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Effects of caffeine on growth and metabolite production of *Cordyceps militaris*

Thanyarat Puttika[a,b], **Waeowalee Choksawangkarn**[c], **Sunita Chamyuang**[a,b], **Amorn Owatworakit**[a,b,*]

[a] School of Science, Mae Fah Luang University, Chiang Rai 57100 Thailand
[b] Microbial Product and Innovation Research Group (MP&I), Mae Fah Luang University, Chiang Rai 57100 Thailand
[c] Department of Biochemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Burapha University, Chonburi 20131 Thailand

*Corresponding author, e-mail: amorn@mfu.ac.th

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ABSTRACT: This study was aimed to address the caffeine response of *Cordyceps militaris*, an entomopathogenic fungi, on growth, development, metabolite production, and caffeine modification. Our results demonstrated that caffeine showed a dosage effect on growth, development, and adenosine production of *C. militaris*. Without caffeine, the mycelial growth rate of the control was 0.42 ± 0.02 cm/day, but at concentrations of 0, 1 and 10 mM, caffeine inhibited the mycelial growth of *C. militaris* with reduced growth rates of 0.37 ± 0.01 and 0.27 ± 0.01 cm/day, respectively; and the number of conidia was also reduced by 23% and 77%, respectively. Caffeine also affected the fungal metabolite production by increasing the extracellular adenosine productivity 5-fold (from 4.34 ± 0.60 µg/g/day to 24.28 ± 0.65 µg/g/day), but there was no change in the cordycepin production ($p < 0.05$). Interestingly, this fungus could metabolize caffeine, resulting in an accumulation of its derivatives, including theophylline (55.23 ± 2.18 µg/ml), theobromine (16.95 ± 0.97 µg/ml), and paraxanthine (12.88 ± 1.22 µg/ml). The findings provided a new understanding of how caffeine affects the growth of *C. militaris*. The information could be useful for the production of bioactive compounds of either adenosine or caffeine derivatives.

KEYWORDS: caffeine, *Cordyceps militaris*, adenosine, cordycepin, N-demethylation

INTRODUCTION

Caffeine is a purine analogue closely related to, adenine and guanine, two essential components involved in a wide range of biological processes in eukaryotic cells [1, 2]. The effect of caffeine has been studied in yeasts [3], filamentous fungi [4], and mushrooms [5, 6]; however, it has not been reported in entomopathogenic fungi. Many reports showed a wide range of pleiotropic effects of caffeine on multiple cell integrity pathways of the fungal cells, either activation or inhibition, including cell proliferation, DNA damage repair, and cell cycle regulation [1, 3, 7]. However, Caffeine has been investigated as an alternate carbon and nitrogen source for caffeine metabolism in filamentous fungi [8] and edible mushrooms such as *Pleurotus ostreatus*, *Lentinula edodes*, and *Flammulina velutipes* cultivated on coffee residues [9, 10], which required N-demethylation and demethylation of caffeine into theophylline and 3-Methylxanthine [8, 11]. Of these, it is noteworthy that theophylline and theobromine, being the active compounds, have gained increased significance in medical applications.

Cordyceps militaris, an entomopathogenic fungi, belongs to the order Hypocreales of the Ascomycota. It is a parasitic fungus that colonizes insect larvae or pupae and whose fruiting bodies burst out of the host. So far, the fungus can be grown in culture and has significant medical benefits [12]; thus, market demand for artificial *C. militaris* has increased. Adenosine (or 3'-deoxyadenosine), a nucleoside analogue, and cordycepin, are notable bioactive compounds of Cordyceps, with significant medical properties, such as anticancer [13], antioxidant [14], anti-influenza virus [15], and anti-inflammatory [16]. From a biotechnological perspective, *C. militaris* is a target source of valuable natural compounds with a range of medicinal uses. The fungus has been developed and successfully cultivated in artificial media in a large-scale production.

Caffeine had previously been observed to impact the production of secondary metabolites and inhibit growth of fungal cells. It participates in the same stress response as xenobiotics such as hydrogen peroxide [2, 3, 17]. A few studies have examined *C. militaris* and discovered that the fungus's oxidative stress response during cultivation and subsequent subculture has an impact on bioactive compound production, mycelial growth, and fruiting bodies [18–20]. The purpose of this study was to investigate how caffeine affects cell growth, metabolite production, and detoxification of caffeine in *C. militaris*. So, we understand how caffeine affects *C. militaris* growth and development, particularly the synthesis of adenosine and cordycepin.

