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Effects of light and heavy metals on *Cordyceps militaris* fruit body growth in rice grain-based cultivation

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Abstract−The objective of this study was to examine the light and heavy metals on the fruit body growth of *Cordyceps militaris* in the rice-based cultivation. Since heavy metals are commonly detected in the paddy field, we investigated the effect of lead, cadmium and mercury on the rice grain-based cultivation of *C. militaris*. Cordycepin and cordycepic acid were determined by HPLC method. The result showed that the best fruit body growth and bioactive complements was obtained in rice I under 12 h light/dark cycle conditions. The effects of heavy metals (Pb, Hg, and Cd) to the fruit body were remarkable-the inhibition carried a dose-dependent behavior.

Key words: Lead, Cadmium, Mercury, Rice Grain-based Cultivation, *Cordyceps militaris*

INTRODUCTION

Heavy metal contamination in soils and plants at polluted sites including heavy industries, metal mining, smelting, and untreated wastewater irrigation areas is commonly found in developing countries [1-3]. These heavy metals accumulate mostly in waters and soils, and their action is long-lasting. By disquieting biochemical and metabolic activities, they lead to alterations in the vital functions of trees, whole forests and soil microorganisms in ecosystems [4]. This issue has increased sharply over the last century due to increasing industrialization, imposing stress on organisms, the bioaccumulation of heavy metals being closely connected with their toxicity which causes a restrained metabolism and growth of the microorganisms. In fact, fungi are capable of accumulating high concentrations of heavy metals, and this may pose serious human health risks in the case of edible mushrooms, especially if they were grown on agro-industrial products that may contain toxic substances such as heavy metals [5]. Accordingly, it is possible that different sources of rice grain contain different levels of the heavy metals, which might affect the growth of fungus. The mycelia growth of *Lentinula edodes* (Shiitake), for instance, is highly sensitive to cadmium (Cd) and mercury (Hg), but less sensitive to zinc (Zn), copper (Cu), and lead (Pb) [5]. In contrast, high concentrations of Pb inhibit the growth of litter-decomposing fungi [6]. The decreasing order of toxicity of select heavy metals on the yeast *Saccharomyces cerevisiae* culture was found to be Cu, Pb, and nickel (Ni). Heavy metal (200 μM) induced a decrease in the number of viable cells by about 50% in the first 5 min for Cu and in 4 h for Pb, while Ni was not toxic up to a 200 μM concentration over a period of 48 h [7]. Nevertheless, mycelia development of *Glomus mosseae* isolate was reduced as much as the amount of Cd or Zn increased in the growth medium [8]. The presence of Ni^{2+}, V^{4+} and Mo^{6+} also inhibited the ferrous oxidation ability of *Acidithiobacillus ferrooxidans* [9].

Fungi Cordyceps genus are a valuable drug in traditional medicine. This mushroom produces cordycepin (3′-deoxyadenosine) and has immunological modulation, anti-cancer, anti-infection and anti-inflammatory activities [10-14]. Recently, mass production of these fungi through artificial cultivation has been successfully established [15]. The optimization of parameters for submerged cultivation of different Cordyceps species, such as temperature, initial pH, carbon and nitrogen levels, and mineral source, have been reported [16,17]. Solid-state cultivation of *Ganoderma lucidum* and *C. sinensis* has been reported in the fermentation on rice-based medium for the production fruiting body [18,19]. This is due mainly to the fruit body containing more bioactive compounds than in the mycelium; investigators have sought to cultivate fungi on a solid-state fermentation [20,21]. Consequently, the *C. militaris* fruit body growth and the production of bioactive compounds with various rice grain-based medium after exposure to the heavy metals Cd, Hg, and Pb was examined in this study.

MATERIALS AND METHODS

1. Chemicals

Lead oxide (PbO), mercury oxide (HgO) and cadmium (Cd) were purchased from Fluka (Buchs, Switzerland). $CdCl_2$ was prepared from Cd by dissolving with HCl and diluted with deionized water.

