Mycobiology 38(2) : 128-132 (2010)
DOI:10.4489/MYCO.2010.38.2.128

Fruit Body Formation on Silkworm by *Cordyceps militaris*

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(Received March 31, 2010. Accepted April 11, 2010)

Injection inoculation protocols for fruit body formation of *Cordyceps militaris* (*C. militaris*) were investigated to improve the incidence of infection in the silkworm species *Bombyx mori* (*B. mori*). Injection, with suspensions of *C. militaris* hyphal bodies into living silkworm pupae, was used to test for fruit body production. Use of Daeseungjam rather than Baegokjam or Keumokjam varieties of *B. mori* is thought to be suitable for infection by *C. militaris*. From mounting, nine-day-old to 11-day-old pupae showed the best incidence of infection with a 100 μL injection volume. Silkworm pupae injected with a hyphal suspension concentration of more than 2×10^5 colony-forming unit (cfu) recorded a greater than 96% incidence of infection. Also, fruit bodies of *C. militaris* were induced and produced at a light intensity between 500 and 1,000 lx.

KEYWORDS : *Bombyx mori*, *Cordyceps militaris*, Fruit body, Hyphal bodies, Injection inoculation

Cordyceps is entomopathogenic fungi that parasitize insects and spiders. There are more than 700 species of entomopathogenic fungi, of which about 300 species have been reported to produce a fruiting body, namely *Cordyceps* mushroom [1, 2]. About 78 species have been collected and identified according to host type and shape of fruit body [3].

Cordyceps mushrooms, "winter-worm summer-grass", have been used as a traditional folk medicine or as a food ingredient to strengthen the immune system and regain energy, similar to the tonic functions of ginseng (*Panax* spp.) for hundreds of years in Far East Asia countries such as Korea, Japan, and China [4]. However, only a few are collected for their medicinal properties. These species are referred to as a pharmaceutical *Cordyceps* mushroom: *Ophiocordyceps sinensis* (formerly *Cordyceps sinensis*), *C. militaris*, *C. ophioglossoides*, *C. sobolifera*, C. *liangshanensis*, and *C. cicadicola* [5]. In general, the two species of *Cordyceps* mushrooms most widely used and valued in traditional Asian medical practice are *O. sinensis* and *C. militaris*. Although O. *sinensis* may be the most famous and expensive fungus, it is comparatively rare and cannot be easily grown in culture; whereas, *C. militaris* occurs worldwide and forms fruiting bodies on unconverted rice grain [6].

There is a well-established cottage industry in Korea to produce silkworm powder or pupae as a dietary supplement or culinary ingredient to improve health. Therefore, an alternative culture method for fruiting body production of *C. militaris* using a silkworm is needed. This study seeks to facilitate and simplify the injection protocols used to produce fruiting bodies in living silkworm. We studied the conditions, including injection volume and light intensity, to achieve infection and fruiting body formation.

Materials and Methods

Host insect. Silkworm larvae of *Bombyx mori* were reared with natural mulberry leaves, as directed by the silkworm rearing guidebook of the National Academy of Agricultural Science, Rural Development Administration (RDA), Korea. In this study, the pupae of the silkworm, *B. mori* were used for inoculation by *C. militaris*.

Fungal strain. *C. militaris*, Cmb233, the mating strain between the single ascospores of Cm186 and Cm209 preserved in the Rural Development Administration, was used for inoculation of the *B. mori* pupae.

Inoculum preparation. One hundred millimeters of potato dextrose broth (PDA) medium was poured into 250 mL flasks and autoclaved at 121°C for 15 min. Each flask was inoculated with the mycelial discs (5 mm) of *C. militaris* from growing margins on PDA medium. The inoculated media were cultured under static conditions at 25°C for 10 days. The cultured media were then homogenized at 120,000 rpm for 5 min with a Homogenizer (AM-11; Nihonseiki Kaisha Ltd., Tokyo, Japan) and filtered through sterilized gauze to remove entangled hyphae.

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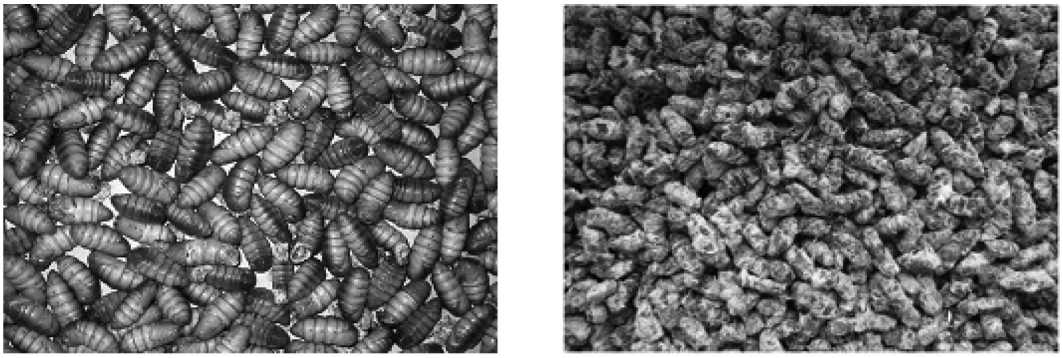

Fig. 1. Pupae of *Bombyx mori* infected with *Cordyceps militaris*. A, Pupae before infection; B, Pupae after infection.

