-term storage of broken spores, and two soils

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## Abstract

Ergosterol is an important fungal-specific biomarker, but its use for fungal biomass estimation is still varied. It is important to distinguish between free and esterified ergosterols, which are mainly located on the plasma membrane and the cytosolic lipid particles, respectively. The present study analyzes free and esterified ergosterol contents in: (1) the fifty-nine strains of culturable fungi isolated from mangrove soil, (2) the broken spores of the fungus *Ganoderma lucidum* stored in capsule for more than 12 years, and (3) the mangrove soil and nearby campus wood soil samples by high performance liquid chromatography (HPLC). The results show that the contents of free and esterified ergosterols varied greatly in fifty-nine strains of fungi after 5 days of growth, indicating the diversity of ergosterol composition in fungi. The average contents of free and total ergosterols from the fifty-nine strains of fungi are 4.4 ± 1.5 mg/g and 6.1 ± 1.9 mg/g dry mycelia, respectively, with an average ergosterol esterification rate of 27.4%. The present study suggests that the fungi might be divided into two classes, one is fungi with high esterification rates (e.g., more than 27%) such as *Nectria* spp. and *Fusarium* spp., and the other is fungi with low esterification rates (e.g., less than 27%) such as *Penicillium* spp. and *Trichoderma* spp. Moreover, the ergosterol esterification rate in the spores of *G. lucidum* is 91.4% with a very small amount of free ergosterol (0.015 mg/g), compared with 41.9% with a higher level of free ergosterol (0.499 mg/g) reported in our previous study in 2007, indicating that free ergosterol degrades more rapidly than esterified ergosterol. In addition, the ergosterol esterification rates in mangrove soil and nearby campus wood soil samples range from 0 to 39.0%, compared with 80% in an old soil organic matter reported in a previous study, indicating the potential relationship between aging degree of fungi or soil and esterification rate. The present study proposes that both free and esterified ergosterols should be analyzed for fungal biomass estimation. When the ergosterol esterification rates in soils are higher, free ergosterol might be a better marker for fungal biomass. It is speculated that the ergosterol esterification rate in soils might contain some important information, such as the age of old-growth forests over time scales of centuries to millennia, besides the senescence degree of fungal mycelia in soils.

## Key points

- *Fungi might be divided into two classes depending on ergosterol esterification rates.*
- *Ergosterol esterification rate of broken spores stored for long time raised evidently.*
- *Both free and esterified ergosterols should be analyzed for fungal biomass estimate.*
- *Free ergosterol is a better marker for fungal biomass with a high esterification rate.*

**Keywords** Fungi · Free Ergosterol · Esterified Ergosterol · Fungal biomass · Mangrove soil · HPLC analysis

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## Introduction

Fungi are an important part of microbial community in soils and play a central role in forest ecosystems, both as the decomposers of organic matter and as the root-associated mediators of underground carbon transport and respiration (Clemmensen et al. 2013, 2015). Fungal biomass in soils could be an important indicator of soil health and interior biogeochemical processes (Beni et al. 2014). To assess the central role of fungi in terrestrial ecosystems, the adequate methods to determine fungal biomass are essential (de Ridder-Duine et al. 2006).

Ergosterol, a 5,7-diene oxysterol, is an indispensable component of fungal cell membrane, and its biosynthesis is important for filamentation (O'Meara et al. 2015). Because of its specificity to fungi, ergosterol has been widely used as a biomarker for providing a better correlation with the metabolically active biomass of fungi and estimating fungal biomass in various samples using appropriate factors of conversion (Nylund and Wallander 1992; Charcosset and Chauvet 2001). Ergosterol as a biomarker for fungal biomass has proved to be a good indicator of fungal activity and decomposition processes under field conditions (Beni et al. 2014). Many research results have shown a strong correlation between ergosterol content and fungal biomass (Beni et al. 2017; Nurika et al. 2018). The analysis of soil ergosterol could be an effective method for detecting the changes of the fungal biomass under different environmental conditions (Montgomery et al. 2000; Ruzicka et al. 2000; Charcosset and Chauvet 2001; Barajas-Aceves et al. 2002; Li et al. 2009; Wallander et al. 2010; Beni et al. 2017; Cheeke et al. 2017), which has become standard in many research groups (de Ridder-Duine et al. 2006).

However, the ergosterol content in fungi varied from 0.8 to 11 mg/g, suggesting that care must be taken when the ergosterol content is used to compare data generated in different field environments (Charcosset and Chauvet 2001). It was proposed that ergosterol and fungal biomass were not always tightly correlated due to the slow decomposition of ergosterol associated with dead hyphae in soils, and thus, ergosterol should be used cautiously as a reliable biomarker for living fungi, especially in periods of quick decline of the fungal biomass (Mille-Lindblom et al. 2004; Zhao et al. 2005; Helfrich et al. 2008).

In fungal cells, as in other eukaryotes, ergosterol is present in two forms, free ergosterol and esterified ergosterol. Ergosterol is biosynthesized in the endoplasmic reticulum through the sequential activity of twenty-five distinct enzymes (Rodrigues 2018), and excess ergosterol is often converted to ergosterol esters, and then stored in cytoplasmic lipid droplets (Henneberry and Sturley 2005). Since free ergosterol is present mainly in the plasma membrane, where it forms microdomains that modulate membrane fluidity, allowing vital processes such as vesicular sorting and traffic, cytoskeleton organization, and asymmetric growth (Caspeta et al. 2014), free ergosterol is more important for cell integrity (Henneberry and Sturley 2005; Shobayashi et al. 2005) and is maintained at the most favorable levels by an equilibrium between ergosterol biosynthesis and storage as ergosterol esters in cytoplasmic lipid droplets (Henneberry and Sturley 2005). The turnover of esterified ergosterol and the esterification of ergosterol are both important for ergosterol homeostasis and distribution (Wagner et al. 2009). It has been suggested that free ergosterol is a better proxy for living fungi than total ergosterol (Yuan et al. 2008; Wallander et al. 2010; Cheeke et al. 2017). Wallander et al. (2010) used two subsamples for estimates of free ergosterol and total ergosterol, respectively. The free ergosterol extracted with methanol was used as a measure of active fungal biomass, while the total ergosterol extracted with 10% KOH in methanol was used as a measure of active and inactive fungal biomass (Wallander et al. 2010; Cheeke et al. 2017). However, a large number of studies did not distinguish between free and esterified ergosterols. Only total ergosterol or free ergosterol was analyzed. Normally, total ergosterol, including free and esterified forms, was most used for fungal biomass estimates in soils (Nylund and Wallander 1992). Thus, many studies have been conducted to determine the fungal biomass in various samples using the total ergosterol method after saponification (Montgomery et al. 2000; Voříšková et al. 2013; Bahr et al. 2013; Sterkenburg et al. 2015; Ekblad et al. 2016; Baldrian et al. 2016; Guhr et al. 2016; Teste et al. 2016; Noll et al. 2016; Žifčáková et al. 2016; Hendricks et al. 2016; Brosed et al. 2017; Beni et al. 2017; Awad et al. 2019).

The ecological environment of sediments in mangrove region is relatively unstable, and the composition of organic matter is more complex compared with terrestrial ecosystems (Guillén-Navarro et al. 2015). However, there are few reports on the use of ergosterol as a biomarker for the determination of fungal biomass in mangrove sediments and soils (Dinesh and Ghoshal-Chaudhuri 2013). On the basis of the fact that measuring the total ergosterol as a proxy for living fungal biomass is misleading since the free and esterified ergosterols might differ in their indicative value of mycelium vitality, the present study analyzed and evaluated the ergosterol fractions and esterification rate in fifty-nine fungal strains isolated from mangrove soil, and presented a temporal analysis of ergosterol fractions and fungal biomass in four typical fungal strains. In addition, the broken spores of the fungus *Ganoderma lucidum* (Yuan et al. 2007a) stored for more than 12 years were analyzed to investigate the relative change of free and esterified ergosterols in the broken and encapsulated spores to evaluate the degradation of free and esterified ergosterols. Moreover, mangrove and wood soils were also analyzed to compare the esterification rate and fungal biomass between mangrove soil and wood soil.

