Growth of Gold Cordyceps (*Cordyceps militaris*) on Pupae of Nanglai Thai Native Silkworm and Eri Silkworm

การเจริญของเห็ดถั่งเช่าสีทอง (*Cordyceps militaris*) บนดักแด้ไหมไทยพื้นบ้านพันธุ์นางลายและไหมป่าอีรี่

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บทคัดย่อ: ศึกษาการเจริญเติบโตของเส้นใยและดอกเห็ดของเชื้อเห็ดถั่งเช่าสีทอง (*Cordyceps militaris*) ในดักแด้ไหมและสารออกฤทธิ์ที่มีสรรพคุณทางยาบนดักแด้ไหมไทยพื้นบ้านพันธุ์นางลาย (*Bombyx mori*)และไหมป่าอีรี่ (*Samia ricini*) ทดสอบกับเชื้อเห็ดถั่งเช่าสีทองจำนวน 2 สายพันธุ์ คือ CMRU-1 และ CM Saraburi โดยทำการทดลอง 3 กรรมวิธี คือ การฉีด การจุ่ม และการทำแผลแล้วจุ่มดักแด้ในเชื้อเห็ดถั่งเช่าสีทอง ผลการทดลองพบว่า กรรมวิธีการฉีดสารแขวนลอยเชื้อเห็ดถั่งเช่าสีทองสายพันธุ์ CMRU-1 เข้าตัวดักแด้ เป็นกรรมวิธีที่ดีที่สุด มีการเจริญเติบโตและสามารถเข้าก่อโรคกับดักแด้ไหมพันธุ์นางลายสูงสุด 72.50 เปอร์เซ็นต์ มีการเจริญของดอกเห็ดสูงสุด 85.50 เปอร์เซ็นต์ และดอกเห็ดมีน้ำหนักสด 42.70 กรัม ในขณะที่เชื้อเห็ดถั่งเช่าสีทองสายพันธุ์ CM Saraburi มีการเจริญเติบโตและสามารถเข้าก่อโรคกับดักแด้ไหมพันธุ์นางลาย 53.00 เปอร์เซ็นต์ และมีการเจริญของดอกเห็ด 88.70 เปอร์เซ็นต์ นอกจากนี้เชื้อเห็ดถั่งเช่าสีทองสายพันธุ์ CM Saraburi มีการเจริญเติบโตและสามารถเข้าก่อโรคกับดักแด้ไหมป่าอีรี่ 26.00 เปอร์เซ็นต์ มีการเจริญของดอกเห็ด 94.20 เปอร์เซ็นต์ และน้ำหนักสดดอกเห็ดอยู่ที่ 24.13 กรัม ในส่วนของการศึกษาสารออกฤทธิ์จากเห็ดถั่งเช่าสีทองที่เจริญบนดักแด้ไหมพบความแตกต่างของสารคอร์ไดซิปินและอะดีโนซินระหว่างส่วนดอกเห็ดและตัวดักแด้ โดยพบความเข้มข้นของสารคอร์ไดซิปินในดอกเห็ดของเชื้อเห็ดถั่งเช่าสีทองสายพันธุ์ CM Saraburi บนดักแด้ไหมป่าอีรี่สูงสุด 445.65 มิลลิกรัม/100 กรัม ในขณะที่ตัวดักแด้พบ 306.41 มิลลิกรัม/100 กรัม เชื้อเห็ดถั่งเช่าสีทองสายพันธุ์ที่พบความเข้มข้นของคอร์ไดซิปินรองลงมาคือ CMRU-1 ที่ตัวดักแด้ไหมพันธุ์นางลายพบ 233.80 มิลลิกรัม/100 กรัม ในส่วนของความเข้มข้นอะดีโนซินพบส่วนดอกเห็ดของเชื้อเห็ดถั่งเช่าสีทองสายพันธุ์ CMRU-1 ที่ขึ้นบนตัวดักแด้ไหมป่าอีรี่ มีความเข้มข้นของอะดีโนซินสูงสุด 13.79 มิลลิกรัม/100 กรัม ส่วนความเข้มข้นของอะดีโนซินรองลงมา คือ สายพันธุ์ CM Saraburi ในดอกเห็ดที่ขึ้นบนตัวดักแด้ไหมป่าอีรี่ 5.930 มิลลิกรัม/100 กรัม