2. Microorganism and Culture Conditions

C. militaris was obtained from COMSUM Biotechology Company (Taoyuan, Taiwan). The seed culture was prepared on potato-dextrose broth (PDB; Difco Lab., Detroit, MI) in a 250-mL flask at 25 °C on a rotary shaker incubator at 25 rpm for 7 days. Rice grain (purchased from the local market or supplied from two local farms), proteins, vitamins and trace elements was mixed with distill water (1 : 1) in 300 mL jars and autoclaved at 121 °C for 30 min. The experiments were performed in jars containing rice grain medium after being inoculated with 5 ml of the seed culture and incubated in the

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dark for 2 weeks with lid loosely covered at 25 ºC over 70% humidity. Subsequently, the main cultures underwent light effect experiments. The treatments consisted of exposure to 600±20 lux light intensities throughout 40 days of cultivation in different light intervals (0, 6, 12, 18, and 24 h exposure/day). The light intensity in each chamber was equipped with incandescent lighting typical of that used in commercial housing. The light fittings and tubes were dusted weekly to minimize dust buildup that would otherwise reduce the intensity. Meanwhile, the PDB was added into the jars every eight days to replenish the nutrients. All experiments were carried out in duplicate and average values were calculated.

3. Experiments with Heavy Metals

To determine the effect of each heavy metal on fruit body growth, media containing different rice sources, the heavy metal (Cd, Pb, and Hg in the concentrations from 1-10 ppm) were inoculated. After incubation for 40 to 45 days, the fruit body weight and height were measured.

4. Analytical Methods

To determine the contents of cordycepin and cordycepic acid, the fruit body sample was dried for 1 h at 85 ºC and extracted by hot distilled water (95 ºC) for 3 h. The supernatant was separated by centrifugation and filtered through 0.45 μm filter membrane. Cordycepin and cordycepic acid standards were obtained from Merck (Darmstadt, Germany) and used as internal controls. Cordycepin and cordycepic acid were detected using the method of Hsu et al. [22]. After filtering through a 0.45 μm filter, samples (10 μL) were subjected to analysis (Hitachi 15000 HPLC, Tokyo, Japan) with a pre-packed Lichrospher 100 RP-18 column (4×250 mm, 5 μm particle size). The mobile phase was a mixture of methanol/0.02 M KH_2PO_4 (15 : 85). Elution was performed at a flow rate of 1 mL/min and detection was determined using a variable-wavelength UV detector at 260 nm. The filtrate obtained as described above was mixed with four volumes of 95% (v/v) ethanol for the recovery of the precipitated exopolysaccharide (EPS), whose method was according to the report of Hsieh et al. [23]. The reducing sugar of EPS was measured by a phenol-sulfuric acid method [24].

RESULTS AND DISCUSSIONS

1. Effects of Light on the Fruit Body Growth and Cordycepin/Cordycepic Acid Contents of *C. Militaris*

Solid-state cultivation of *Ganoderma lucidum* has been reported in the fermentation on stillage grain supplemented with wheat bran, ground rice or sawdust for the production of fruiting body [18]. Therefore, we used rice grain and potato-dextrose broth as a carbon and nitrogen sources, respectively, for yielding maximum mycelium and fruit body growth. In view of the fact that light plays a crucial role in fruiting body growth in solid-state cultures [25-27], various light/dark cycles for the fruit body growth of *C. militaris* were investigated. Fig. 1 depicts the effects of light interval (under 600±20 lux constant exposure) on the production of cordycepin, cordycepic acid, and polysaccharides after 40 days cultivation. Obviously, all the components reached a maximum level under 12 h exposure interval. As a result, the optimal light condition for the maximal fruit body growth was in a 12 h light/dark cycle at 25 ºC followed by 18 h or 24 h of light, and then with 6 h of light. The fruit body growth seems to have become inferior when the duration of light was diminished.

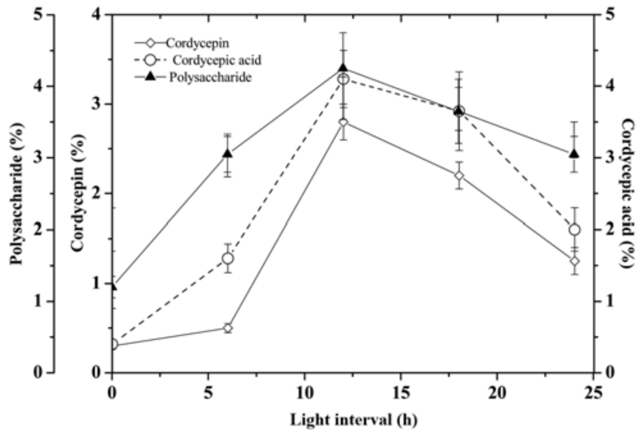

Fig. 1. Light interval dependent of various metabolites production after 40 days cultivation of *C. militaris*.

