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# Isolation and Culture of Entomopathogenic Fungus, *Cordyceps sphecocephala*

**Sung Hee Nam[1,2]*, Chun Ru Li[2], In Pyo Hong[1], Kyu Byoung Sung[1], Seok Woo Kang[1], Mei-Zhen Fan[2] and Zeng-Zhi Li[2]**

[1]*National Institute of Agricultural Science and Technology, Department of Agricultural Biology, R.D.A., Suwon 441-100, Korea*

[2]*Anhui Agricultural University, Hefei, Anhui 230036, P.R. China*

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**In this study, morphology of perithecia, asci, ascospores, *etc.* of *C. sphecocephala* were examined for its telemorphic characteristics. Its colony grew up to 32 mm in diameter on potato dextrose agar (PDA) for 30 days under the condition of 24±1°C. PDBLA and PDBAA media were selected as optimal media for *C. sphecocephala,* on which the growth was 1.5 times as fast as on PDA medium. Moreover, PDBLA medium induced successfully the synnemata of anamorphic state. *C. sphecocephala* was able to be proliferated *in vitro* on both larva and adult of honeybee drone as its substrate. After inoculated onto the drone larva, it produced mycelium at 24±1°C, with the maximum yield up to 67±3 mg on the 50th day.**

**Key words**: *Cordyceps sphecocephala, Apis mellifera,* honeybee, drone

## Introduction

The genus *Cordyceps* is a group of entomopathogenic fungi. Their spores adhere on insect cuticle and produce germ tubes, which then penetrate insect integument and form Hyphal bodies to proliferate in insect haemocoel and kill the insect. Finally, mycelium protrudes the cuticle of the insect cadaver and produce one or several stromata.

In China, some *Cordyceps* spp. have been traditionally used for hundreds of years for immunity modulation, fatigue resistance, longevity elongation, and other functions. Especially, *C. sinensis* has been used for roborant, sedatives, and supplementary therapy to jaundice, opiumism, tuberculosis and cancer, *etc. Beauveria bassiana,* another important entomopathogenic fungus has been utilized for relief of asthma (Tanada and Kaya, 1993), sore throat, and pruritus, and aphonia from apolexy. In Japan, *C. militaris* and *C. ophioglossoides* were identified to have active substances related to anticarcinogenic and immunity modulation functions (Ohmori *et al.*, 1986; Kiho *et al.*, 1996).

The investigation shows that *Paecilomyces tenuipes* cultured on silkworms, has anticarcinogenic bioactivity and modulates liver function and immunity (Cho *et al.*, 1999; Shin *et al.*, 2001). In addition, some species of *Beauveria, Metarhizium,* and *Paecilomyces,* anamorphs of *Cordyceps*, have been successfully used for fungal insecticides to regulate populations of pest insects in the world (Ferron, 1985; McCoy *et al.*, 1988, Deacon, 1998).

*Cordyceps* reported in the world includes over 300 species. Among them, more than 80 have been identified in Korea. Species of *Cordyceps* on wild bees involve *C. sphecocephala, C. oxycephala, C. elongatostromata,* and *C. japonensis* (Shimizu, 1994). *C. sphecocephala* among them, can be easily found during collection season from July to August in whole Korean peninsula (Nam *et al.*, 2005). This species has been known to have vigorous functions, and its anticarcinogenic effect was recently discovered in Japan. However, there are some difficulties in study of its use as a resource due to loss of teleomorphic state and its tardy growth under culture condition.

The purpose of this study is to identify *C. sphecocephala* collected in the field and to developa stable technique

*To whom correspondence should be addressed.
National Institute of Agricultural Science and Technology, Department of Agricultural Biology, R.D.A., Suwon 441-100, Korea. Tel: +82-31-290-8529; E-mail: sunghee@rda.go.kr

for its *in vitro* production on honeybee drone larvae so as to improve the use value of *C. sphecocephala* and honeybee as its substrate.

## Materials and Methods

### Fungus collection and preservation

*Cordyceps sphecocephala* J201 was collected from the Sorak Mountain in Korea and has been preserved in the entomopathogenic fungus collection in Department of Agricultural Biology at the National Institute of Agricultural Science and Technology (NIAST, RDA) in Suwon, Korea.

