Scratching Stimuli of Mycelia Influence Fruiting Body Production and ROS-Scavenging Gene Expression of Cordyceps militaris

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ABSTRACT
The entomopathogenic fungus Cordyceps militaris is a valuable medicinal ascomycete, which degenerates frequently during subsequent culture. To avoid economic losses during industrialized production, scratching stimuli of mycelia was introduced to improve the fruiting body production. The present results indicated that higher yields and biological efficiency were obtained from two degenerate strains (YN1-14 and YN2-7) but not from g38 (an insertional mutant in Rhf1 gene with higher yields and shorter growth periods). Furthermore, the growth periods of the fruiting bodies were at least 5 days earlier when the mycelia were scratched before stromata differentiation. Three ROS-scavenging genes including Cu/Zn superoxide dismutase (CmSod1), Glutathione peroxidase (CmGpx), and Catalase A (CmCat A) were isolated and their expression profiles against scratching were determined in degenerate strain YN1-14 and mutant strain g38. At day 5 after scratching, the expression level of CmGpx significantly decreased for strain g38, but that of CmSod1 significantly increased for YN1-14. These results indicated that scratching is an effective way to promote fruiting body production of degenerate strain, which may be related at least with Rhf1 and active oxygen scavenging genes.

Introduction
Cordyceps militaris (Cordycipitaceae, Hypocreales, Sordariomycetes, and Ascomycota) is a valuable edible and medicinal fungus [1]. The medicinal properties and pharmacological effects include anti-tumor [2], anti-influenza virus [3], and immunomodulation [4], which are similar to another traditional medicinal fungus, Ophiocordyceps sinensis [5]. Because of the decreasing natural populations of O. sinensis and high price in the market [6], and the success of C. militaris stromata cultivation in artificial media [7–8], C. militaris is widely used as a good substitute for O. sinensis for health supplements [9].

In artificial cultivation of C. militaris, four growth stages including mycelia culture, pigment induction, stromata stimulation, and fruiting body production are usually identified [7]. Strain, medium components, culture parameters (temperature, humidity, light, and oxygen etc.), and mating behavior influence the industrialized production of C. militaris and optimization of culture conditions is required for each strain [10–12].

Like many other filamentous fungi, C. militaris frequently degenerates during mass production by the loss or reduction of mycelia growth, pigmentation, stromata differentiation, or fruiting body formation [13]. It is found that degeneration usually accompanies with reduced dehydrogenase activity and increased cellular accumulation of reactive oxygen species (ROS) [14,15]. The accumulation of ROS will attack many kinds of organic macromolecules and cause variety of injuries. Antioxidant enzymes can remove ROS and maintain the dynamic balance of O2− to defense against ROS [16]. Thus, reduction of the fungal cellular ROS levels could probably improve the characteristics of the degenerate strains.

Mycelia stimulus by scratching technique is one of the regular agronomic techniques to improve the cultivation yields in Agaricus bisporus and Pleurotus eryngii [17,18], but in Flammulina velutipes, this technique shows no significantly positive effect on the yield and the harvest time [19]. In Cordyceps, the effects of the scratching technique on mycelial growth, color induction, or fruiting body formation

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were variable [20] and even negative effects of this technique on fruiting body production were recorded [21].

To explore whether the scratching technique can induce a higher yield of fruiting bodies, and whether this induction is correlated to ROS-scavenging system in C. militaris, four strains (two degenerate strains YN1-14 and YN2-7, one normal strain YN2-11, and one insertional mutant g38 in Rhf1 gene) were used to evaluate the effects of scratching technique on the growth potentials, and the expression profiles of three antioxidant genes including Cu/Zn superoxide dismutase (CmSod1), glutathione peroxidase (CmGpx), and catalase A (CmCat A) were analyzed by using quantitative real-time PCR (qRT-PCR).