Nevertheless, longer exposure under the light would also have been harmful to the growth of *C. militaris* and thus reduced the cordycepin/cordycepic acid production (Fig. 1). The result was consistent with known effects of temperature, light and circadian rhythm in bacteria, plants, as well as fungi, arthropods, and vertebrates [28].

Accordingly, the light/dark cycle is a dominant environmental factor that affects many organisms, and different organisms have adapted to its influence in different ways. Fungi are no exception. The conidiation during asexual development and its circadian periodicity in the filamentous fungus such as *Neurospora crassa*, and *Coprinus cinereus* was strongly affected by the light/dark cycle [29]. In addition, light is also required for fruit-body formation in some basidiomycetes. Nevertheless, a strong light or a long exposure time appears to inhibit vegetative growth and fruit-body initiation. The amount of light required varies from species to species. Our observation shows that the equal light/dark interval provided a good fruit body pattern of *C. militaris*. There is a general consensus that, in some phases, light promotes early primordial and gill development, and, in other phases, light either slows or inhibits progression. It was reported that the meiosis progression is controlled by light/dark cycles in *Coprinus cinereus* [29]. Light is essential to propel basidia into karyogamy, and light intensity determines the timing of meiotic events. The higher the light intensities, the faster the fruiting bodies enter karyogamy [29]. The effects of light on the fruit body growth *C. militaris* may follow a similar manipulation, even though the effects of light/dark cycle on sexual reproduction are not as well recognized.

2. Effects of Rice Sources on Cordycepin and Cordycepic Acid Contents of the Fruit Body

Fig. 2 shows the time course of various rice medium on the fruit body weight and fruit body height during the 45 days cultivation. Two rice-based media (rice I and II) demonstrated a better growth pattern both on fruit body weight and fruit body height, especially rice I exhibited a relatively superior result. The fruit body weight and fruit body height observed in the rice I cultivation have almost six and three times higher than that of rice III cultivation, respectively. Interestingly, the different source of rice offered a remarkable different growth pattern. These may partially be due to its different composition of nutrients as well as heavy metal content (Table 1). Yet, nutrient facts of the organic rice are still unavailable due to the

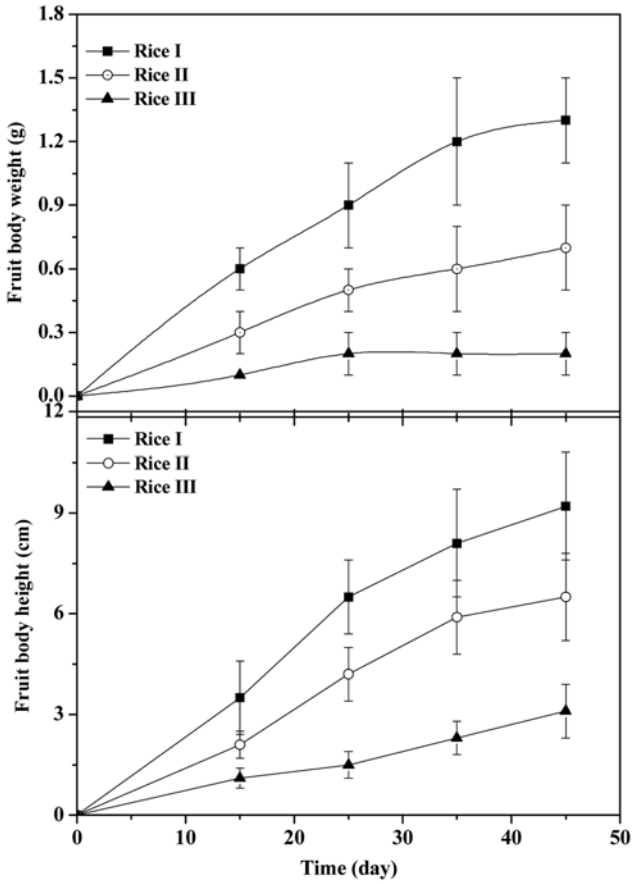

Fig. 2. The time course of the fruit body weight (top panel) and fruit height (bottom panel) of *C. militaris* cultivated in different rice medium. Data represent the mean±SEM from three independent experiments.