Inoculum injection. One hundred microliters of homogenized hyphal bodies were injected into each pupae of the following three varieties of *B. mori*: Daeseungjam (F1 hybrid between the Japanese parental line Jam 125 and the Chinese parental line Jam 126), Baegokjam (F1 hybrid between the Japanese parental line Jam 123 and the Chinese parental line Jam 124), and Keumokjam (F1 hybrid between the Japanese parental line Jam 125 and the Chinese parental line Jam 140). The injection was conducted with a self refilling syringe (Socorex 173; Socorex, Ecublens, Switzerland).

The pupae aged 7-day-old to 11-day-old, from mounting, were injected with a 100 μL volume of the hyphal body suspension of *C. militaris*. One hundred microliters of a hyphal suspension were injected into each haemocoel of head, thorax and abdomen in pupae of the Daeseungjam variety of *B. mori*. To estimate the hyphal body concentration suitable for infection, the hyphal body suspension of *C. militaris* was adjusted with sterilized distilled water to between 10^3 colony forming unit (cfu) to 10^6 cfu (cells/mL). Fifty microliters of each suspension concentration were injected into the thorax haemocoel of *B. mori*. The number of hyphal bodies was counted with a hematocytometer. The hyphal suspension volume of *C. militaris* was adjusted to 0.025 mL, 0.05 mL, 0.075 and 0.1 mL, and each volume was injected into the thorax haemocoel in each *B. mori* pupae. The injection volumes of a hyphal suspension contained 2×10^5 cfu.

Induction of endosclerotium. After injection inoculation, the injected pupae were spread on tissue paper in plastic containers at 20°C until their bodies became hard and mummified by fungal proliferation.

Induction of fruit body. The inoculated pupae were placed on a wet cotton cloth at 1 cm square distance in transparent plastic containers. To induce the fruiting bodies, each container with the inoculated pupae was kept at 20°C under 12 hr light and 12 hr dark, with light intensity at 100, 500 and 1,000 lx, respectively. The containers were supplied with water from time-to-time to prevent excessive drying. The growth chamber for fruit body formation was controlled at 20°C with relative humidity over 90%.

Results

Silkworm variety. To select the silkworm variety suitable for infection of *C. militaris*, three varieties of *B. mori*, Daeseungjam, Baegokjam and Keumokjam were tested.

The pupae infected with *C. militaris* died within 2~3 days and became hard about 7 days after injection (Fig. 1). Infection of *C. militaris* into silkworm pupae was excellent in the Daeseungjam variety with a 100 μL injection (Table 1). Daeseungjam showed the highest infection rate at 90.8% (n = 2,611), followed by Keumokjam with 76.6% (n = 5,586), and Baegokjam with 63.9% (n = 2,489).

Pupal ages. The pupae aged 7-day-old to 11-day-old, from mounting, were used for inoculation by *C. militaris*.

Table 1. Infection of *Cordyceps militaris* by silkworm variety

Silkworm variety	No. of pupae tested	No. of pupae infected	Infection rate (%)
Baegokjam	2,489	1,590	63.9
Daeseungjam	2,611	2,372	90.8
Keumokjam	5,586	4,278	76.6

Table 2. Injection of *Cordyceps militaris* at different pupal age

Injection day[a]	No. of pupae tested	No. of pupae infected	Infection rate (%)
7	30	24	80.0
8	30	23	76.7
9	30	30	100.0
10	30	30	100.0
11	30	30	100.0

[a]Days from mounting.

While all the tested (n = 30) Daeseungjam pupae aged between 9-day-old and 11-day-old were infected, the infection rate of treated pupae aged 7-day-old and 8-day-old was 80.0% and 76.7%, respectively (Table 2).

Injection process. Each haemocoel of head, thorax and abdomen of 9-day-old pupae was injected to try to facilitate and simplify the injection process. Infection rate of pupae injected with a 100 μL volume ranged from 99.4% of thorax (n = 175) to 97.6% of head (n = 164) in the Daeseungjam variety. In Table 3, the injection (anatomical) parts of pupae showed significantly less infection.