คำสำคัญ: เห็ดถั่งเช่าสีทอง ดักแด้ไหม สารออกฤทธิ์ คอร์ไดซิปิน

Abstract: Growth and infection ability of Golden *Cordyceps militaris* on 2 varieties of silkworm pupae, Thai native silkworm (*Bombyx mori*) and Eri silkworm (*Samia ricini*) were tested with two strains of *Cordyceps militaris*; CMRU-1 and CM Saraburi, by injection, soaking, and stabbing and soaking pupae methods. The mycelial suspension injection was the best method of inoculation. The injection of CMRU-1 strain of *C. militaris* with mycelial suspension on Nanglai silkworm pupae has shown the highest infection rate with 72.50 percent and 85.50 percent of fruiting body formation and the highest fresh weight of fruiting body with 42.70 g, followed by CM Saraburi strain of *C. militaris* on Nanglai silkworm pupae that showed the infection rate with 53.00 percent and 88.70 percent of fruiting body formation. In addition, the injection of *C. militaris* strain CM Saraburi on Eri silkworm pupae has shown the highest infection rate with 26.00 percent, 94.20 percent of fruiting body formation and the fresh weight of fruiting body with 24.13 g. The content of bioactive components, cordycepin and adenosine, in *C. militaris* between the fruiting body and the pupae were different. The highest cordycepin amount was found in CM Saraburi in fruiting body of Eri silkworm pupae was 445.65 mg/100 g and in Eri silkworm pupae (insect body) *was* found 306.41 mg/100 g. The amount in CMRU-1 in Nanglai silkworm pupae (insect body) was 233.80 mg/100 g. Adenosine contents has shown the highest on fruiting body of CMRU-1 in Eri silkworm pupae was 13.79 mg/100 g followed by CM Saraburi in fruiting body of Eri silkworm pupae was 5.93 mg/100 g.

Keywords: Gold cordyceps, silkworm, chemical component, cordycepin

Introduction

From 80,000 known fungi, only 5% of them provided many benefits to the human (Isaka *et al*., 2005). This can be seen in traditional medicine of China, *Cordyceps sinensis*, the fungus that infected on silkworm (Lepidoptera: Bombycidae) and produced fruiting body on the caterpillar's head that has effective medical and pharmacological properties (Hong *et al*., 2007). *C. sinensis* can be called in the name as "DongChongXiaCao" (Zhu *et al*., 1998) that has a fruiting body and has grown on the dead or living caterpillars. The classification of the cordyceps is placed in family Clavicipitaceae (Hong *et al*., 2007), has a cylindrical asci, thickened ascus, and filiform ascospores (Sung *et al*., 2007) in the black-blade shape (Zhu *et al*., 1998). The extract from cordyceps is the polysaccharides called as Cordycepin (Paterson, 2008) and other altered nucleosides which are antiviral and have many benefits to pharmacological and medical properties.

Cordyceps militaris is the fungus that has ability of insect pupae infection and effective medical and pharmacological properties likes *C. sinensis* but it is able to develop in laboratory easier than *C. sinensis* and is able to grow in other hosts which are silkworms and other media. The commercial products of *C. militaris* have been available on healthy food retailers as oral liquid cordyceps tonic, tonic cordyceps wine for kidney reinforcing, cordyceps health beer, and cordyceps capsules. In recent years, as increasing attention of the function of *C. militaris*, foreign manufacturers have shown an interest, so the exportation of cordyceps products has been increasing. Presently Japan, Korea, Malaysia including Thailand and others countries have been increasing their research and

development of cordyceps as the functional food (Zhou *et al*., 2009).