Table 1. Heavy metal and pesticides contents of different rice sources

Contents (ppm)	Rice I	Rice II	Rice III
Pb	N.D.[b]	0.02	0.11
Cd	0.01	0.01	0.08
Hg	N.D.	0.002	0.005
P	N.D.	0.01	0.05
Cl	N.D.	N.D.	0.03

[a]The legal limit of heavy metals in rice: Cd, 0.5 ppm; Hg, 0.05 ppm; Pb, 0.2 ppm, Department of Health, Executive Yuan, ROC
[b]N.D. not detectable. P: organic phosphate pesticide; Cl: organic chloride pesticide

complexity of the growth conditions. The present work demonstrated that the under optimal light condition the highest fruit body growth together with the cordycepin production was achieved. At 12 h of light/dark cycle at 25 °C and 70% humidity for 45 days of cultivation with rice I, the highest production of cordycepin (2.8%=28 mg/g)/cordycepic acid (4.1%=41 mg/g) was detected in fruit body of *C. militaris*. In parallel, around 4.0% (40±5 mg/g) of cordycepin was found in the solid medium with mycelium of *C. militaris* (in submerged culture). Both values were better than that of the natural Cordyceps (0.16±0.04 mg/g) and those reported elsewhere [30,31].

Table 2. Cordycepin/cordycepic acid contents of *C. militaris* fruit body from different rice-based cultures

Culture time (days)	Cordycepin/Cordycepic acid (%)		
	Rice I	Rice II	Rice III
15	0.7[b]/2.0[d]	N.D.[a]/0.3[b]	N.D.[a]/0.1[a]
25	1.3[c]/2.7[d]	0.8[c]/1.2[c]	0.3[b]/0.3[b]
35	2.0[d]/3.4[e]	0.9[c]/1.3[c]	0.3[b]/0.4[b]
45	2.8[e]/4.1[f]	1.1[d]/1.6[d]	0.5[c]/0.6[b]

[a]N.D. not detectable; different superscript letter among the group or incubation time indicates a significant difference, $p<0.01$. Data represent the mean from three separated experiments

Table 2 summarizes the results of cordycepin and cordycepic acid production from the fruit body of *C. militaris* in various rice cultures. No cordycepin was detected in the case of rice II and III until day 25. It can also found that rice I exhibited the best production of cordycepin/cordycepic acid after 45 days cultivation followed by rice II and then rice III. These results also reflected on its growth of fruit body as stated above. Moreover, the polysaccharide (43±4 mg/g) was detected in the fruit bodies after 45 days of cultivation with rice I (*c.f.* Fig. 1). Apparently, rice I provided an outstanding performance on fruit body formation as well as the bioactive components production.

3. Effect of Heavy Metals on the Fruit Body Growth

In the present study, we used rice grain and potato-dextrose broth as the carbon and nitrogen sources to produce a maximum mycelium and fruit body growth, respectively. As mentioned above, at the 12 h of light/dark cycle condition, the rice I provided the highest fruit body growth and cordycepin production among the rice grain-based cultivation (Fig. 2). The contents of heavy metals were quite different among the rice grain from different sources (Table 1). As can be seen from this table, rice I had no detectable Pb, and Hg was found as well as trace amount of Cd (0.01 ppm) were detected. In contrast, more significant levels of Pb (0.11 ppm), Cd (0.08 ppm) and Hg (0.005 ppm) were discovered in rice III. A valid pattern of inferences may partially be due to the rare heavy metal content in rice I. Although the trace amount of heavy metals in the rice grain III in the market was regulated for human consumption, it was inferior to the rice I and II for fruit body growth (*c.f.* Fig. 2). In fact, rice produced in Taiwan is monitored for heavy metals and pesticides by a government agency. However, sporadic incidences of Cd and Hg contamination (over 0.5 ppm and 0.05 ppm, respectively) have been reported in particular parts of Taiwan farmland owing to the industrial waste mismanagement [32]. The bulk of literature also warns against the cumulative effects of prolonged heavy metal exposure; regular consumption of rice by local populations might pose potential health problems [1-3].