### Fungus isolate

A fresh mature stroma was disinfected by 2% sodium hypochloride and taped to the inner surface of lid of a petri dish containing water agar. The dishes were incubated at 24±1°C for 3~4 days. Then, the isolated ascospores from stroma were transferred to a PDA medium for 30 days.

The specimen was observed by using a stereoscopic microscope and microscope, BX40 Olympus. The host cadavers, stromata, perithecia, asci, secondary spores and hyphae were examined for characterization.

### Medium screening for fungal growth

Six media, PDA, PDBLA, BLA, PDBAA, BAA, and SLA (Nam *et al.*, 2001), were prepared to culture *C. sphecocephala.* A piece of colony 3 mm in diameter of the strain was taken from the growing margin on the medium and placed upside down at the center of the six media, respectively. All the inoculated media were incubated at 24±1°C, in darkness. After 14 days, the colonies were measured for mycelial growth. Duncan's multiple range test was run to check the growth differences. The media compositions are shown in Table 1.

**Table 1.** Composition of media used in this study

| No. | Medium | Compounds (g/*l*) |
|---|---|---|
| 1 | PDA | Potato dextrose broth 24 g, agar 15 g |
| 2 | PDBLA | PD 12, powder of frozen dried honeybee larvae 12 g, agar 15 g |
| 3 | BLA | Powder of frozen dried honeybee larvae 24 g, agar 15 g |
| 4 | PDBAA | PD 12 g, powder of frozen dried honeybee adult 12 g, agar 15 g |
| 5 | BAA | Frozen larvae 24 g, agar 15 g |
| 6 | SLA | Powder of silkworm larvae 24 g, agar 15 g |

### Inoculum preparation and inoculation of honeybee drone cadavers

The colony on the agar-solidified medium was punched with a cork bore 3 mm in diameter and soaked in 150 m*l* PD medium (Potato Dextrose 24 g, distilled water 1) and then cultured at 24±1°C for 14 days.

Twenty g of honeybee drone larvae and adults, respectively, were put into 20 m*l* cylindrical bottle and sterilized at 121°C for 20 minutes. Body fluid which flowed out of the host cadavers was removed from the cylindrical bottle with a pipette. Then, 1 m*l* spawn was inoculated into the cadavers in the cylindrical bottle, respectively, and shaken sufficiently so that the spawn could be smeared on the cuticle of the cadavers evenly. Rootage period, cultivation period, synnema formation, colony color and dried weight of mycelium were examined.

## Results

### Macroscopic and microscopic description of *C. sphecocephala* and its anamorphic state

A single stroma arise from the thorax of its adult wasp host. It is cotton bud shaped, whitish yellow, and up to 31 mm in length. It is divided into two parts distinctly, a fertile head on the upper part and a stalk on the lower part. The stalk consists of parallel strands of septate, tightly packed hyphae (Fig. 1). Perithecia are arranged obliquely in the head part of stroma, 700~950×180~269 μm. Numerous asci discharged from the perithecia can be observed under microscope, filiform, hyaline, 330~430×6.3~7.0 μm including apical cap. Matured ascospores are gradually broken into secondary ascospores, hyaline, fusoid, 9.0~10.5×1.5~2.0 μm. The colony on PDA grown from ascospores is up to 32 mm in diameter under 24±1°C on the 30th day, white cream, with its reverse side white cream to pale grey brown. Mycelium on the medium is agglutinated tightly, sterile and indistinguishable morphologically, 2.8-3.3 μm in diameter. Growth is very slow in comparison with other *Cordyceps* spp. such as *C. militaris.*

### Mediam of agar-solidified medium for *C. sphecocephala* cultivation

Six media were investigated to screen the optimum medium. *C. sphecocephala* grew to 21±1.0, 20±1.5 mm in diameter, respectively on the PDBLA and PDBAA, over 14 days under 24±1°C. The growth on these 2 media was about 1.5 times as fast as on PDA, with significant difference to those on all the other media (Fig. 2). On the other hand, the growth on the BLA and BAA was as slow as on PDA. Besides, growth on SLA medium grew significantly slowly than on BLA and BAA, suggesting