Additionally, traditional chinese medicals are able to develop many cordyceps products through modern technology. The products have been mainly focused on the following aspects: enhancing physique, anti-aging, protecting the heart, improving sleep, increasing appetite, increasing immunity, etc. For instance, *C. militaris* mycelial powder and the capsule of *C. militaris* mycelial powder had been authorized as a Chinese national drug in April 2003. Jilin Northeast Tiger Pharmaceutical Co., Ltd. reported to the State Ministry of Health to declare classes of new drugs, which have been approved and called Xinkeqi capsules (Zhou *et al*., 2009).

Sericulture, silk farming can make incomes to Thai farmers around the country, especially in north-eastern region of Thailand. The silkworms encouraged to culture by Queen Sirikit Sericulture Center are native varieties, poly into bivoltine varieties, and bivoltine varieties (Matmathatip and Campeerawat, 2013). Tayutivutikul *et al*. (1998) reported about the method of mulberry leaf utilization for rearing single cross hybrid silkworm founded that the greater yield percentage mounting, cocoons, good cocoons, normal pupae and cocoon shells were obtained from rearing the silkworms with individual mulberry variety of BR. 51 and Nakornratchasima 60. Moreover, Eri silkworm (*Samia ricini*) is a new interested for industrial sericulture because silk is white and size of cocoon are bigger than mulberry silkworm and oil extracted from pupa has anti-oxidant properties (Thamee and Rattanapitigorn, 2011). Pupa of Eri silkworm can used to be human food products as well.

Since *C. militaris* grows on silkworm and a very large number of silkworm pupa are available in northern Thailand as a by-product of sericulture, therefore we used silkworm pupa as host to culture this fungus in this study under the objective of examining fungal mycelia and fruiting body of gold cordyceps and determining content of bioactive components, cordycepin and adenosine in gold cordyceps on those silkworm pupae.

Materials and Methods

Host insects: Thai native silkworm (*Bombyx mori*), Nanglai and Eri silkworm (*Samia ricini*).

Fungal strains: Two fungal strains (CMRU-1 and CM Saraburi) were cultivated by the original mycelia of *Cordyceps militaris*.

Inoculum and injection preparation: Gold cordyceps mycelia were cultivated in PDA using 0.5 mm mycelial discs grown in a Petri dish (Hong *et al*., 2010). The gold cordyceps inoculated media were cultivated under static conditions at 22 °C for 20-25 days and used as parent strains. After that, the cultures of gold cordyceps strains in the Sabouraud medium were prepared into 250 ml flasks and then were inoculated each flask with the mycelial discs of the gold cordyceps parent strain from growing margins on PDA medium (Hong *et al*., 2010). Then culture was put on the rotative shaker at the rotative velocity of 150 rpm at 20-25 °C for culturing the mycelia for 2-3 weeks, and kept at 22-25 °C (Zhenxiang, 2004).

Gold cordyceps inoculation: The 6-day-old pupa of Thai native silkworm and Eri silkworm were inoculated with 10^6 spores/ml of gold cordyceps suspension all this experiment. There are 3 inoculation methods, injection, soaking, and

stabbing and soaking silkworm pupae with mycelial suspension. In each treatment 200 silkworm pupae were used in each species.

Treatment 1: Injection method

Gold cordyceps mycelial suspension at concentration of 10^6 spores/ml of mycelial suspension for 0.05-0.1 ml was injected into spiracle of pupae using self-refilling syringe.

Treatment 2: Soaking method

Silkworm pupae in each species were soaked in 10^6 spores/ml of mycelial suspension for 3-5 minutes and allowed them dry by air.

Treatment 3: Stabbing and soaking method

Silkworm pupae were stabbed 10 times with the sterile self-refilling syringe and then stabbed silkworm pupae were soaked in 10^6 spores/ml of mycelial suspension for 3-5 minutes, allowed them to dry by air.

Induction of gold cordyceps endosclerotium

After injection, soaking infection, and stabbing and soaking treatments, the pupae were put in transparent plastic boxes and covers with lids and kept at 22-25 °C (Zhenxiang, 2004) until their bodies became hard and mummified for 7-9 days after treatments (Hong *et al*., 2010).