## Materials and methods

### Soil samples and the spores of *G. lucidum*

The mangrove wetland soil samples were collected randomly in July 2017 from the mangrove (22°26' N, 113°38′ E) in the Qi'Ao-Dangan Island Provincial Nature Reserve located in Qi'Ao Island, Zhuhai, China. The soil samples from the upper 5 cm were placed in a box with ice, returned to the laboratory, and stored in the refrigerator (4 °C) for the succedent fungal isolation and ergosterol extraction. The soil samples from the nearby campus woods (22°21' N, 113°35′ E) were also collected for comparison purposes.

In addition, the broken and encapsulated spores of *G. lucidum* as a dietary supplement for human health (Liu et al. 2002) were obtained in 2007 and have been stored in the laboratory at room temperature.

### Chemicals and reagents

HPLC-grade methanol and dichloromethane were purchased from CNW Technologies GmbH (Duesseldorf, Germany). Ergosterol standard (98% purity) for HPLC measurement was obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA). Water was purified using a Millipore Simplicity system (Billerica, MA, USA). Potato dextrose agar (PDA) and potato dextrose broth (PDB) were obtained from Guangdong Huankai Microbial Sci. & Tech. Co., Ltd. (Guangzhou, China).

### Culture and purification of fungi

Soil samples were first ground into powder using sterile mortar and pestle. Soil sample powder (10 g) was mixed with 100 mL of sterile distilled water. After stirring for 30 min at 180 rpm on a shaking table and leaving to rest for 10 min, the supernatant was diluted by employing serial decimal dilution method up to $10^{-3}$ in triplicate. Every diluted sample (100 μL) was spread on Petri plates containing Rose Bengal medium (glucose 10 g/L, peptone 5 g/L, potassium dihydrogen phosphate 1 g/L, magnesium sulfate 0.5 g/L, agar 15 g/L, Bengal red 0.1 g/L, chloramphenicol 0.1 g /L, pH 6.0 ± 0.2, autoclaved at 121 °C for 20 min). All the plates were incubated at 28 °C and checked each day for the appearance of new colonies until fungal growth appeared. Each new colony was individually isolated and cultured on new PDA medium (potato dextrose agar medium, potatoes extract 300 g/L, glucose 20 g/L, agar 15 g/L, sterile distilled water, pH 6.0 ± 0.2, autoclaved at 121 °C for 20 min) supplemented with 0.1 g/L chloramphenicol.

### Growth of the fungi

Isolated fungi were grown on PDA at 28 °C for producing spores. The spores of the fungi grown in PDA were washed off with sterilized normal saline. One milliliter of the spore suspension was inoculated into 250 mL Erlenmeyer flasks containing 100 mL of potato dextrose broth (PDB) medium (potato extract 300 g/L, glucose 20 g/L, sterile distilled water, pH 5.6 ± 0.2, autoclaved at 121 °C for 20 min) at 28 °C for 5 days on a constant temperature shaking incubator at 180 rpm. Every strain was cultured in three repetitions. In addition, a few representative fungi were cultured under the same conditions in three repetitions for up to 25 days. Regular sampling was carried out. These cultures were filtered on the filter paper (0.45 μm), and the wet mycelia were collected and then dried by lyophilization. The dry mycelia were used to determine the fungal biomass and ergosterol composition.

### Fungal identification

Representative isolates of each morphospecies were identified by sequencing the internal transcribed spacer (ITS) regions of each isolate. Genomic DNA was extracted from isolates grown on PDA at day 8. DNA extraction, amplification, and sequence were performed commercially at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China). The amplifications for fungi were performed using 2 × Taq Plus Master Mix (Vazyme, Nanjing, China), employing 5 pmol of each primer ITS1 and ITS4 and 0.1 μg of template DNA in a total volume of 20 μL. The amplifications were carried out on a MG96G thermal cycler (Long-Gene, Hangzhou, China). The program for amplification was as follows: a first denaturation step at 95 °C for 5 min was followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 32 s, with a final extension at 72 °C for 10 min. The amplified PCR products were sequenced using ABI 3730XL Sequencer (Applied Biosystems, Foster City, USA). The sequences of the ITS regions were compared with the existing species sequences in GenBank using BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

### HPLC analysis of free ergosterol and ergosterol esters in the culturable fungi, the broken spores of *G. lucidum*, and the soil samples

The present study used the non-alkaline extraction method, including the shaking of fungal and soil samples with glass beads to disrupt the fungal mycelia (Gong et al. 2001) and the extraction of free and esterified ergosterols using the mixture solvent (Yuan et al. 2006, 2007a, b, 2008). Freeze-dried fungi, the broken spores of *G. lucidum*, and the soil samples were ground into powder and extracted by a Bead Ruptor 24 Elite bead mill homogenizer (OMNI International, Kennesaw,

Georgia, USA). The free and esterified ergosterols were extracted with the mixture of methanol and dichloromethane (75:25, v/v) followed by sonication for 10 min and centrifugation at 12000×*g* for 5 min. The extraction procedure of each sample was repeated three times, and the total extract of each sample was sampled for HPLC analysis.

HPLC was conducted on a Waters liquid chromatograph equipped with a 1525 binary pump and a 2996 photodiode array detector (Waters Corporation, Milford, MA, USA). A Waters Symmetry C18 column (150 × 3.9 mm; 5 μm) was used for the separation and analysis of ergosterol and its esters. The mobile phase consisted of solvent A (methanol: water, 80: 20, v/v) and solvent B (methanol: dichloromethane, 75: 25, v/v). The following gradient procedure was used: 50% of B for 5 min; a linear gradient from 50 to 90% of B for 10 min; and 90% of B for 10 min. The flow rate was set at 1.0 mL/min. The detection wavelength of the PDA detector was set between 240 and 340 nm. Ergosterol and ergosterol esters were detected at a wavelength of 280 nm and quantitated by comparing the values of their peak area with the ergosterol standard (Yuan et al. 2006, 2007a, b, 2008). Aliquots of 20 μL were directly injected into the HPLC by manual injector. All injections were repeated three times.

### Statistical analysis

All experiments were repeated at least three times, and the data were expressed as the mean values with standard error of the mean of three replicates on the dry weight basis. The one-way analysis of variance (ANOVA) was carried out by using the Microsoft Excel 2016 software (Microsoft, Redmond, WA, USA).

## Results

### Identification of fungal isolates

Fifty-nine strains of fungi were isolated from Qi'Ao Island mangrove soil. Genomic DNA was extracted and ITS region was amplified and sequenced. The obtained results were compared with those in the GenBank database, and the results are shown in Table 1. Consensus sequence data of the fungi were submitted to the GenBank database, and the GenBank accession numbers are also shown in Table 1. The results indicated that the identified fungal strain belonged to 13 genera as follows: *Penicillium* (17 strains), *Fusarium* (16 strains), *Nectria* (10 strains), *Trichoderma* (4 strains), *Gliocephalotrichum* (3 strains), *Hypocrea* (2 strains), *Aspergillus* (1 strain), *Pseudallescheria* (1 strain), *Schwanniomyces* (1 strain), *Geotrichum* (1 strain), *Cylindrocladiella* (1 strain), *Xylogone* (1 strain), and *Xenoacremonium* (1 strain).

### Analysis of free ergosterol and ergosterol esters in the fifty-nine strains of fungi

Free ergosterol and ergosterol esters in the fifty-nine strains of fungi after 5 days of cultivation were analyzed using the HPLC method. The contents of free ergosterol, ergosterol esters, total ergosterol, and the esterification rate are shown in Table 2. The results show that the contents of free, esterified, and total ergosterol vary greatly in different fungal strain samples, ranging from 2.28 to 10.05 mg/g dry mycelia for free ergosterol, from 0 to 9.67 mg/g dry mycelia for esterified ergosterol, and from 2.36 to 12.92 mg/g dry mycelia for total ergosterol.