MATERIALS AND METHODS

Fungal strain and culture condition

Cordyceps militaris strain ATCC 34165 was purchased from American Type Culture Collection (ATCC, USA). The fungal mycelium was cultured on potato dextrose

agar (PDA) (Himedia, India) at 20 °C for 3–4 weeks and kept at 4 °C before further use in the experiment.

Fungal mycelial cultivation

The fungal mycelium was cultured on PDA with a supplement of caffeine (Sigma, USA) at 1 and 10 mM concentrations and kept at 20 °C for 3 weeks. Similarly, a control was prepared without caffeine supplement. The fungal growth rates were calculated by dividing the mycelial colony diameter (in centimeters) with the cultivation time (in days) [21].

Determination of conidial production

Fungal conidia were collected from the 3-week-old mycelium cultured on PDA using the protocol of Wang et al [22] with some modification. The area of the hyphal colony was normalized by selecting the mycelial from the edge of the fungal colony in each treatment. Ten agar plugs (8 mm in diameter) were transferred into 5 ml of 0.01% Tween 80 (Vivantis, Malaysia) and rotary shaken at 125 rpm for 1 h. Mycelium part was removed from spore suspension by filtering through a double layer of cheesecloth. A hemocytometer was used to count the number of conidia under microscope at 400× magnification.

Metabolite analysis and productivity

Analysis of adenosine and cordycepin using High-Performance Liquid Chromatography (HPLC)

Amounts of adenosine and cordycepin were quantified from crude extracts of mycelium and culture media using 1 g of freeze-dried mycelium in 20 ml of deionized water at 60 °C for 3 h [20] and 5 g of solid fungal growth media, respectively. Before conducting HPLC analysis, the crude extracts were filtered using a 0.2-µm filter membrane. Stock standard solutions (2 mg/ml) of adenosine and cordycepin (Sigma) were prepared. HPLC analysis was performed on the serial dilutions of the stock solution, with concentrations of 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 250 µg/ml. The crude extract was quantified by plotting peak area against concentration. One microliter of crude extracts was analyzed by reversed-phase column (Water, USA) (C18, 4.6 × 250 m, 5-µm) with a flow rate of 0.2 ml/min at 30 °C. The condition was isocratic 95:5 (0.2% formic acid in water (A):methanol (B)). Both adenosine and cordycepin were monitored at 260 nm. The productivity of adenosine and cordycepin (expressed as µg/g dry weight/day) was determined by subtracting the amount of adenosine or cordycepin (in mycelia and extracellular adenosine in medium) at day 21 with day 1 and divided by cultivation time (total 21 days) [21].

Quantification of caffeine and its derivatives using Liquid Chromatography-Mass spectrometry (LC-MS) analysis

The crude extract samples were analyzed using a Nexera X2 System, which included the SIL-30AC autosampler and LCMS-8060 triple quadrupole mass spectrometer from Shimadzu Corporation in Kyoto, Japan. The mass spectrometer had an electrospray ionization-positive and -negative source. A Shim-pack LC column (100 × 2.1 mm and a particle size of 3.0 µm) (Shimadzu, Ltd.) was used. The temperature of the column oven was kept constant at 30 °C throughout the entire analytical process. A volume of 1 µl of each sample was injected into the column and eluted using a binary solvent gradient consisting of 0.1% formic acid in water (solvent A) and methanol (solvent B) at a flow rate of 0.3 ml/min. The separation process was conducted at 30 °C, employing a gradient program that spanned the following time intervals: from 0 to 0.5 min, the solvent composition was 30% B; from 0.5 to 3.5 min, the solvent composition transitioned from 30% B to 100% B; from 3.5 to 5.0 min, the solvent composition remained at 10% B; and from 5.01 to 6 min, the solvent composition shifted from 100% B back to 30% B. Quantitative analysis was done by using an Agilent QQQ mass spectrometer with positive ionization and dynamic multiple reaction monitoring (MRM) mode. For the mass spectrometry analysis, various parameters were set: desolvation line temperature, 250 °C; heat block temperature, 400 °C; drying gas flow rate, 10.0 l/min; and nebulizer gas flow rate, 3.0 l/min. Other specific parameters about the analytes after derivatization were provided in Table 1. However, precise quantification of the compounds was carried out by using standard caffeine and caffeine derivatives (Sigma) with concentrations of 0.05, 0.1, 1, 5, and 10 µg/ml.

Statistical analysis

The experiments were performed in three replicates, and statistical analysis was conducted using SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance and Duncan's multiple-range tests were used to determine significant differences. *p*-values below 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Effects of caffeine on mycelial growth and conidia production

We investigated the effect of caffeine on *C. militaris* by cultivating the fungal mycelium on PDA with different caffeine concentrations (0, 1, and 10 mM) for three weeks at 20 °C, and then investigating mycelial growth, morphology, and conidial number. The results showed that caffeine has a significant effect on the mycelial

Table 1 Multiple reaction monitoring (MRM) parameters of caffeine and its derivatives.