Induction of gold cordyceps fruiting body

Mummified pupae were cultured to induce fungal fruiting body in cultured boxes. Gold cordyceps mummies were transferred individually to transparency multi squared boxes and incubated at 22-25 °C, a 12:12 day: light photoperiod for 30-45 days (Hong *et al*., 2010).

Determination of the bioactive components

The gold cordyceps culture in silkworm pupae was dried using vacuum drying machine for 5 hours at 60 °C, stored in a plastic box for the analysis of bioactive components such as cordycepin and adenosine using HPLC method (Huang *et al*., 2009).

Results and Discussions

The growth of gold cordyceps on PDA was fast at the first 3 days and full grown on a Petri dish at day 21. The mycelial dises were able to use as inoculum in Sabouraud medium for 14-21 days (Figure 1).

Silkworm varieties and fungal strains: Two silkworm pupae varieties, Nanglai and Eri were selected, for infection of 2 *C. militaris* strains, CMRU-1 and CM Saraburi. The pupae that infected by *C. militaris* died in 2-3 days and became hard within 7-9 days after injection (Figure 2). The injection of CMRU-1 on

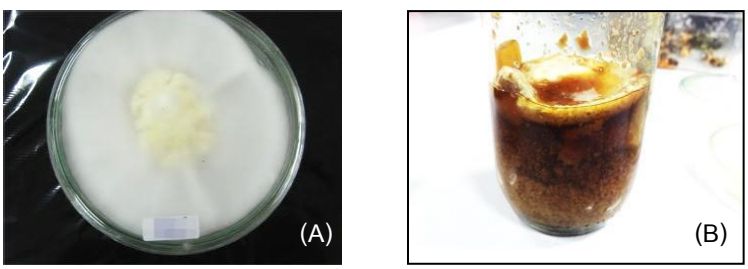

Figure 1 Growth of gold cordyceps on Potato Dextrose Agar and Sabouraud medium on day 21 (A), and gold cordyceps suspension in Sabouraud medium at day 22 (B)

Nanglai pupae showed the highest infection rate with 72.50 percent and 85.50 percent of fruiting body formation (Table 1) and the highest fresh weight of fruiting body with 42.70 g followed by the injection of CM Saraburi cordyceps strain on Nanglai pupae showed 53.00 percent infection rate and 88.70 percent of fruiting body formation. Whereas, the injection of CM Saraburi on Eri pupae has shown the highest infection rate with 26.00 percent, fruiting body formation at 94.20 percent and the fresh weight of fruiting body at 24.13 g (Table 2).

In injection method, the injected Nanglai silkworm pupae by both fungal strains became harder or mummies earlier than Eri silkworm pupae did, due to the smaller in size. Light exposure is able to induce fruiting body formation on mummied pupae and can be harvested during 15-18 days. Bioactive content of cordycepin and adenosine, the cultured gold cordyceps on silkworm pupae were determined (Figure 3).

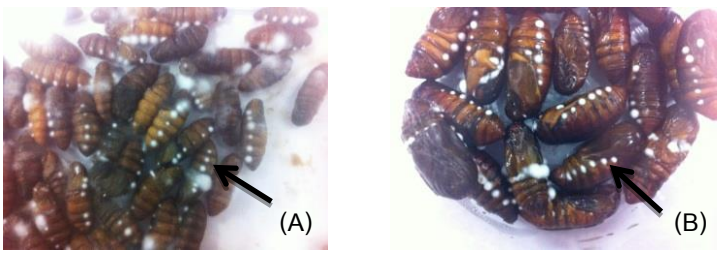

Figure 2 Harden pupae infected by *C. militaris* after 7-9 days of inoculation on Nanglai silkworm pupae (A), and Eri silkworm pupae (B)

Table 1 Infection and fruiting body formation rate of *C. militaris* on silkworm pupae by mycelial suspension injection method at the concentration of 10^6 spores/ml