2. Materials and methods

2.1. Fungal strains

Four C. militaris strains were used in this study. Two degenerate strains YN1-14 and YN2-7 were characterized by fluffy mycelia and reduced ability of stromata formation. Strain YN2-11 was a strain with normal fruiting body production, whereas strain g38 was an insertional mutant in Rhf1 gene with faster stromata development and higher yields [22]. All the strains were preserved in Guangdong Institute of Applied Biological Resources, Guangzhou, China.

2.2. Fungal culture conditions

The culture conditions for mycelia in solid PPDA medium (200 g potato, 20 g glucose, 10 g tryptone, 1.5 g KH2PO4, 0.5 g MgSO4, 20 mg vitamin B1, 15 g agar powder, and distilled water to 1000 mL) and liquid PPDA medium (without agar powder) and for fruiting body production in rice medium were described previously [7]. All the test strains were cultured on solid PPDA at 23 °C for 7 days. Then, the mycelia from PPDA were transferred to 250 mL flasks containing 100 mL liquid PPDA medium. The flasks were then incubated at 23 °C on a 150-rpm shaker for 8 days and the resulting mycelia culture was used as a liquid inoculum. For fruiting body production, the liquid inoculum was diluted with three volumes of sterile water, and 15 mL was added into each 300 mL glass bottle containing with 20 g pearl rice, 0.5 g powder of silkworm pupae, and 25 mL nutrient solution (20 g glucose, 2 g KH2PO4, 1 g MgSO4, 1 g ammonium citrate, 5 g peptone, 20 mg vitamin B1, and distilled water to 1000 mL). The bottles with the substrates were sterilized at 121 °C for 30 min and cooled at room temperature before inoculation. After dark cultivation at 23 °C with the relative humidity of 60–70% for 12 days, the substrates were entirely colonized by the mycelia and then followed by illuminated cultivation (250 lx, 12 h/day) to induce pigmentation, stromata development and fruiting body formation. About 6–8 weeks were required for fruiting body maturation.

2.3. Mycelia scratching

The aerial mycelia of four strains on the surface of the substrates in each bottle were mechanically scratched by a metal scraper. Mycelia were scratched at different mycelia growth stages: (1) when mycelia entirely colonized the substrates 8 days post inoculation (S1), (2) when the mycelia turned light yellow 3 days post illumination (S2), and (3) mycelia turned yellow and became twist 6 days post illumination (S3). The mycelia without scratching were regarded as controls (NS). Ten bottles with three replicates to each treatment were established for each strain. The culture conditions were set as described above. The production parameters (the numbers, fresh weights per bottle and mature periods of fruiting bodies) were recorded at the end of the harvest period (when the end of the fruiting body swelling to a rod and its surface covered with white powder, the fruiting body is deemed to be mature). Biological efficiency is defined as the ratio of dry weight of fruiting body per dry weight of the

| Table 1. Primers used in this study. |
|------------------------------------|----------------------------------------------|
| Primers | Sequences (5'–3') | Notes                                      |
| CmSod1-F | ATGGTCAAAGCAGTCTGCCGTCCTC | Isolation of CmSod1, CmGpx, and CmCat A  |
| CmSod1-498R | CTACAAAGCAGTCTGCCGTCCTC | qRT-PCR for gene expression analysis |
| CmGpx-F | ATGGTCAAAGCAGTCTGCCGTCCTC | |
substrates. The fresh fruiting bodies and substrates were freeze-dried by using Savant Mldulyo freeze drier (Thermo Fisher Scientific, Waltham, MA, USA). Data were analyzed using one-way ANOVA and paired sample t-tests. The significant differences were determined by least significance difference (LSD) test ($p = 0.05$) with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

### 2.4. Genomic DNA and total RNA extraction

The mycelia of strains YN1-14 and g38 from liquid medium (PPDA without agar powder) at $23^\circ C$ on a 150-rpm shaker for 7 days were harvested by centrifugation and washed twice with sterile distilled water for genomic DNA and total RNA extraction. The genomic DNA was extracted based on the manual of DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). The total RNA was extracted according to the instruction of the RNasy Plant Mini Kit (Qiagen). Extraction was followed by DNase treatment using the RNase-Free DNase Set (Qiagen). The first strand cDNA was synthesized using $1 \mu g$ total RNA, oligo-dT primer based on the manual of the instruction of the PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Shiga, Japan). The synthesized cDNA was stored at $-20^\circ C$ for further use.

