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RESEARCH ARTICLE

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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.

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Introduction

Cordyceps militaris (Cordycipitaceae, Hypocreales, Sordariomycetes, and Ascomycota) is a valuable edible and medicinal fungus [1]. The medicinal properties and pharmacological effects include antitumor [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. militarlis* 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 O^{2-} 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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Supplemental data for this article can be accessed on the publisher's website.

Table 1. Primers used in this study.

Primers	Sequences (5′–3′)	Notes
CmSod1-F	ATGGTCAAAGCAGTCTGCGTTCTCC	Isolation of CmSod1, CmGpx, and CmCat A
CmSod1-498R	CTACAAAGCAGTTAGCAAGCCTGTCG	
CmGpx-F	ATGTCTTCTGCCGCGTCCTTTTAC	
CmGpx-632R	CACTCGGCTTTTTTGTTAATCTCGG	
CmCatA-F	ATGAAGGCGGCGCAAATTAAAGC	
CmCatA-2175R	ATACGCAACAAGATCAACCAGCCCAT	
rpb1-F	CTGTTCCCCCTCCTCCTGTG	qRT-PCR for gene expression analysis
rpb1-R	ATGTTGCGGCGATCCTTCTC	
tef1-F	GTCAAGGAAATCCGTCGTGGTAA	
tef1-R	GCAGGCGATGTGAGCAGTGTG	
CmSod1-135F	CTTCCACATCCACACCTTT	
CmSod1-268R	TCTTGATGTTGCCGAGATC	
CmGpx-305F	ACTTTGTCATCCTCGGCTTT	
CmGpx-417R	CAAGATGGGAAAGGTGACG	
CmCatA-979F	GACGAGTACTTTACCGAGAC	
CmCatA-1173R	GCGGTTAAAGTTCATCATGG	

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 KH_2PO_4, 0.5 g $MgSO_4$, 20 mg vitamin B_1, 15 g agar powder, and distilled water to 1000 mL) and liguid 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 KH_2PO_4, 1 g $MgSO_4$, 1 g ammonium citrate, 5 g peptone, 20 mg vitamin B_1, 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 at dark (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 2. Effects of scratching on fruiting body production of *C. militarlis* strains at different mycelia growth stages.

Strains	Treatments[a]	Fruiting body weight per bottle (g)	Numbers of fruiting bodies per bottle	Biological efficiency[b] (%)	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
	NS	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 ± 2.32bc	24.03 ± 0.43a	50.00 ± 0.00b
	S2	16.14 ± 0.24a	64.40 ± 3.82ab	24.13 ± 0.31a	50.10 ± 0.18b
	S3	15.78 ± 0.12a	69.00 ± 5.37a	24.32 ± 0.34a	55.30 ± 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 ± 4.07ab	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 ± 0.44a	56.50 ± 0.37a
	NS	9.14 ± 0.56c	19.70 ± 0.90c	15.56 ± 0.91b	52.20 ± 0.29b

[a]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.
[b]Biological efficiency, the ratio of dry weight of fruiting body (g) per dry weight of the substrates (g).

substrates. The fresh fruiting bodies and substrates were fieeze-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 °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 RNeasy 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 μg 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 °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 °C; 5 cycles of 30 s at 94 °C, 45 s at 62 °C, 1.5 min at 72 °C; 10 cycles of 30 s at 94 °C, 45 s at 60 °C (−0.5 °C/cycle), 1.5 min at 72 °C; 25 cycles of 30 s at 94 °C, 45 s at 55 °C, 1.5 min at 72 °C; and 7 min at 72 °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 °C for total RNA extraction as described above. The integrity of the total RNA was checked and 1 μg 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

Table 3. Details of the primer pairs for qRT-PCR analysis.

Gene name (abbreviation)	Amplicon length (bp)	Efficiency (%)	Slope	R^2
RNA polymerase II large subunit (*rpb1*)	152	98.5	−3.358	0.999
Translation elongation factor 1-alpha (*tef1*)	162	99.3	−3.339	0.999
Copper-zinc superoxide dismutase gene (*CmSod1*)	134	110.7	−3.112	0.994
Glutathione peroxidase (*CmGpx*)	113	107.3	−3.158	0.999
catalase A (*CmCat A*)	195	110	−3.110	0.990

triplicate in a 25-mL final volume and the thermal cycling conditions were the same as described before [24]. Cycle threshold (Ct) values were averaged for each triplicate by SPSS software. Samples were normalized using the geometric mean of *rpb1* and *tef1* Ct values. The expression levels were calculated using the $2^{-\triangle\triangle Ct}$ method for each biological replicate. 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. *CmCat A* 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

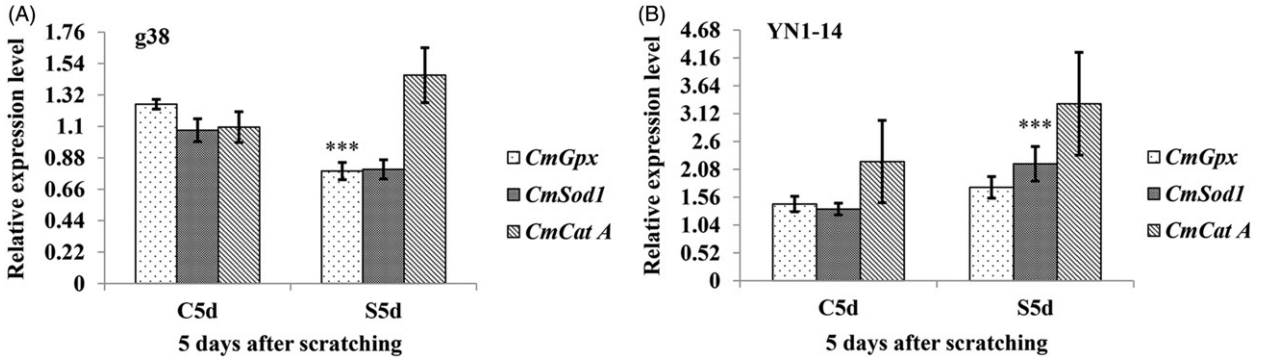

Figure 1. qRT-PCR analysis of *CmSod1*, *CmGpx*, and *CmCat A* at 5 days after scratching stimuli of *Cordyceps militaris* mycelia. (a) g38 strain. (b) YN1-14 strain. C5d: 5 days without scratching. S5d: 5 days after scratching. Data represented the means of three replicates. The vertical bars represented standard errors and the asterisk indicated significant difference ($p < 0.05$).

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 *Rhf1* gene, the expression pattern of *CmGpx* was down-regulated. The *Rhf1* gene was involved in the fruiting body production of *C. militaris* fungus and that silencing the *Rhf1* 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 *Rhf1* 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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