The lowest free ergosterol level (2.28 mg/g dry mycelia) was detected in *Aspergillus* sp. QA36, and the highest level (10.09 mg/g dry mycelia) was found in *Penicillium* sp. QA17, which has the lower esterified ergosterol level (0.05 mg/g dry mycelia). *Fusarium* sp. QA55 and *Nectria* sp. QA42 have the higher contents of ergosterol esters (6.25 mg/g and 9.69 mg/g dry mycelia, respectively), while the lowest contents of ergosterol esters were detected in *Gliocephalotrichum* sp. QA12 (not detected), *Gliocephalotrichum* sp. QA11 (0.03 mg/g), *Gliocephalotrichum* sp. QA18 (0.07 mg/g), *Penicillium* sp. QA17 (0.05 mg/g), *Penicillium* sp. QA16 (0.08 mg/g), and *Aspergillus* sp. QA36 (0.08 mg/g).

The higher total ergosterol levels (> 8.5 mg/g dry mycelia) were found in *Fusarium* sp. QA32, *Fusarium* sp. QA4, *Fusarium* sp. QA34, *Fusarium* sp. QA55, *Nectria* sp. QA42, *Penicillium* sp. QA17, *Pseudallescheria* sp. QA59, and *Xenoacremonium* sp. QA56. The lower total ergosterol levels (< 3 mg/g dry mycelia) were detected in *Aspergillus* sp. QA36, *Gliocephalotrichum* sp. QA11, and *Gliocephalotrichum* sp. QA12. These results indicated the diversity of ergosterol composition in fungi.

Table 3 shows the average contents of free, esterified, and total ergosterols and the ergosterol esterification rates in *Fusarium* spp., *Nectria* spp., and *Penicillium* spp. and all 59 strains of fungi. As can be seen from Table 3, the total ergosterol contents of *Fusarium* spp., *Nectria* spp., and *Penicillium* spp., the most abundant culturable fungus genera in this mangrove soil, show the approximate average values of 6.12, 6.58, and 6.05 mg/g dry mycelia, respectively, close to the total ergosterol average content (6.12 mg/g dry mycelia) of all 59 strains of culturable fungi, while the average contents of free and esterified ergosterols of *Fusarium* spp., *Nectria* spp., *Penicillium* spp., and all 59 strains of culturable fungi are different. Table 3 also shows that the average content of free ergosterol and the ergosterol esterification rates of all 59 strains of culturable fungi are 4.44 mg/g dry mycelia and 27.4%, respectively.

The ergosterol esterification rates were also different in the different fungal strains ranging from 0 to 75.0%. Figure 1, a scatter diagram, distinctly shows an obvious difference in the

**Table 1** The strain codes, GenBank accession number, closest species, and coverage of fifty-nine strains of fungi isolated from Qi'Ao Island mangrove soil

| Strains | Accession no. | Closest species (accession no.) | Identity (%) |
|---|---|---|---|
| QA-1 | MN701662 | *Penicillium* sp. (KT336528.1) | 100 |
| QA-2 | MN701663 | *Penicillium* sp. (KT336528.1) | 100 |
| QA-3 | MN701664 | *Nectria* sp. (FJ037731.1) | 99 |
| QA-4 | MN701665 | *Fusarium solani* (KF918582.1) | 99 |
| QA-5 | MN701666 | *Penicillium cataractum* (KT887847.1) | 100 |
| QA-6 | MN701667 | *Penicillium cataractum* (KT887847.1) | 100 |
| QA-7 | MN701668 | *Nectria* sp. (FJ037731.1) | 99 |
| QA-8 | MN701669 | *Trichoderma reesei* (KU377472.1) | 100 |
| QA-9 | MN701670 | *Fusarium solani* (KF918582.1) | 99 |
| QA-10 | MN701671 | *Fusarium solani* (KF918582.1) | 99 |
| QA-11 | MN701672 | *Gliocephalotrichum simplex* (JQ688045.1) | 100 |
| QA-12 | MN701673 | *Gliocephalotrichum* sp. (KY413724.1) | 100 |
| QA-13 | MN701674 | *Trichoderma reesei* (KM246746.1) | 100 |
| QA-14 | MN701675 | *Trichoderma harzianum* (KT030885.1) | 100 |
| QA-15 | MN701676 | *Debaryomyces pseudopolymorphus* (EF198011.1) | 100 |
| QA-16 | MN701677 | *Penicillium cataractum* (KT887847.1) | 100 |
| QA-17 | MN701678 | *Penicillium cataractum* (KT887847.1) | 100 |
| QA-18 | MN701679 | *Gliocephalotrichum simplex* (JQ688045.1) | 100 |
| QA-19 | MN701680 | *Fusarium solani* (KF918582.1) | 99 |
| QA-20 | MN701681 | *Penicillium cataractum* (KT887847.1) | 100 |
| QA-21 | MN701682 | *Nectria* sp. (FJ037731.1) | 99 |
| QA-22 | MN701683 | *Nectria* sp. (FJ037731.1) | 99 |
| QA-23 | MN701684 | *Nectria* sp. (FJ037731.1) | 99 |
| QA-24 | MN701685 | *Fusarium solani* (KF918582.1) | 99 |
| QA-25 | MN701686 | *Penicillium cataractum* (KT887847.1) | 100 |
| QA-26 | MN701687 | *Fusarium solani* (KF918582.1) | 99 |
| QA-27 | MN701688 | *Penicillium cataractum* (KT887847.1) | 100 |
| QA-28 | MN701689 | *Fusarium solani* (KF918582.1) | 100 |
| QA-29 | MN701690 | *Nectria* sp. (FJ037731.1) | 99 |
| QA-30 | MN701691 | *Penicillium* sp. (KT336528.1) | 100 |
| QA-31 | MN701692 | *Fusarium solani* (KF918582.1) | 99 |
| QA-32 | MN701693 | *Fusarium solani* (KF918582.1) | 99 |
| QA-33 | MN701694 | *Fusarium solani* (KF918582.1) | 99 |
| QA-34 | MN701695 | *Fusarium solani* (KU939058.1) | 100 |
| QA-35 | MN701696 | *Nectria* sp. (FJ037731.1) | 100 |
| QA-36 | MN701697 | *Aspergillus niger* (MH064151.1) | 100 |
| QA-37 | MN701698 | *Penicillium* sp. (KT336528.1) | 100 |
| QA-38 | MN701699 | *Penicillium simplicissimum* (KU059955.1) | 100 |
| QA-39 | MN701700 | *Fusarium solani* (KF918582.1) | 100 |
| QA-40 | MN701701 | *Penicillium cataractum* (KT887847.1) | 100 |
| QA-41 | MN701702 | *Penicillium* sp. (KT336528.1) | 100 |
| QA-42 | MN701703 | *Nectria* sp. (FJ037731.1) | 99 |
| QA-43 | MN701704 | *Penicillium* sp. (KT336528.1) | 100 |
| QA-44 | MN701705 | *Nectria* sp. (FJ037731.1) | 100 |
| QA-45 | MN701706 | *Hypocrea lutea* (JN943361.1) | 99 |
| QA-46 | MN701707 | *Penicillium simplicissimum* (KU059955.1) | 100 |
| QA-47 | MN701708 | *Fusarium solani* (KF918582.1) | 99 |
| QA-48 | MN701709 | *Fusarium solani* (KF918582.1) | 99 |

**Table 1** (continued)

| Strains | Accession no. | Closest species (accession no.) | Identity (%) |
|---|---|---|---|
| QA-49 | MN701710 | *Galactomyces* sp. (HG936031.1) | 100 |
| QA-50 | MN701711 | *Penicillium cataractum* (KT887847.1) | 100 |
| QA-51 | MN701712 | *Cylindrocladiella camelliae* (JN943098.1) | 100 |
| QA-52 | MN701713 | *Trichoderma* sp. (KT192427.1) | 100 |
| QA-53 | MN701714 | *Fusarium solani* (KF918582.1) | 100 |
| QA-54 | MN701715 | *Nectria* sp. (FJ037731.1) | 99 |
| QA-55 | MN701716 | *Fusarium solani* (KF918582.1) | 99 |
| QA-56 | MN701717 | *Xenoacremonium recifei* (KM231834.1) | 100 |
| QA-57 | MN701718 | *Xylogone sphaerospora* (KU194337.1) | 100 |
| QA-58 | MN701719 | *Hypocrea lutea* (JN943361.1) | 99 |
| QA-59 | MN701720 | *Pseudallescheria boydii* (JQ668664.1) | 100 |

ergosterol esterification rates in different fungi. As shown in Table 2 and Fig. 1, the ergosterol esterification rates were much higher in *Fusarium* spp. ranging from 22.4 to 63.1%, *Nectria* spp. ranging from 16.2 to 75.0% and *Pseudallescheria* sp. QA59 with 40.3% than that in *Penicillium* spp. ranging from 0.5 to 22.3%, *Trichoderma* spp. ranging from 9.8 to19.5%, and other fungal strains less than 26.2%.