Compound	Retention time	Molecular formula	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
Caffeine	7.415	$C_8H_{10}N_4O_2$	195.00	138.15 110.10	−19.0 −22.0
Paraxanthine (1,7-dimethylxanthine)	5.474	$C_7H_8N_4O_2$	181.10	124.05	−21.0
Theophylline (1,3-dimethylxanthine)	5.147	$C_7H_8N_4O_2$	181.15	124.05	−19.0
Theobromine (3,7-dimethylxanthine)	3.816	$C_7H_8N_4O_2$	181.10	163.10 138.10 110.05	−19.0 −19.0 −22.0
3-methylxanthine	2.590	$C_6H_6N_4O_2$	167.10	149.05	−20.0
7-methylxanthine	2.277	$C_6H_6N_4O_2$	167.10	124.10	−20.0
Xanthine	1.569	$C_5H_4N_4O_2$	153.00	135.95 110.05	−14.0 −20.0

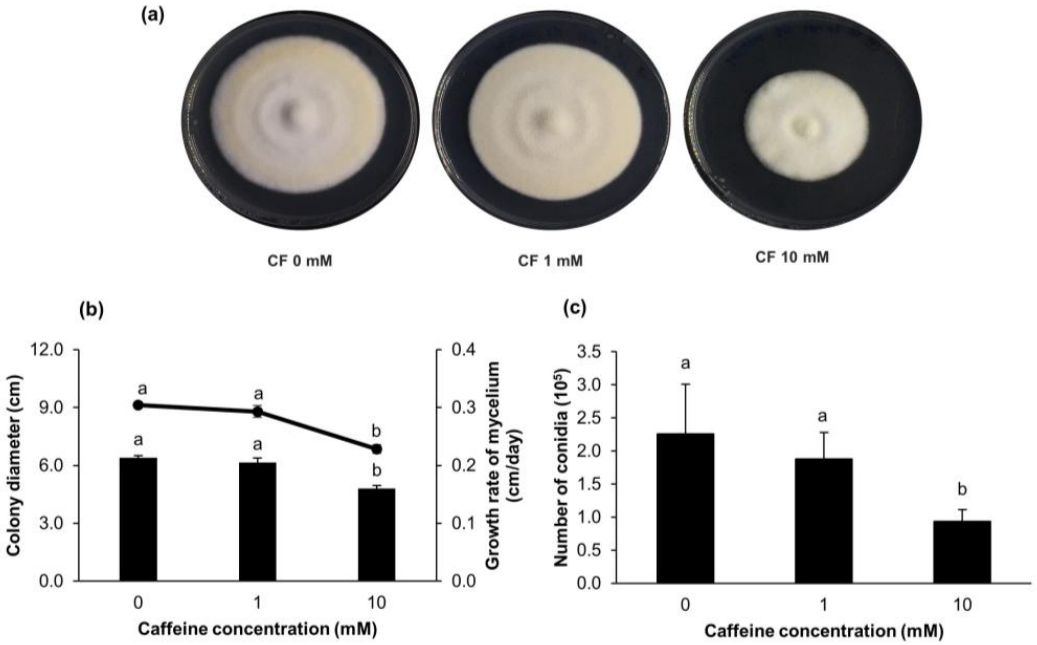

Fig. 1 Effects of different concentrations of caffeine (CF) on the mycelial growth of *C. militaris* cultivated on PDA for 3 weeks by reducing: (a, b), mycelial colony diameter; (b), mycelial growth rate; and (c), number of conidia. Different letters above the bars indicate significant differences ($p < 0.05$).

growth and development of *C. militaris*, as illustrated in Fig. 1a,b. In the presence of caffeine at concentrations of 1 mM and 10 mM, the fungal growth rates were reduced to 0.37 ± 0.01 cm/day and 0.27 ± 0.01 cm/day, respectively, as compared with 0.42 ± 0.02 cm/day of the control (without caffeine). Moreover, Fig. 1c shows that caffeine at concentrations of 1 mM and 10 mM reduced the conidial number by 23% and 77%, respectively, with an initial conidial concentration of 1.45×10^6 conidia/ml. These findings suggest that caffeine inhibited the growth and the development of *C. militaris* with a concentration dependent response.