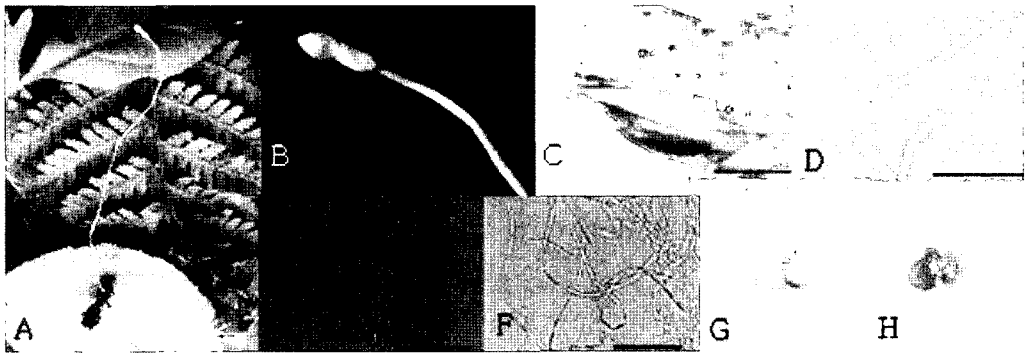


**Fig. 1.** *Cordyceps sphecocephala* (Kl.) Sacc. A. a stroma from an ant (J201); B. fertile head; C. perithecia (bar=500 μm); D. asci showing thickened apical cap (bar=50 μm); E. fusiform ascospores (bar=50 μm); F. mature ascospores (bar=50 μm); G. obverse of colony on PDA after 30 days at 24±1°C; H. reverse of the colony.

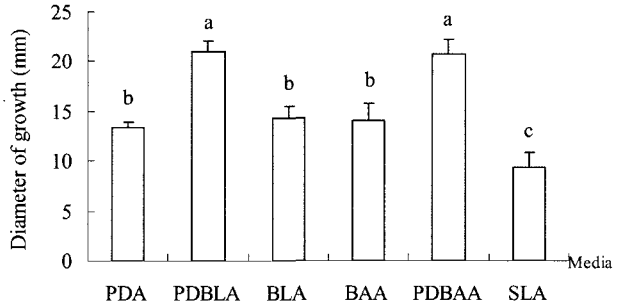


**Fig. 2.** The mycelial growth of *C. sphecocephala* on different media over 14 days, Note: The coloums were from the Mean and Standard error of three replicate plates. Values in the small figure followed by the same letters are not significantly different (Duncan's Multiple Range Tests, $P=0.05$).

that SLA medium was inadequate for *C. sphecocephala,* although it was a proper medium for some entomopathogenic fungi (Nam *et al.*, 2005). The colony appearances on the five media were all irregular in shape. The shape of elevation and margins on the other media was all different from those on PDA, but the colony shapes on all five media were convex and undulate (Table 2). Synnemata ware very easy to induce on the PDBLA.

**Proliferation of J201 on Honeybee Drones**

Honeybee drone larvae and adults were inoculated with cultured liquid spawn of *C. sphecocephala* and incubated under various temperature conditions, respectively. Cultivation period, formation of synnemata, color of synnemata and the dried weight were examined to evaluate the proliferation capability of *C. sphecocephala.* Rootage period, which represents the required period that mycelium of *C. sphecocephala* covering honeybee cuticle entirely after inoculation, varied from a minimum of 25 days to a maximum of 35 days, depending on treatment temperature. The cultivation period, which means the

**Table 2.** Cultural characteristics of *C. sphecocephala* on different media

| Fungus | Medium | Colonies on agar plates | | |
|---|---|---|---|---|
| | | Form | Elevation | Margin |
| *C. sphecocephala* | PDA | Irregular | Pulvinate | Curled |
| | PDBLA | Irregular | Convex | Undulate |
| | BLA | Irregular | Convex | Undulate |
| | PDBAA | Irregular | Convex | Undulate |
| | BAA | Irregular | Convex | Undulate |
| | SLA | Irregular | Convex | Undulate |

whole period from inoculation to synnema formation, was checked in 5 days interval (Table 3). The results showed that the cultivation period tended to be shortened under the higher temperature. Treating temperature of 30°C shortened approximately 20~30 cultivation days in comparison with the 15°C treatment. However, when the cultivation temperature went up above 30°C, the mycelium yield decreased significantly and no symmemata formed. The amount of the mycelium yield was significantly higher ($P=0.05$) by using the honeybee drone larvae as substrate, than by using the adults. Only with the larvae as substrate at 20 and 25°C, produced synnemata and achieved the highest yield significantly higher than at the other temperatures. At 25°C, the yield was not significantly lower than that at 20, but cultivation period was shortened by 5 days. Therefore, the optimum temperature for proliferation of *C. sphecocephala* was 25°C and the drone larva was more suitable to be utilized as the substrate than the adults.