## Analysis of free ergosterol and ergosterol esters conducted across a time scale in four strains of fungi during the cultivation

Because *Fusarium*, *Nectria*, *Penicillium*, and *Trichoderma* are the most abundant culturable fungus genera isolated from mangrove soil, and have the higher or the lower ergosterol esterification rates, respectively, the biomass and the contents of free ergosterol and ergosterol esters in four strains of fungi, *Penicillium* sp. QA5, *Trichoderma* sp. QA14, *Fusarium* sp. QA34, and *Nectria* sp. QA44, during the cultivation of about 600 h, were analyzed, and the results are shown in Fig. 2.

During the culture of *Penicillium* QA5 (Fig. 2a), the cell dry weight kept increasing up to 240 h and then dropped linearly. The free ergosterol content showed a relatively small fluctuation ranging from 6.75 to 8.71 mg/g dry mycelia before 240 h of cultivation, and an obvious increase in the free ergosterol content was observed, reaching a maximum (17.78 mg/g dry mycelia) after 520 h of cultivation corresponding to the decrease in biomass (Fig. 2a). In contrast, the content of ergosterol esters kept at a lower level throughout the growth, and increased until reaching a maximum (1.01 mg/g dry mycelia) after 280 h of cultivation, lagging behind slightly the decrease in biomass. The maximal value of total ergosterol content in *Penicillium* QA5 during the culture is 18.25 mg/g dry mycelia with an esterification rate of 2.59%.

For *Trichoderma* sp. QA14 (Fig. 2b), the cell dry weight kept increasing rapidly up to 88 h and then reduced slowly and steadily. The free ergosterol content showed a sharp reduction from 7.79 to 4.96 mg/g dry mycelia, and then remained relatively unchanged (4.80–5.38 mg/g dry mycelia). Whereafter, an approximately linear increase in the free ergosterol content from 5.89 to 8.86 mg/g dry mycelia was observed from 140 to 604 h of cultivation. In contrast, the content of ergosterol esters kept at a lower level with a maximum value of 1.13 mg/g dry mycelia throughout the growth similar to *Penicillium* sp. QA5. The maximal value of total ergosterol content in *Trichoderma* sp. QA14 during the culture is 9.59 mg/g dry mycelia with an esterification rate of 12.05%.

For *Fusarium* sp. QA34 (Fig. 2c), the cell dry weight kept increasing up to 267 h and then reduced slowly. The free ergosterol content initially decreased from 4.61 to 3.33 mg/g dry mycelia and remained relatively constant (3.33–3.59 mg/g dry mycelia), and then increased from 3.47 to 11.73 mg/g dry mycelia after 522 h of cultivation. Meanwhile, the content of ergosterol esters quickly increased from 0.25 to 3.51 mg/g dry mycelia after 100 h of cultivation, then slowly increased and reached a maximum value of 4.94 mg/g dry mycelia and finally began to fall slightly (Fig. 2c). The maximal value of total ergosterol content in *Fusarium* sp. QA34 during the culture is 15.73 mg/g dry mycelia with an esterification rate of 25.42%.

For *Nectria* sp. QA44 (Fig. 2d), the cell dry weight kept increasing rapidly up to 156 h and then dropped linearly. The free ergosterol content initially decreased quickly from 6.58 to 3.53 mg/g dry mycelia and remained relatively constant (3.48–3.87 mg/g dry mycelia), and then began to increase from 3.77 to 5.15 mg/g dry mycelia after 480 h of cultivation, and remained unchanged. Meanwhile, the content of ergosterol esters quickly increased from 0.34 to 2.89 mg/g dry mycelia after 100 h of cultivation, then slowly increased and reached a maximum value of 3.77 mg/g dry mycelia and finally began to

**Table 2** Ergosterol composition of fifty-nine strains of fungi isolated from Qi'Ao Island mangrove soil

| Fungal strains | Ergosterol contents (mg/g) | | | Esterification rate (%) |
|---|---|---|---|---|
| | Free | Esterified | Total | |
| *Fusarium* sp. QA4 | 4.530 ± 0.127 | 5.212 ± 0.061 | 9.742 | 53.5 |
| *Fusarium* sp. QA9 | 2.937 ± 0.024 | 1.692 ± 0.019 | 4.629 | 36.6 |
| *Fusarium* sp. QA10 | 3.005 ± 0.025 | 1.160 ± 0.018 | 4.165 | 27.9 |
| *Fusarium* sp. QA19 | 3.197 ± 0.047 | 2.209 ± 0.041 | 5.406 | 40.9 |
| *Fusarium* sp. QA24 | 3.111 ± 0.114 | 1.323 ± 0.022 | 4.434 | 29.8 |
| *Fusarium* sp. QA26 | 3.323 ± 0.029 | 4.063 ± 0.042 | 7.386 | 55.0 |
| *Fusarium* sp. QA28 | 3.118 ± 0.032 | 2.488 ± 0.078 | 5.606 | 44.4 |
| *Fusarium* sp. QA31 | 3.498 ± 0.084 | 1.322 ± 0.052 | 4.820 | 27.4 |
| *Fusarium* sp. QA32 | 6.500 ± 0.126 | 2.277 ± 0.175 | 8.777 | 25.9 |
| *Fusarium* sp. QA33 | 3.713 ± 0.179 | 1.074 ± 0.044 | 4.787 | 22.4 |
| *Fusarium* sp. QA34 | 3.463 ± 0.064 | 2.922 ± 0.007 | 6.385 | 45.8 |
| *Fusarium* sp. QA39 | 5.179 ± 0.084 | 1.674 ± 0.087 | 6.853 | 24.4 |
| *Fusarium* sp. QA47 | 3.342 ± 0.075 | 1.327 ± 0.017 | 4.669 | 28.4 |
| *Fusarium* sp. QA48 | 3.648 ± 0.049 | 1.683 ± 0.037 | 5.331 | 31.6 |
| *Fusarium* sp. QA53 | 3.435 ± 0.023 | 1.632 ± 0.016 | 5.067 | 32.2 |
| *Fusarium* sp. QA55 | 3.659 ± 0.062 | 6.248 ± 0.126 | 9.907 | 63.1 |
| *Nectria* sp. QA3 | 2.559 ± 0.088 | 1.489 ± 0.056 | 4.048 | 36.8 |
| *Nectria* sp. QA7 | 3.326 ± 0.034 | 0.642 ± 0.008 | 3.968 | 16.2 |
| *Nectria* sp. QA21 | 3.781 ± 0.050 | 3.381 ± 0.047 | 7.162 | 47.2 |
| *Nectria* sp. QA22 | 2.808 ± 0.019 | 3.006 ± 0.026 | 5.814 | 51.7 |
| *Nectria* sp. QA23 | 3.499 ± 0.095 | 1.721 ± 0.028 | 5.220 | 33.0 |
| *Nectria* sp. QA29 | 4.192 ± 0.108 | 1.372 ± 0.168 | 5.564 | 24.7 |
| *Nectria* sp. QA35 | 3.138 ± 0.062 | 3.585 ± 0.290 | 6.723 | 53.3 |
| *Nectria* sp. QA42 | 3.229 ± 0.104 | 9.686 ± 0.107 | 12.915 | 75.0 |
| *Nectria* sp. QA44 | 3.684 ± 0.008 | 3.152 ± 0.053 | 6.836 | 46.1 |
| *Nectria* sp. QA54 | 3.951 ± 0.079 | 3.594 ± 0.145 | 7.545 | 47.6 |
| *Penicillium* sp. QA1 | 5.205 ± 0.207 | 0.852 ± 0.030 | 6.057 | 14.1 |
| *Penicillium* sp. QA2 | 4.361 ± 0.087 | 0.549 ± 0.010 | 4.910 | 11.2 |
| *Penicillium* sp. QA5 | 7.988 ± 0.061 | 0.406 ± 0.024 | 8.394 | 4.8 |
| *Penicillium* sp. QA6 | 3.700 ± 0.079 | 0.861 ± 0.051 | 4.561 | 18.9 |
| *Penicillium* sp. QA16 | 7.537 ± 0.080 | 0.077 ± 0.002 | 7.614 | 1.0 |
| *Penicillium* sp. QA17 | 10.045 ± 0.093 | 0.047 ± 0.001 | 10.092 | 0.5 |
| *Penicillium* sp. QA20 | 4.449 ± 0.026 | 0.780 ± 0.006 | 5.229 | 14.9 |
| *Penicillium* sp. QA25 | 3.997 ± 0.094 | 1.145 ± 0.016 | 5.142 | 22.3 |
| *Penicillium* sp. QA27 | 3.852 ± 0.097 | 0.853 ± 0.080 | 4.705 | 18.1 |
| *Penicillium* sp. QA30 | 4.905 ± 0.034 | 0.532 ± 0.015 | 5.437 | 9.8 |
| *Penicillium* sp. QA37 | 5.143 ± 0.043 | 1.001 ± 0.029 | 6.144 | 16.3 |
| *Penicillium* sp. QA38 | 5.228 ± 0.059 | 1.253 ± 0.044 | 6.481 | 19.3 |
| *Penicillium* sp. QA40 | 3.814 ± 0.084 | 0.837 ± 0.034 | 4.651 | 18.0 |
| *Penicillium* sp. QA41 | 5.046 ± 0.080 | 0.594 ± 0.016 | 5.640 | 10.5 |
| *Penicillium* sp. QA43 | 4.664 ± 0.037 | 0.317 ± 0.032 | 4.981 | 6.4 |
| *Penicillium* sp. QA46 | 5.644 ± 0.119 | 0.531 ± 0.030 | 6.175 | 8.6 |
| *Penicillium* sp. QA50 | 6.306 ± 0.141 | 0.315 ± 0.007 | 6.621 | 4.8 |
| *Trichoderma* sp. QA8 | 4.061 ± 0.062 | 0.743 ± 0.015 | 4.804 | 15.5 |
| *Trichoderma* sp. QA13 | 4.245 ± 0.045 | 0.979 ± 0.023 | 5.224 | 18.7 |
| *Trichoderma* sp. QA14 | 5.028 ± 0.048 | 0.858 ± 0.008 | 5.886 | 14.6 |
| *Trichoderma* sp. QA52 | 4.303 ± 0.061 | 1.044 ± 0.024 | 5.347 | 19.5 |