### 2.5. Isolation and sequence analysis of CmSod1, CmGpx, and CmCat A

RT-PCRs were performed to isolate *CmSod1*, *CmGpx*, and *CmCat A* genes using the designed primers (Table 1) with the PCR conditions: 2 min at $95^\circ C$; 5 cycles of $30 s$ at $94^\circ C$, $45 s$ at $62^\circ C$, 1.5 min at $72^\circ C$; 10 cycles of $30 s$ at $94^\circ C$, $45 s$ at $60^\circ C$ ($-0.5^\circ C/cycle$), 1.5 min at $72^\circ C$; 25 cycles of $30 s$ at $94^\circ C$, $45 s$ at $55^\circ C$, 1.5 min at $72^\circ C$; and 7 min at $72^\circ C$. These primers were designed on the base of the transcriptomic sequencing data (unpublished data). Homology analysis of the genes and amino acid sequences was conducted at BLAST server. Multiple sequence alignments were performed using the ClustalW program. Phylogenetic trees were reconstructed using MEGA5.1 software with the method neighbor-joining. The reliability of the resulting topologies was tested by running 1000 bootstrap replicates.

### 2.6. Sample collection

Mycelia were sampled for mRNA expressions at different growth stages after inoculation, including colonization of mycelia on the substrates (C0d), 5 days after scratching (S5d), and 5 days without scratching (C5d). Three samples from each developmental stage were collected and stored at $-80^\circ C$ for total RNA extraction as described above. The integrity of the total RNA was checked and $1 \mu g$ total RNA was used to synthesize cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara) following the manufacturer’s instructions.

### 2.7. qRT-PCR analysis

Two housekeeping genes *rpb1* and *tef1* were selected as internal controls to normalize the mRNA expression data [23]. All primer sets (Table 1) were designed with the software Primer 3 (version 0.4.0) and Primer Premier 5.0. Primers were tested with RT-PCR to confirm amplification of the desired size products (Supplemental Figure S1). qRT-PCR was performed on a Stratagene MX3000P qPCR system (Stratagene, Santa Clara, CA, USA) and SYBR® Premix Ex Taq II (Tli RNaseH Plus; Takara, Japan). A standard curve of amplification efficiency for the primer sets was generated with a fivefold dilution series of cDNA from C5d. Samples were assayed in

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**Table 2. Effects of scratching on fruiting body production of *C. militaris* strains at different mycelia growth stages.**