Consequently, further studies on the effects of these heavy metals (supplemented in medium) for the fruit body growth were conducted. The inhibition of fruit body weight and fruit body height by individual Pb, Cd, and Hg supplement exhibited a dose-dependent manner (Table 3). All the heavy metals revealed a significant diminishment in the fruit body weight and fruit body height. In the process of adapting to the toxic effect of heavy metals, the fungi demonstrates a fruit body growth decreased at beginning, followed

Table 3. The Effects of heavy metals concentrations on the fruit body weight and fruit body height of *C. militaris*

Concentration (ppm)	Pb		Hg		Cd	
	Weight (g)	Height (cm)	Weight (g)	Height (cm)	Weight (g)	Height (cm)
0	1.5±0.4	9.2±1.3	1.5±0.6	9.6±2.3	1.5±0.3	9.2±2.1
1	0.7±0.2	3.5±1.1	0.8±0.3	4.4±1.1	0.6±0.2	4.1±0.9
2	0.5±0.1	2.5±0.7	0.6±0.2	3.2±0.5	0.5±0.2	2.9±0.5
5	0.3±0.1	2.2±0.5	0.4±0.2	2.6±0.5	0.3±0.1	2.5±0.3
10	0.2±0.1	0.3±0.1	0.2±0.1	0.8±0.4	0.1±0.0	0.2±0.1

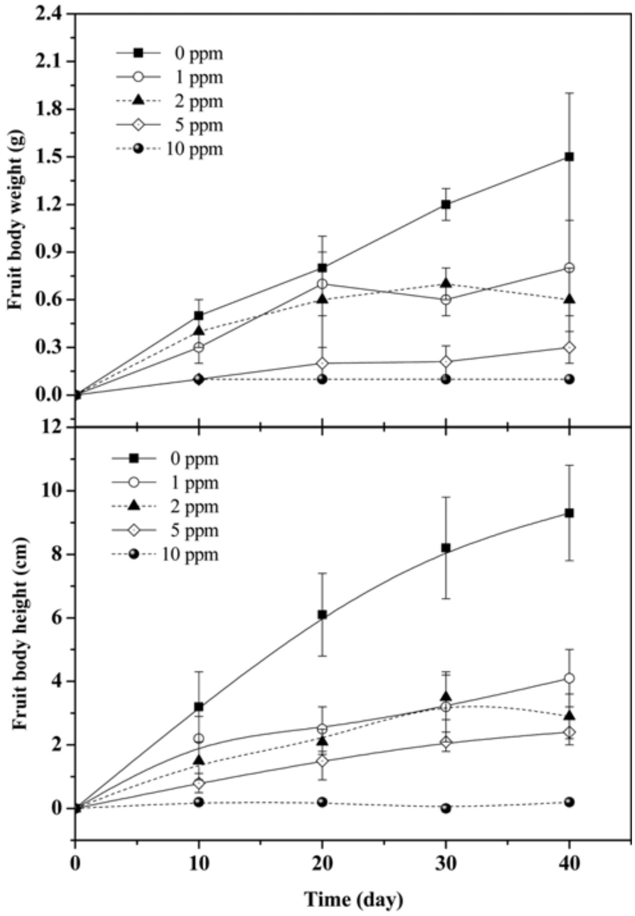

Fig. 3. The time course of the fruit body growth of *C. militaris* in rice based culture under different mixed heavy metals (ratio 1 : 1 : 1) concentration. Data represent the mean±SEM from three independent experiments.

by a decline in fruit body growth relative to the control in the rest of the cultivation period. No synergetic effects were observed when all the heavy metals were mixed together (Fig. 3). Table 4 summarizes the effects of additive metal concentration on the content of cordycepin, cordycepic acid, polysaccharide, and free amino acids in the *C. militaris* fruit body. The inhibition pattern also exhibits a dose-dependent manner. Almost 99% of the all components were inhibited under 10 ppm of various metals added. Our observation suggests that the heavy metal used herein had significant effects on the bioactive components production of *C. militaris*.