**Table 2** (continued)

| Fungal strains | Ergosterol contents (mg/g) | | | Esterification rate (%) |
|---|---|---|---|---|
| | Free | Esterified | Total | |
| *Hypocrea* sp. QA45 | 5.981 ± 0.052 | 1.352 ± 0.021 | 7.333 | 18.4 |
| *Hypocrea* sp. QA58 | 5.304 ± 0.067 | 1.734 ± 0.030 | 7.038 | 24.6 |
| *Gliocephalotrichum* sp. QA11 | 2.882 ± 0.140 | 0.027 ± 0.003 | 2.909 | 0.9 |
| *Gliocephalotrichum* sp. QA12 | 2.701 ± 0.129 | ND | 2.701 | 0 |
| *Gliocephalotrichum* sp. QA18 | 4.450 ± 0.097 | 0.069 ± 0.002 | 4.519 | 1.5 |
| *Pseudallescheria* sp. QA59 | 5.629 ± 0.042 | 3.799 ± 0.118 | 9.428 | 40.3 |
| *Xenoacremonium* sp. QA56 | 6.654 ± 0.065 | 2.003 ± 0.039 | 8.657 | 23.1 |
| *Xylogone* sp. QA57 | 5.628 ± 0.078 | 1.518 ± 0.061 | 7.146 | 21.2 |
| *Aspergillus* sp. QA36 | 2.281 ± 0.110 | 0.081 ± 0.007 | 2.362 | 3.4 |
| *Cylindrocladiella* sp. QA51 | 5.948 ± 0.027 | 1.189 ± 0.032 | 7.137 | 16.7 |
| *Debaryomyces* sp. QA15 | 6.874 ± 0.069 | 0.976 ± 0.020 | 7.850 | 12.4 |
| *Galactomyces* sp. QA49 | 4.513 ± 0.049 | 1.602 ± 0.094 | 6.115 | 26.2 |

ND indicates below the lowest limit of detection (LOD)

fall slightly (Fig. 2d). The maximal value of total ergosterol content in *Nectria* sp. QA44 during the culture is 8.56 mg/g dry mycelia with an esterification rate of 39.85%.

Figure 3 shows the changes of the ergosterol esterification rates in the fungi *Trichoderma* sp. QA14, *Penicillium* sp. QA5, *Fusarium* sp. QA34, and *Nectria* sp. QA44 during the growth. The results show that the ergosterol esterification rates in *Fusarium* sp. QA34 and *Nectria* sp. QA44 quickly increased to more than 40% after 100 h of cultivation, remained relatively constant, and then began to fall slightly. For the fungi *Penicillium* sp. QA5 and *Trichoderma* sp. QA14, the ergosterol esterification rates kept fluctuation at a lower level with a maximum value of 10.5% and 14.6%, respectively.

## Analysis of free ergosterol and ergosterol esters in the broken spores of *G. lucidum* stored in capsule for more than 12 years

The ergosterol composition in the broken spores of *G. lucidum* stored in capsule for more than 12 years from 2007 to 2019 was analyzed, and the results in the present study and our previous study (Yuan et al. 2007a) are shown in Table 4. The results show that while the content of free ergosterol markedly decreased from 0.499 to 0.015 mg/g dry spores, the content of esterified ergosterol decreased from 0.360 to 0.159 mg/g dry spores during storage. The ergosterol esterification rate in the spores has obviously increased from 41.9 to 91.4% during the storage of more than 12 years.

## Analysis of free ergosterol and ergosterol esters in mangrove soil samples and wood soil samples

The ergosterol composition in 8 mangrove soil samples from Qi'Ao Island and 5 soil samples from the nearby campus woods was analyzed, and the average contents are shown in Table 5. The results show that the average free ergosterol content of 8 mangrove soil samples (1.75 ± 1.54 μg/g dry soil mass) is lower obviously than that of 5 wood soil samples (5.43 ± 1.35 μg/g dry soil mass). Moreover, all wood soil samples contain ergosterol esters (0.886 ± 0.696 μg/g dry soil mass) with the esterification rates ranging from 7.0 to 39.0%, and the average esterification rate is 14.0%, but no esterified ergosterol was found in all mangrove soil samples.

**Table 3** The average contents of free, esterified, and total ergosterols and ergosterol esterification rates of fifty-nine strains of fungi isolated from Qi'Ao Island mangrove soil

| Fungi | Average contents of ergosterols (mg/g) | | | Esterification rate (%) |
|---|---|---|---|---|
| | Free | Esterified | Total | |
| *Fusarium* spp. | 3.729 ± 0.936 | 2.394 ± 1.521 | 6.123 ± 1.892 | 39.1 |
| *Nectria* spp. | 3.417 ± 0.923 | 3.163 ± 1.897 | 6.580 ± 2.210 | 48.1 |
| *Penicillium* spp. | 5.405 ± 1.605 | 0.644 ± 1.761 | 6.049 ± 1.991 | 10.7 |
| All 59 strains of fungi | 4.444 ± 1.474 | 1.675 ± 1.667 | 6.119 ± 1.930 | 27.4 |