Fungal strains	No. of pupae tested		No. of pupae infected		Infection Rate (%)		Fruiting Body Formation Rate (%)	
	Nanglai silkworm	Eri silkworm	Nanglai silkworm	Eri silkworm	Nanglai silkworm	Eri silkworm	Nanglai silkworm	Eri silkworm
CMRU-1	200	200	145	46	72.50	23.00	85.50	93.40
CM Saburi	200	200	106	52	53.00	26.00	88.70	94.20

Table 2 Weight of fruiting body formation of *C. militaris* on silkworm pupae after 30-45 days of mycelial suspension injection method at the concentration of 10^6 spores/ml

Fungal strains	Nanglai silkworm pupae		Eri silkworm pupae		Net weight (g.)	
	Fresh weight (g.)	Dry weight (g.)	Fresh weight (g.)	Dry weight (g.)	Nanglai silkworm	Eri silkworm
CMRU-1	42.70	19.48	22.59	16.76	62.18	39.35
CM Saraburi	18.94	14.95	24.13	15.98	33.89	40.11

The cordycepin amount found in fruiting body of CM Saraburi from infected Eri silkworm pupae was 445.65 mg/100 g and from Eri silkworm pupae was 306.41 mg/100 g. Meanwhile, those CMRU-1 from infected Nanglai silkworm pupae was 233.86 mg/100 g. The adenosine content on fruiting body of CMRU-1 from infected Eri silkworm pupae was 13.79 mg/100 g followed by in fruiting body of CM Saraburi from infected Eri silkworm pupae was 5.930 mg/100 g (Table 3).

In the soak infection method, 3 days after infection, hyphae of *C. militaris* were white in color, fluffy cotton (Figure 4) but at 15 days after infection, the hyphae of CM Saraburi on Nanglai and Eri silkworm pupae were collapse and no growth at all, meanwhile, the CMRU-1 on Nanglai silkworm pupae were able to develop when exposed to light for 24 hrs. However, the fruiting body was not produced.

In stab and soak infection method, the hyphae of *C. militaris* were able to grow on 2 varieties of silkworm pupae in 3-9 days after infection, however at day 15, the hyphae of CMRU-1 on Nanglai and Eri silkworm pupae were collapse (Figure 5). The same pattern was found in hyphae of CM Saraburi on Nanglai pupae at day 27 after infection.

In this study, the gold cordyceps strains played an important role of fungal growth because fungal strains had to develop vigorous hyphae which were able to infect into the host (pupa) and fruiting body could grow effectively on a good length. According to Hong *et al*. (2010), the intensity of light used to induce the fruiting body of *C. militaris* was between 500-1,000 lx, which could induce the length of fruiting bodies ranged from 61.00 to 76.00 mm. The fruiting bodies produced in this light condition

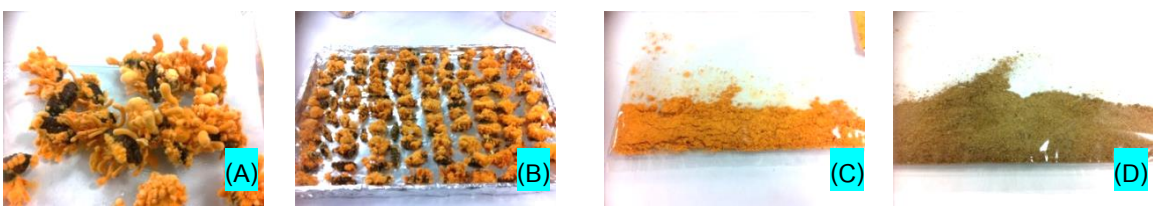

Figure 3 Gold cordyceps preparation for cordycepin and adenosine analyses. Fresh gold cordyceps mushroom on pupae (A) Dried gold cordyceps cultured on silkworm pupae (B) Ground gold cordeyceps fruiting bodies (C) Ground gold cordyceps grown on silkworm pupae

Table 3 Content of cordycepin and adenosine of *C. militaris* cultured in silkworm pupae for 45 days