The incidence of infection of *C. militaris* in silkworm was excellent in pupae injected with a hyphal suspension of 2×10^6 concentration (ascospore/mL). But, the infection rate steeply declined to 7.7% in 2×10^4 cfu and 4.0% in 2×10^3 cfu (Table 4).

Regardless of injection volume (Table 5), injection of a hyphal body suspension with a concentration over 2×10^5 cfu into silkworm pupae did not show a difference in incidence of infection. This result showed that a volume of 75 μL with over 2×10^5 cfu was suitable for infection by *C. militaris* into the pupal thorax of *B. mori,* because silkworm fluid squirted from pupa in the injection of 100 μL.

Influence of light intensity. Primodia of fruit bodies were not induced in dark culture conditions (Table 6). At 100 lx, fruit bodies were produced from all pupae tested (n = 30). The individual number of fruit bodies ranged from 3 to 10 (mean = 6.1) on a pupa. Fruit bodies with 5 individual numbers accounted for 26.7% (n = 8). Fruit bodies produced in this condition were generally short and thin (maximum 37.7 mm and 2.51 mm in length and diameter, respectively). At 500 lx, fruit bodies were produced from all pupae tested (n = 30), and the individual number of fruit bodies ranged from 3 to 5 (mean = 3.5) on a pupa. Seventy percent of fruit bodies had 2 or 3 individuals (n = 21). Fruit bodies produced in this 500 lx light condition were thick and long. The length of fruit bodies ranged from 65.0 to 76.0 mm (69.5 ± 3.95 mm), and their diameters ranged from 2.43 to 3.94 mm (3.10 ± 0.63 mm). At

Table 3. Infection of *Cordyceps militaris* by anatomical part of inoculation

Inoculation part	No. of pupae tested	No. of pupae infected	Infection rate (%)
Head	164	160	97.6
Thorax	175	174	99.4
Abdomen	142	141	99.3

Table 4. Infection of *Cordyceps militaris* by concentration of hyphal body suspension

CFU	No. of pupae tested	No. of pupae infected	Infection rate (%)
2×10^6	192	188	97.3
2×10^5	84	81	96.4
2×10^4	298	23	7.7
2×10^3	173	7	4

CFU, colony-forming unit (ascospore/mL).

Table 5. Infection of *Cordyceps militaris* by injection volume

Injection volume (mL)	No. of pupae tested	No. of pupae infected	Infection rate (%)
0.1	50	49	98.0
0.075	50	50	100.0
0.05	50	50	100.0
0.025	50	49	98.0

Table 6. Effect of light intensity on growth of fruit body in *Cordyceps militaris*

Light intensity (lx)	Fruiting body		
	No. of individuals	Length (mm)	Diameter (mm)
100	6.1 ± 2.41	34.1 ± 2.42	1.70 ± 0.38
500	3.5 ± 1.29	69.5 ± 3.95	3.10 ± 0.63
1,000	4.8 ± 2.92	69.7 ± 7.99	2.41 ± 0.80

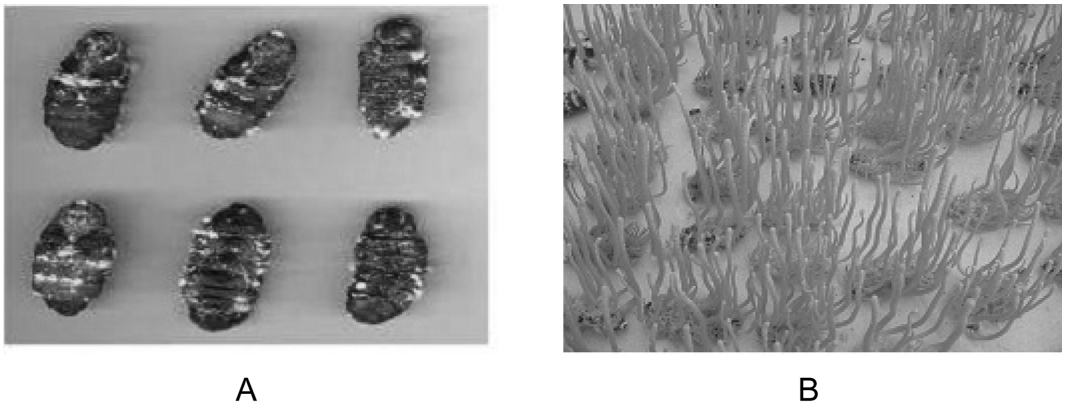

Fig. 2. Stomata on pupae of *Bombyx mori*. A, Primodia formation on pupae; B, Fruity bodies on pupae.