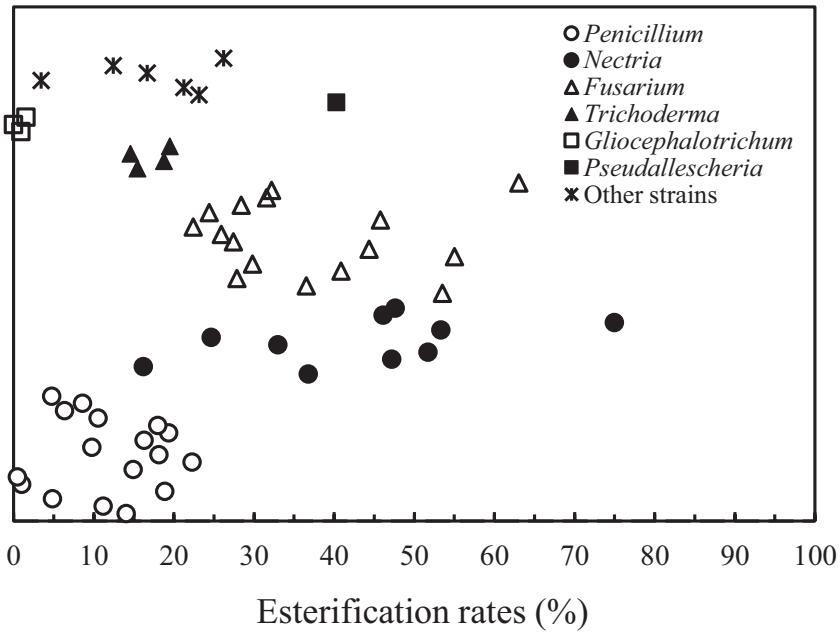


**Fig. 1** The scatter diagram of ergosterol esterification rate of 59 strains of culturable fungi

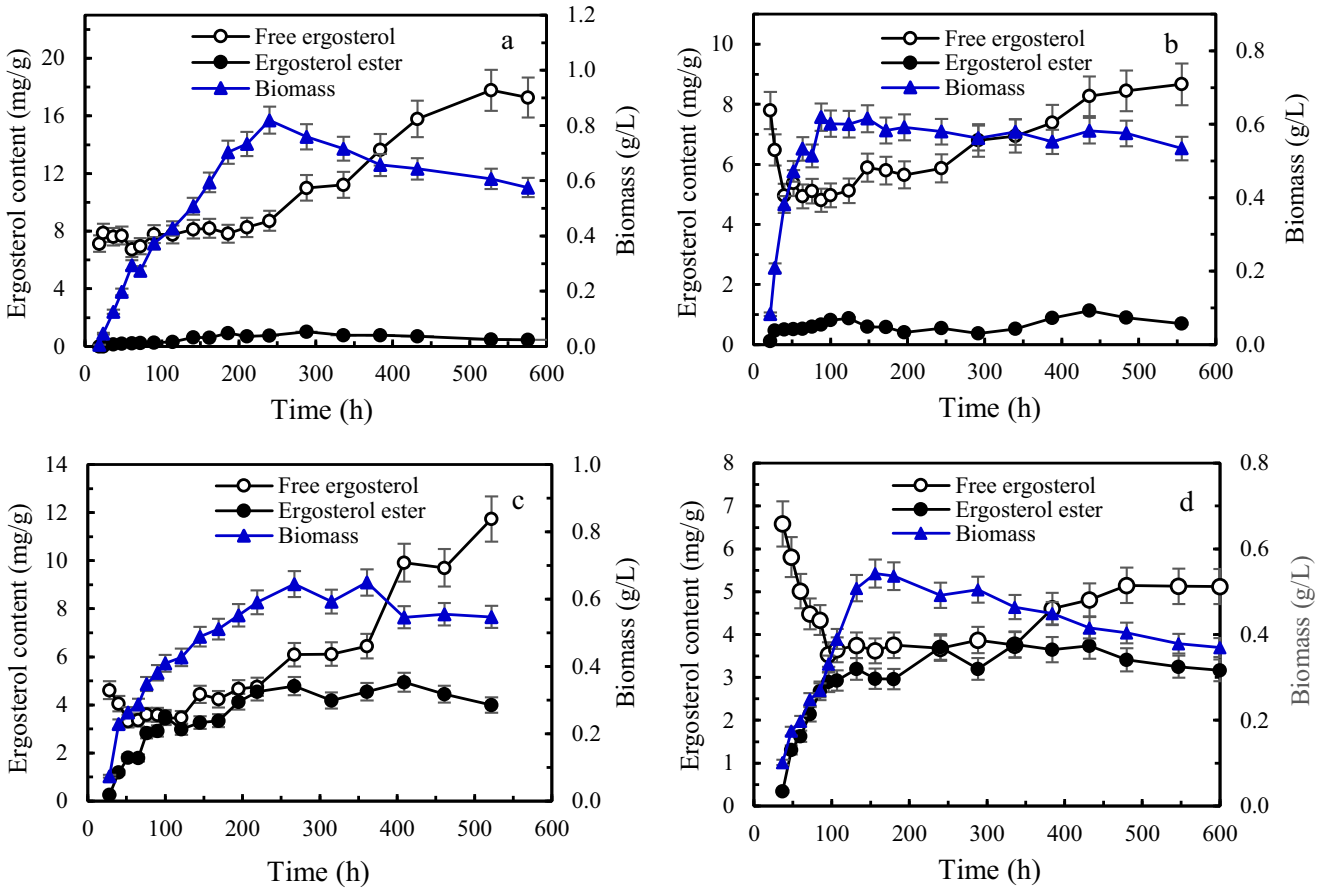


**Fig. 2** Changes in the biomass and the contents of free and esterified ergosterols during the growth of *Penicillium* sp. QA5 (**a**), *Trichoderma* sp. QA14 (**b**), *Fusarium* sp. QA34 (**c**), and *Nectria* sp. sp. QA44 (**d**). ($n$ = 3, RSD < 10%)

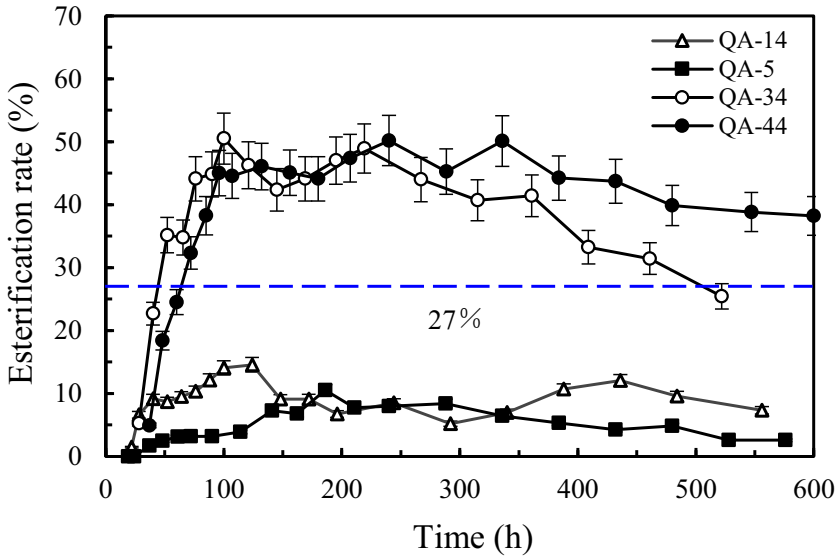


**Fig. 3** Changes in the ergosterol esterification rate during the growths of *Penicillium* sp. QA5 (■), *Trichoderma* sp. QA14 (Δ), *Fusarium* sp. QA34 (○), and *Nectria* sp. QA44 (●) ($n = 3$, RSD < 10%)

Using a conversion factor (225 μg dry fungal biomass $\mu g^{-1}$ ergosterol) of 4.44 mg/g dry fungal biomass based on the average free ergosterol content obtained in this study, the average fungal biomass of 8 mangrove soil samples is 0.39 mg/g dry soil mass, and the average fungal biomass of 5 wood soil samples is 1.22 mg/g dry soil mass (Table 5).

## Discussion

### Diversity of free and esterified ergosterols in fifty-nine strains of culturable fungi isolated from mangrove soil

As shown in Table 1, the fifty-nine strains of fungi isolated from the mangrove soil belonged to 13 genera, and the most abundant culturable fungus genera were *Penicillium* (28.8%), *Fusarium* (27.1%), *Nectria* (16.9%), and *Trichoderma* (6.8%). Therefore, most of the fungi isolated in this study are the most frequent genera in mangroves (de Souza Sebastianes et al. 2013; D'Souza and Rodrigues 2013) and in other habitats (Montgomery et al. 2000; Bosso et al. 2017).