Strians of gold Cordyceps and silkworm	Samples	Bioactive ingredient (mg/100g)	
		cordycepin	adenosine
CMRU-1, Nanglai	Fruiting body	130.34	0
	Insect body (Pupa)	233.86	3.980
CMRU-1, Eri	Fruiting body	175.60	13.790
	Insect body (Pupa)	230.00	3.400
CM Saraburi, Nanglai	Fruiting body	113.50	0
	Insect body (Pupa)	158.77	1.940
CM Saraburi, Eri	Fruiting body	445.65	5.930
	Insect body (Pupa)	306.41	4.510

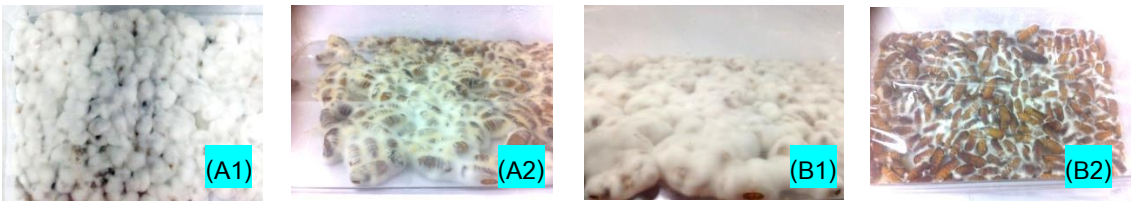

Figure 4 Gold cordyceps hyphae grown on silkworm pupae inoculated by soaking with gold cordyceps mycelial suspension on Nanglai silkworm pupae after soaking for 3 days (A1), 15 days (A2) on Eri silkworm pupae after soaking for 3 days (B1) and 15 days (B2)

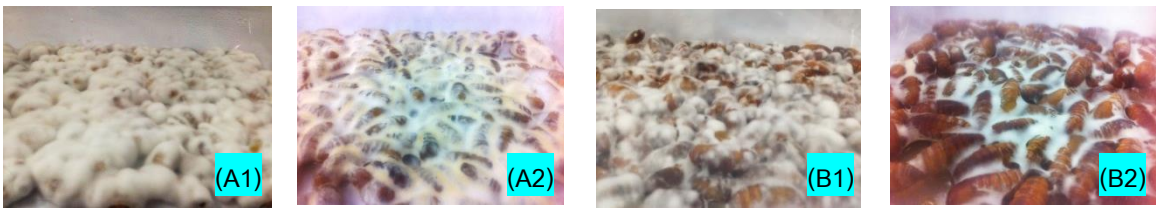

Figure 5 Gold cordyceps hyphae grown on silkworm pupae inoculated by stabbing and soaking with gold cordyceps mycelial suspension on Nanglai silkworm pupae after stabbing and soaking for 3 days (A1), 15 days (A2) on Eri silkworm pupae after stabbing and soaking for 3 days (B1) and 15 days (B2)

were thick and long, and cylindrical or clavate shape with orange in color. In this experiment, the same results were found and the older silkworm pupae tended to be contaminated by airborne fungi and infected by flies. Bioactive components, cordycepin and adenosine, in this study showed closely amount to the report from Huang *et al*. (2009) that found the average contents of cordycepin and adenosine in artificial cultural *C. militaris* fruiting bodies were 265.4 and 245.0 mg/100g, respectively.

Conclusions

The best infection was injection methods of gold cordyceps of *C. militaris* hyphal suspension into the haemocoel of silkworm pupae. Growth and infection ability of gold cordyceps on Thai native silkworm (Nanglai) and Eri silkworm pupae by injection of CMRU-1 fungal strain showed the highest infection rate on Nanglai silkworm pupae at 72.50 percent, formation of fruiting body was 85.50 percent and the highest fresh weight of fruiting body was at 44.70 g. The highest cordycepin concentration was found in fruiting body of CM Saraburi from infected Eri silkworm pupae at 445.65 mg/100 g and the highest adenosine concentration was found in fruiting body of CMRU-1 from infected Eri silkworm pupae at 13.79 mg/100 g.

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