1000 lx, fruit bodies were produced from all pupae tested (n = 48), and the individual number of fruit bodies per pupa ranged from 1 to 10 (mean = 5.1). Fruit bodies with 2 or 3 individuals accounted for 36.7% (n = 18). Fruit bodies produced in this 1000 lx light condition were generally thick and long. The length of fruit bodies ranged from 61.5 to 72.4 mm (69.7 ± 7.99 mm), and their diameters ranged from 1.90 to 4.20 mm (2.41 ± 0.80 mm, n = 30). Fruit bodies produced in this experiment were orange in color and cylindrical or clavate in shape (Fig. 2).

Discussion

C. militaris has long been used as a medicinal mushroom in oriental countries, because of its biological and pharmaceutical activities, generally attributed to the presence of important bioactive ingredients such as adenosine, cordycepin and N^6-(2-hydroxyethyl) adenosine [7, 8].

The fruit bodies of wild *C. militaris* are expensive, because of host specificity and rarity in nature; they grow extremely slow in nature, their growth is restricted to a specific area, and their sizes are very small. It is very difficult to collect sufficient quantities for use as a drug or in functional foods. Synnemata formation on silkworm larvae of *B. mori* by percutaneous infection using conidiaspores of *Isaria tenuipes* has been reported [9]. However, this spray inoculation, using ascospores of *C. militaris* to silkworm larvae was quite poor in the incidence of infection. Therefore, an alternative infection process such as injection inoculation is needed.

Spores of the *Cordyceps* species usually attach to the larval state of the insect and penetrate into the larval body. The mycelium develops inside the body of the insect, feeding on its nutrients until it has taken over the complete organism filling the caterpillar with its hyphae. After the insect is completely mummified and devoid of nutrients, the fruiting body grows out of the insect body filled with the *Cordyceps* mycelium [10, 11].

The silkworm pupae of *B. mori* infected with *C. militaris* died within 2~3 days, became hard in about 7 days after injection, and produced fruit bodies (Fig. 1). According to Sung, wild fruit bodies of *C. militaris* collected in Korea were orange in color, cylindrical or clavate in shape, and ranged from 25 to 45 mm in length [3]. Our results agree with these findings, indicating that fruit bodies produced in this experiment are similar to wild fruit bodies.

By injection of hyphal bodies, infection of *C. militaris* into silkworm pupae was excellent in the Daeseungjam variety of *B. mori*, followed by the Keumokjam and Baegokjam varieties. This suggests that the silkworm variety of *B. mori* could affect the production of *C. militaris* fruit bodies.

Shanor derived fruit bodies of *C. militaris* by forcible introduction, with a needle, of cultured hyphae into the haemocoel of lepidopteran pupae [12]. In this study, we used the hyphal body instead of hyphae for inoculation. Hyphal body for inoculation, which could be more easily obtained in liquid culture, is another one of the vegetable stages of *C. militaris*.

In the spray or dipping inoculation method, the ascospores require the following stages for infection: germination, penetration to cuticle and formation of hyphal bodies in the haemocoel. Our process may take a shorter period to produce fruit bodies on pupae of *B. mori,* because the injection of hyphal bodies skips these three stages.

Injection tests on *B. mori* pupae of different ages were conducted to facilitate and simplify the inoculation process. Incidence of infection showed a significant difference according to the number of days from mounting. This result showed that a silkworm suitable for infection by *C. militaris* was after 9 days from mounting.

The injection of this fungus into head, thorax and abdomen of pupae appeared to show similar infection rates. This result indicates that the injection parts of pupae are less important in the infection process.

The incidence of infection of *C. militaris* into silkworm pupae was excellent in hyphal bodies at a 2×10^6 concentration (ascospore/mL).

It is thought that a concentration greater than 2×10^5 cfu is suitable for infection of *C. militaris*.

Injection volume of hyphal bodies into pupae is not significant in incidence of infection if the concentration is greater than 2×10^5 cfu, although the optimum condition may depend on strain. These findings suggest that a volume of 75 μL with over 2×10^5 cfu is suitable for infection of *C. militaris* into the pupal thorax of *B. mori*, because silkworm fluid squirted from pupa in the injection of 100 μL.

Primodia of *C. militaris* fruit bodies are not induced in dark conditions. At 500 lx and 1000 lx, fruiting bodies are long, thick and were produced from all pupae tested.

Sung reported that the fruit bodies of *C. militaris* were induced and grown at 500 lx on unconverted rice grain substrate [13]. Our results agree with these findings, though using different substrates.

We consider that hyphal body injection into silkworm pupae of *B. mori* is a suitable method, although it needs the use of special equipment for inoculation.

Acknowledgements

This research was supported by a grant from the Korean Rural Development Administration (Agenda Program, 200901FHT010101514).

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