The present study analyzed the contents of free and esterified ergosterols in the fifty-nine strains of fungi after 5 days of cultivation. As can be seen from Table 2, the contents of free, esterified, and total ergosterols varied greatly in different fungal strains, ranging from 2.28 to 10.05 mg/g, from 0 to 9.67 mg/g, and from 2.36 to 12.92 mg/g dry mycelia, respectively, indicating the diversity of ergosterol composition in fungi. These results in the contents of total ergosterols are roughly in agreement with previous studies, but the previous studies did not distinguish between free and esterified ergosterols. Gessner and Chauvet (1993) analyzed the total ergosterol contents of fourteen strains of aquatic hyphomycete species grown in liquid culture and found that the contents of ergosterol in fungal mycelium ranged from 2.3 to 11.5 mg/g dry mycelia. Pasanen et al. (1999) proposed that the average total ergosterol content of filamentous fungi varied from 2.6 to 14.0 mg/g dry mycelia. Barajas-Aceves et al. (2002) determined the total ergosterol content in the saponified methanol extracts of 20 white-rot fungus isolates and found that the values ranged from 2.4 to 13.1 mg/g fungal biomass. Klamer and Bååth (2004) analyzed the total ergosterol content ranging from 1 to 24 mg/g dry mycelia in eleven species of common fungi from compost, and suggested that the ergosterol content was related to the hyphal diameter of the fungi, and fungi with thinner hyphae had higher ergosterol concentrations, explaining the large interspecific variation in the content of ergosterol. Ermakova and Zuev (2017) proposed that fungal cell membranes could generally contain high proportions of sterols ranging from 2 to 50 mol%, depending on the cell type and the stages of membrane growth.

**Table 4** The comparison of the free and esterified ergosterol contents in the broken spores of *G. lucidum* with the previous results by Yuan et al. (2007a)

| Samples | Contents of ergosterol (mg/g dry spores) | | | Esterification rate (%) |
|---|---|---|---|---|
| | Free | Esterified | Total | |
| Spores (Yuan et al. 2007a) | 0.499 ± 0.012 | 0.360 ± 0.012 | 0.859 | 41.9 |
| Spores stored for over 12 years | 0.015 ± 0.001 | 0.159 ± 0.007 | 0.174 ± 0.006 | 91.4 |

**Table 5** Average contents of ergosterols of 8 mangrove soil samples from Qi'Ao Island and 5 soil samples from the nearby campus woods

| Samples | Average contents of ergosterol (μg/g dry soil mass) | | | Esterification rate (%) | Average fungal biomass (mg/g dry soil mass) |
|---|---|---|---|---|---|
| | Free | Esterified | Total | | |
| Mangrove soils | 1.748 ± 1.538 | ND | 1.748 ± 1.538 | 0 | 0.39 |
| Wood soils | 5.430 ± 1.348 | 0.886 ± 0.696 | 6.316 ± 0.850 | 14.0 (7.0–39.0) | 1.22 |

ND indicates below the lowest limit of detection (LOD)

The present results indicated that the fungal species, strain, and growth stage could affect the ergosterol composition. The previous studies have shown that other factors such as the growth conditions and nutritional status could affect the ergosterol content in the fungal biomass (Barajas-Aceves et al. 2002; Brosed et al. 2017; Nurika et al. 2018).

As can be seen from Table 3, the average contents of total ergosterol from 16 strains of *Fusarium*, 10 strains of *Nectria*, and 17 strains of *Penicillium* are 6.123 ± 1.892, 6.580 ± 2.210, and 6.049 ± 1.991 mg/g dry mycelia, respectively, which are all close to the average content of total ergosterol of 6.119 ± 1.930 mg/g dry mycelia from 59 strains of fungi. In addition, the average content of free ergosterol of 59 strains of fungi is 4.444 ± 1.474 mg/g dry mycelia, which is not much different from that of three genera of fungi. Therefore, the present study proposes the conversion factors of free and total ergosterol to fungal biomass of 4.44 and 6.12 mg/g fungal biomass, respectively, based on the average free and total ergosterol content from 59 strains of fungi.

Gessner and Chauvet (1993) gave an average ergosterol concentration of 5.5 mg/g dry mycelia from fourteen strains of aquatic hyphomycete species. Djajakirana et al. (1996) gave an average ergosterol concentration of 5.1 mg/g dry mycelia calculated from literature data. Montgomery et al. (2000) gave an average total ergosterol concentration of 4 mg/g dry mycelia from the six species of fungi isolated from soil and plant matrices. Newell (2001) proposed a mean ergosterol content of 6.2 mg/g mycelial mass with only 8% coefficient of variation. Klamer and Bååth (2004) analyzed the total ergosterol content in eleven species of common fungi from compost and proposed that the average content of total ergosterol was 3.1 mg/g dry mycelia. Brosed et al. (2017) analyzed the total ergosterol concentration of 16 fungal strains belonging to 8 species of aquatic hyphomycetes and found that the average ergosterol content was 5.0 mg/g dry mycelia. Wallander et al. (2013) suggested that the average concentration of total ergosterol of 4.5 mg/g dry mycelia was used to determine fungal biomass in soils. Baldrian et al. (2013, 2016) assumed that fungal biomass contained 3.8 mg/g ergosterol. Ekblad et al. (2016) and Hagenbo et al. (2018) proposed a total ergosterol content of 3 mg/g dry mycelia for fungal biomass estimation. Hendricks et al. (2016) used a conversion factor of 5 mg/g fungal biomass based on the average total ergosterol content of published estimates for ectomycorrhizal fungal species. Generally, the ergosterol content varied greatly, suggesting that care must be taken when the ergosterol content was used to compare data generated in different field environments (Charcosset and Chauvet 2001). Therefore, the use of ergosterol as fungal-specific biomarker is still disputable (Barajas-Aceves et al. 2002), and the use of a standard conversion factor for all fungi is inapposite (Nurika et al. 2018). The basic imperfections of the method are the difference in the ergosterol content depending on interspecies variation and growing conditions (Nylund and Wallander 1992), implying that a specific conversion factor needs to be determined and applied for any given fungus (Nurika et al. 2018).

In the present study, as can be seen from Fig. 1, the higher ergosterol esterification rate in the genera *Fusarium* and *Nectria*, and the lower ergosterol esterification rate in the genera *Penicillium* and *Trichoderma* were found, indicating that different genera of fungi might have different levels of ergosterol esterification rates. Therefore, in the present study, the contents of free ergosterol and ergosterol esters in four fungal strains *Penicillium* sp. QA5, *Trichoderma* sp. QA14, *Fusarium* sp. QA34, and *Nectria* sp. QA44 during the cultivation of about 600 h were analyzed. For *Penicillium* sp. QA5 (Fig. 2a) and *Trichoderma* sp. QA14 (Fig. 2b) with a lower esterification rate, the esterification rates kept fluctuation at a lower level with a maximum value of less than 15% (Fig. 3). In contrast, for *Fusarium* QA34 (Fig. 2c) and *Nectria* sp. QA44 (Fig. 2d) with a higher esterification rate, the esterification rates quickly increased to more than 40%, remained relatively constant, and then began to slightly fall (Fig. 3). The decrease of the esterification rates is due to the increase of free ergosterol, especially for *Fusarium* QA34, rather than the decrease of ergosterol esters. In fact, the change in the content of ergosterol ester is relatively small compared with free ergosterol upon entry of the culture into the stationary phase. Accordingly, it might be assumed that, although the ergosterol composition of fungi is diversity, the fungi might be divided into two classes, one is fungi with higher esterification rate (e.g., more than 27%) such as *Nectria* spp., *Fusarium* spp., and *Pseudallescheria* sp. QA-59, and the other is fungi with lower esterification rate (e.g., less than 27%) such as *Penicillium* spp., *Trichoderma* spp., *Hypocrea* spp.,

*Gliocephalotrichum* spp., *Xenoacremonium* sp. QA56, *Aspergillus* sp. QA36, *Debaryomyces* sp. QA15, and *Galactomyces* sp. QA49. The results indicate that the high variation in the ergosterol concentration and different esterification rates among the fungal strains show different functional trait of fungi.