The results were consistent with previously reported findings that the higher the levels of caffeine (ranging from 0.01% to 1%), the more significant inhibition of fungal growth was observed in various filamentous fungi, including *Rhizoctania solani*, *Fusarium oxysporum*, *Sclerotinia sclerotiorum*, *Glomerella cingulate*, *Trichoderma* spp., and *Monacrosporium ambrosium* [6]. The growth suppression of the fungus might be attributed to the effects of crucial enzymes involving in cell wall synthesis, modification of cell

membrane, preservation of cell integrity, or enhancing stress resistance. For example, the chitin synthase enzyme, together with endo-1,3(4)-beta-glucanase and glucan transferase enzymes, play an important role in the synthesis of fungal cell wall. Furthermore, caffeine has been shown to undermine the integrity of the fungal cell membrane, resulting in the leakage of intracellular contents and consequent cell death in *Colletotrichum gloeosporioides* [22]. In contrast, the fungal enzyme endo-1,3-glucanase has been exploited as a target for inquiry into antifungal medications, such as apigenin and luteolin, which block the activity of fungal endo-1,3-β-glucanase [23].

Alternatively, the delayed growth rate of *C. militaris* might involve cell cycle regulation by caffeine binding to nucleotide receptors, hence inhibiting DNA replication during the G2 phase of the cell cycle (see Fig. 1a,b). In addition, it has been reported of inhibitory effect on Target-of-Rapamycin (TOR) pathway, which regulates cell growth and metabolism, in response to environmental stimuli and stress conditions [24, 25]. For example, Schizosaccharomyces pombe upregulated the expression of antioxidant protein ABC transporter in response to coffee and hydrogen peroxide exposure [1, 26–28]. *C. militaris* can respond to stress by increasing antioxidant enzymes like superoxide dismutase and glutathione peroxidase during successive cultures [18, 19].

Effects of caffeine on extracellular adenosine and cordycepin production

Adenosine and cordycepin are the major active compounds in *C. militaris*. Dried mycelia and culture media from all treatments were collected and extracted with distilled water at 60 °C for 3 h and then determined the productivity of adenosine and cordycepin using HPLC analysis (Table 2). The results demonstrated that caffeine could promote the production of extracellular adenosine but not the cordycepin (Table 2). At caffeine concentrations of 1 mM and 10 mM, the extracellular adenosine productions were 4.94 ± 0.67 µg/gDW/day and 24.28 ± 0.65 µg/gDW/day, respectively, with a 5-fold increase. In contrast, the adenosine production in mycelia treated with caffeine was decreased. Interestingly, caffeine did not affect the productivity of cordycepin in both the fungal mycelia and the culture media ($p < 0.05$) (Table 2). This suggested that caffeine appeared to have more influence on *C. militaris* growth and development than in secondary metabolite production.

The promotion of caffeine on the extracellular adenosine production in *C. militaris* suggested that the production might be regulated by the equilibrium of the compound's transport and production. Alternatively, the changes of fungal cell membrane and cell wall might affect the adenosine transport. As caffeine affected the levels of adenosine in *C. militaris* (Fig. 1a); hence, adenosine production could be linked to cell growth and development and cell balance, but not the cordycepin production [20, 34]. Similarly, the different levels of caffeine also affected the synthesis of a secondary metabolite, ochratoxin A1, in *Aspergillus ochraceus* [4].

Capability of *C. militaris* in caffeine modification

The caffeine (1,3,7-Trimethylxanthine) modification of *C. militaris* through demethylation pathway contributes to the formation of six derivatives: paraxanthine (1,7-Dimethylxanthine), theophylline (1,3-Dimethylxanthine), theobromine (3,7-dimethylxanthine), 3-Methylxanthine, 7-Methylxanthine, and xanthine (Fig. 2a) [32–34]. *C. militaris* catalyzes caffeine by N-demethylation, and the results of the catalyzation are, for example, paraxanthine, theophylline, and theobromine. In this study, theophylline was a major demethylated caffeine product in both the mycelia (16.71 ± 0.52 µg/ml) and the culture media (38.52 ± 1.66 µg/ml). In addition, concentrations of theobromine and paraxanthine were found at 16.95 ± 0.97 and 12.88 ± 1.22 µg/ml, respectively, whereas only trace amounts of xanthine were detected. It was noted that caffeine concentrations might directly link to demethylation levels in *C. militaris*.