| Strains | Treatments | Fruiting body weight per bottle (g) | Numbers of fruiting bodies per bottle | Biological efficiency (%) | Fruiting body mature periods after inoculation (d) |
|---------|------------|------------------------------------|-------------------------------------|--------------------------|-----------------------------------------------|
| g38     | S1         | 15.70 ± 0.18a                       | 95.70 ± 1.31a                       | 24.08 ± 0.42a            | 47.00 ± 0.00a                                 |
|         | S2         | 15.79 ± 0.19a                       | 95.50 ± 0.85a                       | 24.02 ± 0.27a            | 47.20 ± 0.19a                                 |
|         | S3         | 15.86 ± 0.22a                       | 96.50 ± 1.42a                       | 24.29 ± 0.35a            | 47.20 ± 0.24a                                 |
|         | N5         | 15.74 ± 0.26a                       | 96.60 ± 1.78a                       | 23.73 ± 0.37a            | 47.00 ± 0.00a                                 |
| YN2-11  | S1         | 15.93 ± 0.18a                       | 57.80 ± 3.22b                       | 24.03 ± 0.45a            | 50.00 ± 0.00b                                 |
|         | S2         | 16.14 ± 0.24a                       | 64.40 ± 3.82b                       | 24.13 ± 0.31a            | 50.10 ± 0.18b                                 |
|         | S3         | 15.78 ± 0.12a                       | 69.00 ± 5.37a                       | 24.32 ± 0.34a            | 50.30 ± 0.28a                                 |
|         | NS         | 14.36 ± 0.25b                       | 50.90 ± 2.33c                       | 22.06 ± 0.48b            | 55.20 ± 0.24a                                 |
| YN1-14  | S1         | 15.91 ± 0.31a                       | 69.38 ± 4.07b                       | 23.91 ± 0.41a            | 50.20 ± 0.19c                                 |
|         | S2         | 16.11 ± 0.21a                       | 64.88 ± 2.47b                       | 24.13 ± 0.40a            | 50.20 ± 0.19c                                 |
|         | S3         | 15.81 ± 0.16a                       | 74.63 ± 1.80a                       | 24.14 ± 0.34a            | 57.50 ± 0.34a                                 |
|         | NS         | 8.18 ± 0.93b                        | 22.00 ± 2.81c                       | 13.09 ± 1.20b            | 56.20 ± 0.24b                                 |
| YN2-7   | S1         | 15.65 ± 0.23a                       | 37.40 ± 1.51b                       | 24.19 ± 0.33a            | 48.20 ± 0.20c                                 |
|         | S2         | 15.24 ± 0.46ab                      | 48.40 ± 0.87a                       | 24.18 ± 0.44a            | 48.30 ± 0.21c                                 |
|         | S3         | 14.10 ± 0.31b                       | 50.80 ± 2.23a                       | 24.19 ± 0.46a            | 56.50 ± 0.37a                                 |
|         | NS         | 9.14 ± 0.56c                        | 19.70 ± 0.90c                       | 15.56 ± 0.91b            | 52.20 ± 0.29b                                 |

*NS: non-scratched; S1: scratched after mycelia fully grew in the substrates; S2: scratched after mycelia turned light yellow; S3: scratched after mycelia turned yellow and became twist.

Biological efficiency, the ratio of dry weight of fruiting body (g) per dry weight of the substrates (g).
ANOVA using SPSS 16.0. The significant differences indicated. All the data were analyzed by one-way ANOVA using SPSS 16.0. The significant differences were determined by LSD test (p = 0.05).

3. Results

3.1. Effects of scratching on fruiting body production

Higher yields and biological efficiencies, and shortened fruiting body mature periods after inoculation were obtained from three strains YN1-14, YN2-7, and YN2-11 except g38 after scratching stimuli of mycelia at three vegetative stages (Table 2; Supplemental Figure S2). The periods for harvesting mature fruiting bodies of YN1-14 and YN2-7 were shortened at least 5 days when the mycelia were scratched at S1 and S2, compared with those at S3 stage (Table 2). The fruiting body numbers per bottle were significantly higher from g38 than those from YN1-14 and YN2-7 (p < 0.01, p < 0.01). Therefore, scratching stimulus of mycelia at S1 and S2 stages was an effective way to improve fruiting body yields of *C. militaris*.

3.2. mRNA expression of CmSod1, CmGpx, and CmCatA under scratching stimuli of mycelia

The sequences of CmSod1, CmGpx, and CmCatA from *C. militaris* were isolated and deposited in GenBank under accession number KX911468, KX911469, and KX911470, respectively. The open reading frame of CmSod1 was 498 bp encoding a polypeptide of 165 amino acids with a predicted molecular mass of 17.1 kDa and theoretical isoelectric point of 6.07. CmGpx gene encoded a mature protein of 170 amino acids with a calculated molecular mass of 18.9 kDa and theoretical isoelectric point of 6.03. CmCatA gene was 2178 bp, encoding a protein with 725 amino acids. No signal peptide was predicted from these three sequences. CmSod1 was a cytoplasmic Cu, Zn Sod. Three cysteines (Cys 6, Cys 58, and Cys 147) were found in the mature Sod1, and two of them (Cys 58 and Cys 147) were coincided with the cysteines known to be involved in an internal disulfide bond. The CmGpx protein contained a normal cysteine residue (Cys 39) instead of a selenocysteine at the catalytic site. The CmCat A protein contained histidine residue (His 75) and asparagine residue (Asn 148) at the catalytic site and tyrosine residue (Tyr 362) at the metal binding site. Phylogenetic trees (Supplemental Figure S3) based on the amino acid sequences similarity showed CmSod1, CmGpx, and CmCat A shared the high degrees of homology to those derived from proteins from other Ascomycota fungi, suggesting that the functions of these three genes could be relatively conservative in evolution.