## Degradation of free and esterified ergosterols in the broken spores stored in capsule similar to a simulated ecologic niche

Our previous study on the change of ergosterol composition in the dry fungal powder (the agaric fungi *Agrocybe aegerita* and *Termitomyces albuminosus*) after 6 months of storage showed that the esterified ergosterol was more stable than the free form, and the free ergosterol might be partly converted into the esterified ergosterol (Yuan et al. 2008). Another previous study on the analysis of ergosterol composition in the medicinal fungus *G. lucidum* showed that the contents of free ergosterol and esterified ergosterol in the broken and encapsulated spores of *G. lucidum* were 0.499 mg/g and 0.360 mg/g spores, respectively, with an esterification rate of 41.9% (Yuan et al. 2007a ). After more than a decade, as can be seen from Table 4, the retained same batch of the broken and encapsulated spores similar to a simulated ecologic niche was analyzed again, and the contents of free ergosterol and esterified ergosterol were found to have changed to 0.015 mg/g and 0.159 mg/g, respectively, with an esterification rate of 91.4%. The fact that the esterified ergosterol is more stable than the free form is further proved in the present study. These results indicate that the free and esterified ergosterols differ in their turnover rates in dead fungi and the broken spores, and this question is worth further investigation (Baldrian et al. 2013).

Moreover, the difference in turnover rates of the free and esterified ergosterols was also affirmed in the study on fungal biomass in old forest soil. de Ridder-Duine et al. (2006) and Wallander et al. (2010) found the trend towards an increase in the proportion of esterified ergosterol in older soil organic matter. In newly formed fungal mycelia, the ergosterol was mostly in free form; therefore, a part of ergosterol that had been esterified in older soil organic matter was unlikely to be connected with the active fungi. Ergosterol in inactive fungi might thus be conserved in soils rich in soil organic matter in a more stable esterified form (Wallander et al. 2010, 2013). Wallander et al. (2010) reported that, in the oldest soil organic matter from the mineral horizon, only 20% of the ergosterol was in free form, indicating that a portion of ergosterol had become esterified form in older soil organic matter. In addition, in a research on a chronosequence of boreal forested islands as a terrestrial net sink in the global carbon cycle, Clemmensen et al. (2013) measured the free and esterified ergosterols as the biomarkers for fungal biomass throughout each soil profile. Interestingly, it was found that total ergosterol was roughly similar on all islands, but free ergosterol was about 20 times more plentiful on large than on small islands. In contrast, esterified ergosterol was more plentiful on smaller islands, which had not burned in the past 5000 years, indicating older mycelia with slower biomass turnover, and impaired degradation and preservation of fungal residues in late successional forests (Clemmensen et al. 2013). Labidi et al. (2014) have also used free ergosterol content as a good proxy for the estimation of living saprophytic fungi in soil. Cheeke et al. (2017) suggested that the free ergosterol was used as a measure of active fungal biomass, and the total (free and esterified) ergosterol concentration was used to quantify the fungal biomass (active and inactive biomass). Although it has been approved that free ergosterol may be a better indicator for living fungi (Wallander et al. 2010; Clemmensen et al. 2013), a large number of studies still use the total ergosterol concentration to evaluate the fungal biomass, indicating that the estimation of fungal biomass by total ergosterol is acceptable in some cases, e. g., with lower esterification rates. When the ergosterol esterification rate in soil samples is relatively higher, free ergosterol may be a better marker for the estimation of fungal biomass. Wallander et al. (2013) proposed that the differentiation between free and esterified ergosterols might provide some additional information about the vitality of the fungal mycelium. The relation between free and esterified ergosterols could possibly be used as the biomarkers for the ratio of active and inactive fungi in soil, and this possibility would be very useful and deserves further studies (Wallander et al. 2013).

## Comparison of ergosterol composition and esterification rate between mangrove soil and wood soil

The ergosterol measurement was seen as a valuable tool to quantify soil fungal biomass and provide a relatively simple measure to record the response of fungi to soil treatments (de Ridder-Duine et al. 2006; Wallander et al. 2010). In the present study, as can be seen from Table 5, the average contents of free, esterified, and total ergosterols in five wood soil samples are $5.430 \pm 1.348$ μg/g, $0.886 \pm 0.696$ μg/g, and $6.316 \pm 0.850$ μg/g dry soil mass, respectively. The average ergosterol esterification rate of five wood soil samples is 14.0%, ranging from 7.0 to 39.0%, compared with 80% in an old soil organic matter reported by Wallander et al. (2010), indicating the potential relationship between aging degree of fungi in soil and ergosterol esterification rates. In contrast, the average free ergosterol content ($1.748 \pm 1.538$ μg/g dry soil mass) of 8 mangrove soil samples is distinctly lower than that of five wood soil samples, and even no esterified ergosterol is found in the mangrove soil samples, indicating the presence of only newly formed fungal mycelia with a lower growth rate and the oligotrophication of this relatively young mangrove soils as

compared with the old ones. Dinesh and Ghoshal-Chaudhuri (2013) showed that mean ergosterol concentration in the mangroves and the adjacent plantation developed by clearing the mangrove forests were 3.1 μg/g and 0.51 μg/g, respectively, indicating a possible change of soil from eutrophication to oligotrophication.

The previous studies showed that total ergosterol contents in soils ranged from 0.75 to 12.94 μg/g soil (Djajakirana et al. 1996). Djajakirana et al. (1996) showed that the mean total ergosterol content in grassland and forest soils was around 5.5 μg/g, and that in the arable soils was 2.14 μg/g. Grant and West (1986) showed that total ergosterol content in grassland and arable soils were 0.99–2.06 μg/g soil. Hendricks et al. (2016) gave the total ergosterol content of 0.43 μg/g soil. Voříšková et al. (2013) showed that the highest ergosterol content was 272 μg/g dry soil mass. However, no esterified ergosterol content in soils was reported in these studies.

In conclusion, in the present study, fifty-nine strains of fungi were isolated from the mangrove soil. The contents of free, esterified, and total ergosterol varied greatly in the fifty-nine strains of fungi, indicating the diversity of ergosterol composition in fungi. The average contents of free and total ergosterol from fifty-nine strains of fungi are 4.44 mg/g and 6.12 mg/g dry mycelia, respectively, with an average esterification rate of 27.4%. The present study assumes that these fungi might be divided into two classes, one is fungi with high esterification rates (e.g., more than 27%) such as *Nectria* spp. and *Fusarium* spp., and the other is fungi with low esterification rates (e.g., less than 27%) such as *Penicillium* spp. and *Trichoderma* spp. Moreover, the ergosterol esterification rate of 5 wood soil samples and 8 mangrove soil samples ranged from 0 to 39%, compared with 80% in an old soil organic matter in previous study. The increase in the ergosterol esterification rate in the older soil samples are caused by the potential further conversion from free to esterified ergosterol and the more rapid degradation of free ergosterol than esterified ergosterol. Therefore, the present study suggests that the free ergosterol and esterified ergosterol as the biomarkers should be simultaneously analyzed for the estimation of fungal biomass. The average content of free ergosterol of 4.44 mg/g or total ergosterol of 6.12 mg/g dry mycelia may be used to determine fungal biomass in soils, but the ergosterol esterification rate in soil samples, especially for the old forested soils, is a requisite parameter, which may contain a lot of important information, including the judgment of the senescence degree of fungal mycelia in soil, and even the comparison or discrimination of the age of the old forests over time scales of centuries to millennia.

**Authors' contributions** SJD, JP, and JPY conceived and designed research. SJD, YLJ, CXZ, YNL, and WLP conducted experiments. SJD, YLJ, QZ, QQW, XYD, and JHW analyzed the data. JP and JPY supervised the works at the Guangzhou and Zhuhai laboratories, respectively, and contributed to data interpretation. YLJ, SJD, JP, and JPY wrote the manuscript. All authors have read and approved the final version of the manuscript.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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