Many studies reported the caffeine biotransformation by N-demethylation in bacteria, filamentous fungi, and mushrooms [8, 11]. Fig. 2 demonstrates the identification and quantification of demethylated caffeine products of *C. militaris*. It was found that the major products: theophylline (1,3), theobromine (3,7), and paraxanthine (1,7), were in the initial steps of the demethylation pathway. Hence, it could be concluded that *C. militaris* exhibited a preference of N-demethylation at the caffeine's methyl group position (Fig. 2a). It was noted that a significant accumulation of theophylline (Fig. 2b) might be caused by caffeine inhibition of the mycelial growth and development. However, caffeine was also carbon and nitrogen sources for mycelial growth [30]. In addition, Fig. 2b shows that the higher the caffeine concentration, the higher amount of the demethylated products were found. Our finding suggested that caffeine treatment could be an alternative method for synthesizing key caffeine derivatives to be used as bioactive compounds. However, further studies are necessary to explore the mechanisms of how caffeine affecting the growth and the development of this fungus. For example, transcriptome or proteome analysis might provide insightful information into the functions of genes in response to caffeine.

CONCLUSION

Caffeine showed a dosage effect on the growth, the development, and the adenosine production of *C. mil-*

Table 2 Effects of caffeine concentrations on the productivity of adenosine and cordycepin in *C. militaris*.

Productivity	Caffeine concentration (mM)		
(μg/g/day)	0	1	10
Adenosine in mycelia	32.35 ± 1.37^{a}	23.77 ± 2.18^{b}	22.89 ± 1.49^{b}
Extracellular adenosine	4.34 ± 0.60^{B}	4.94 ± 0.67^{B}	24.28 ± 0.65^{A}
Cordycepin in mycelia	463.40 ± 21.09^{a}	470.01 ± 35.93^{a}	513.94 ± 15.70^{a}
Extracellular cordycepin	218.89 ± 21.76^{A}	220.04 ± 36.67^{A}	240.48 ± 34.47^{A}

Different letters indicate significant differences (Duncan/s multiple range tests, $p < 0.05$).

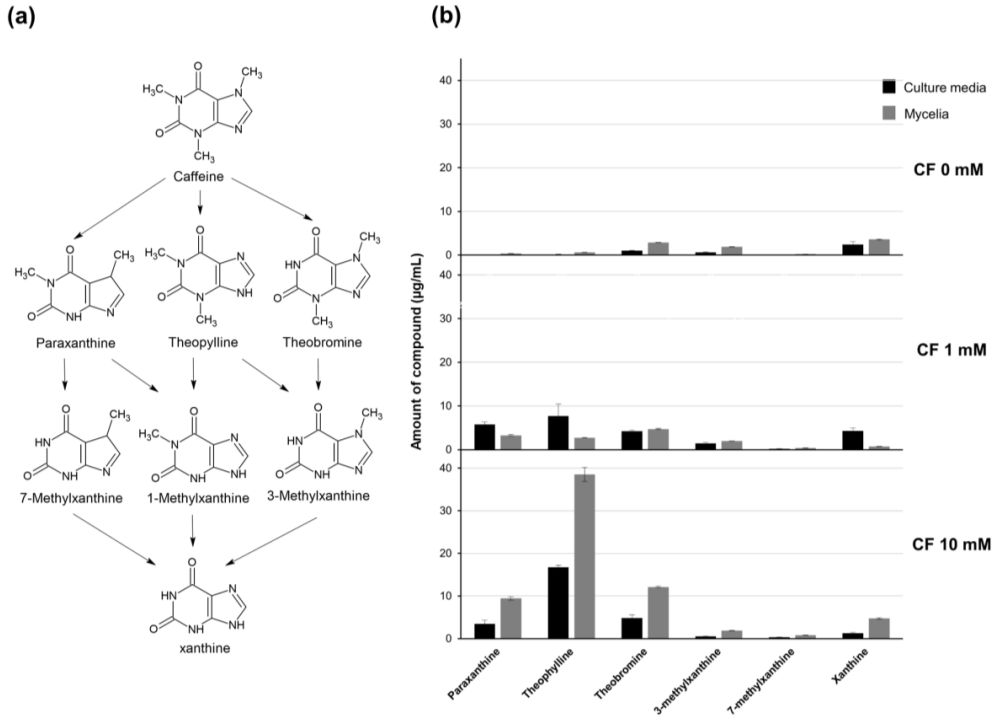

Fig. 2 (a), Demethylation pathway of caffeine modification [8]; and (b), quantification of the six demethylated caffeine products extracted from fungal mycelia and culture media of *C. militaris* treated with different concentrations of caffeine (CF).

itaris. When the concentration of caffeine was increased, the fungus exhibited a decrease in mycelial growth and an increase in adenosine productivity; however, caffeine had no effects on the productivity of cordycepin. Additionally, *C. militaris* could modify caffeine to derivatives by demethylation pathway. Caffeine demethylated derivatives, especially paraxanthine, theophylline, and theobromine, could be significant bioactive compounds with potential uses in functional foods and medicine.

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