Furthermore, Cd and Hg are usually the most toxic heavy metals for basidiomycetes. In our experiments, the Hg exhibited a relatively slight tolerance to the fruit body height (from 9.6 cm to 0.8 cm). Impaired growing ability for fruit body *C. militaris* may be owing to the bulging of the cells due to insufficient chitin formation; the inability of the cultures to grow beyond the germination stage, particularly in presence of higher concentrations of heavy metals, could be due to the inhibition of chitin synthesis as seen in the case of *Penicillium* [33]. The chitin biosynthesis is induced as a response mechanism of the fungal cell of *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium chrysogenum* to stress, thus making it more resistant to heavy metals, as reported [34]. When chitin synthesis is affected, growing hyphae tend to lyse and form pronounced bulges unless the osmolarity of the medium is increased; inability of the cells to increase chitin levels in response to cell wall damage resulted in cell lysis, indicating the importance of the chitin response to prevent cell death [35]. However, the actual mechanisms of the tolerance of the *C. militaris* fruit body growth to heavy metals still need to be clarified. As agro-industrial products are generally complex mixtures of various xenobiotics including heavy metal salts, the inhibitory effects investigated in this study must be taken into consideration during their utilization. In conclusion, the formation of fruit body from *C. militaris* together with the production of metabolites was sensitive to the source of medium and its heavy metal contents.

CONCLUSIONS

The light/dark cycle clearly affects the fruiting body production of *C. militaris*. The best results were obtained under 12 h light/dark cycle condition. Meanwhile, the maximal fruit body growth and the production of cordycepin was obtained from the rice I, which contains no detectable Pb and Hg, and only trace of Cd. During the 45 days cultivation, rice I had better fruit body weight and height than rice II and rice III. The highest amount of cordycepin/cordycepic acid was detected in the fruit body of *C. militaris* with rice I medium after 45 days of cultivation. The influence of tested heavy metals (Pb, Hg, and Cd) was manifest on the fruit body growth. The inhibition of the fruit body growth of *C. militaris* was proportional to the amount of heavy metals presented in the rice grain (dose-dependent). Complete inhibition on fruit body growth was expected beyond 10 ppm concentration. Furthermore, the growth of fruit body because of more bioactive components was found in the fruit body rather than in the mycelium of Cordyceps. However, due to the difficulties of cultivating of fruit body, there was no report on the mass production of cordycepin by the Cordyceps fruit body. Instead, artificial cultivation of the Cordyceps mycelium has been successfully established. The resistance to heavy metals and other toxic compounds

Table 4. The effects of additive metal concentration on the contents of cordycepin, cordycepic acid, and polysaccharide in *C. militaris* fruit body

Metal	Component (%)	Additive concentration (ppm)				
		0	1	2	5	10
Hg	Cordycepin	2.3±0.5	1.8±0.3	0.6±0.1	0.3±0.1	0.1±0.0
	Cordycepic acid	3.8±0.5	2.9±0.4	1.1±0.2	0.6±0.1	0.1±0.0
	Polysaccharide	3.7±0.5	2.8±0.3	1.8±0.3	1.3±0.2	0.3±0.0
Pb	Cordycepin	2.3±0.3	1.5±0.3	0.6±0.2	0.2±0.0	0.1±0.0
	Cordycepic acid	3.8±0.4	2.5±0.3	1.0±0.2	0.5±0.1	0.1±0.0
	Polysaccharide	3.7±0.5	2.4±0.3	1.5±0.3	1.1±0.2	0.2±0.0
Cd	Cordycepin	2.3±0.4	1.4±0.2	0.5±0.1	0.2±0.0	0.1±0.0
	Cordycepic acid	3.8±0.5	2.3±0.3	0.9±0.2	0.5±0.0	0.1±0.0
	Polysaccharide	3.7±0.4	2.2±0.3	1.4±0.2	1.0±0.3	0.2±0.0

could be particularly dangerous with microorganisms because of the possible accumulation of such materials during growth and fruiting body production. The information obtained is considered fundamental and useful to the development of optimal *C. militaris* cultivation process for production of cordycepin.

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