The expression profiles of CmSod1, CmGpx, and CmCat A at 5 days without scratching and 5 days after scratching were compared in strains g38 and YN1-14 by qRT-PCR using rpb1 and tef1 as internal controls. The correlation coefficients of all PCRs were superior to 0.99 and the amplification efficiency of five genes ranged from the lowest for rpb1 (98.5%) to the highest for CmSod1 (110.7%; Table 3). For g38 strain, the expression level of CmGpx in S5d significantly decreased (Figure 1(a)). For YN1-14, the expression level of CmSod1 significantly increased in S5d compared with C5d (Figure 1(b)). These results indicated that scratching negatively influenced the expression level of CmGpx in g38 mutant, and significantly increased that of CmSod1 in YN1-14 strain.

4. Discussion

The insect-born fungus *C. militaris* is an important medicinal fungus. Degeneration is a major constraint for artificial cultivation of this valuable *C. militaris* fungus. Mycelia stimuli by scratching technique have been recognized as a useful agronomic behavior in *A. bisporus* and *P. eryngii* culture but not in *F. velutipes* [17–19]. The scratching technique has a positive effect on the total yield of fruiting bodies in *A. bisporus*, *P. eryngii*, and *P. flabellatus* [17,18,25]. In this study, the scratching technique was introduced to evaluate its effect on the degenerated strain with reduced ability of stromata formation and mutant strain g38 with higher yield and shorter growth period at different mycelia growth stages. The present results showed that scratching
stimuli of mycelia could significantly improve fruiting body production and shorten production periods for the degenerate strains characterized by fluffy mycelia and reduced ability of stromata formation. However, scratching stimuli of mycelia did not improve fruiting body production and production period of g38 mutant. Higher yield was obtained from YN2-11 in the three scratched groups than that of g38. These results indicated that scratching was an effective way to improve the fruiting body production, especially for the degenerate strains. Therefore, scratching would be taken into consideration to reduce the economic loss when C. militaris strains exhibited reduced stromata differentiation in commercial production. In practice, scratching stimuli of mycelia should be carried out when the mycelia fully grow in the substrates or mycelia turn yellow slightly, to obtain higher yield and shorter growth period.

In A. bisporus, the scratching technique creates an open structure in the casing layer to induce the higher yield [17]. Previous studies indicate that degeneration of C. militaris cultures is related with cellular accumulation of ROS [15]. To reveal whether ROS-scavenging system was involved in the improved characteristics of YN1-14 strain by scratching stimuli of mycelia, the expression profiles of three main genes encoding antioxidant enzymes were determined. For YN1-14 strain, the expression level of CmSod1 significantly increased after 5 days of mycelia stimuli by scratching. Previous studies also indicated that transcript levels of genes encoding antioxidant enzymes could be strongly induced under various environmental stresses and the activated gene expressions could contribute to the improved stress tolerance in plants [26,27]. But for the insertional mutant g38 in Rhfl gene, the expression pattern of CmGpx was down-regulated. The Rhfl gene was involved in the fruiting body production of C. militaris fungus and that silencing the Rhfl gene could improve the formation and yields of fruiting bodies during the commercialization of this medicinal fungus [22]. The fruiting ability of degenerate C. militaris strain could be restored by overexpression an antioxidant glutathione peroxidase (Gpx) gene from Aspergillus nidulans to increase oxidative stress tolerance [28]. It seems that the association between Rhfl and CmGpx in regulating the fruiting body production needs further study.

Disclosure statement

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of the country in which they were performed.

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