## Discussion

According to a report on *C. sphecocephala* in Japan

**Table 3.** Characteristics of *C. sphecocephala* proliferated on the honeybee substrate

| Substrate | Temp. | Rootage (days) | Cultivation period (days) | Formation of synnemata | Color* | Dried weight (mg) |
|---|---|---|---|---|---|---|
| Honeybee larva | 15 | 35 | 60 | - | YB | $71 \pm 2^{a**}$ |
| | 20 | 30 | 55 | + | WY | $69 \pm 2^{a}$ |
| | 25 | 25 | 50 | + | WY | $67 \pm 3^{a}$ |
| | 30 | 25 | 30 | - | W | $52 \pm 3^{c}$ |
| Honeybee adult | 15 | 35 | 60 | - | YB | $50 \pm 3^{c}$ |
| | 20 | 35 | 55 | - | YB | $45 \pm 1^{d}$ |
| | 25 | 30 | 50 | - | WY | $56 \pm 2^{b}$ |
| | 30 | 25 | 40 | - | WY | $48 \pm 2^{cd}$ |

*Color: YB: yellowish brown, WY: whitish yellow, W: white. Dried weight: frozen dried weight of the mycelium growth from the honeybee substrate

**Values in the small figure followed by the same letters are not significantly different (Duncan's Multiple Range Tests, $P=0.05$).

(Shimizu, 1994), the specimens could be easily collected from July to September, which was a similar period in Korea (Nam *et al.*, 2005). One of the specimens usually protruded no fewer than 14 stromata from a host's bodies. Their exterior shape is similar to those of *C. tricentri* on true bugs and *C. myrmecophila* on ants.

The head of stroma of *sphecocephala* on *Vespa auraria* developed into a cylindrical shape, instead of a spindle shape. The species was reported by Kobayasi (1940) and Shimizu (1994), but there was no mention about its anamorphic state. Therefore, description of the characteristics of teleomorphic state and anamorphic state in this study will provide a useful tool for accurate identification of this species in the future studies. Although the genus *Cordyceps* has a very long history of utilization, the proper medium for cultivation of this species hasnot been developed yet. Simply, PDA, SDAY and Czapek media usually are not proper for cultivation of *Cordyceps* spp., and especially not proper for mass production.

In this study, six media, PDA, PDBLA, BLA, PDBAA, BAA and SLA were investigated for screening the optimal medium for *Cordyceps*. Among them, BLA and BAA have a similar efficiency to PDA. For the growth of a microorganism, the nutrient in a culture medium is a major factor that influences the results of susceptibility tests (Meletiadis, 2001). Therefore, it could be evaluated as a remarkable result since the media only contained the powder of insect without any supplements of nutrition components. Also the principal ingredient of the SLA medium consisted of the powder of silkworm larvae. Although the majority of entomopathogenic fungi grew on the SLA well, the *C. sphecocephala* showed a tardy growth on the SLA. That is evidence that most species of *Cordyceps* have a restricted host range and rigid host specificity (Evans, 1982). In addition, PDBLA and PDBAA media adding PD broth could promote growth of *C. sphecocephala* 1.5 times as high as PDA.

As to the cultural characteristics on six media, colony shape was all irregular on all media and elevation and margins were pulvinate and curved on PDA, but convex and undulate on the other five media. Entomopathogenic fungi showed peculiar cultural characteristics on the agar-solidified media according to its species and the medium applied. Therefore, the cultural characteristics on different media can be utilized to identify unknown species.

*C. sphecocephala* can grow on both drone larva and adult. But, its optimal condition for growth was on larva under the 25°C over 50 days. On the drones, a few synnemata, which was the stroma-like stalks, were induced and produced maximal $67 \pm 3$ mg mycelium by dry weight.

Drones do not have any function in the apicultural industry. A drone of *A. mellifera* needs 24 days to develop from an egg to a fully developed male adult and may live as an adult for several months. Drones do not work and their only function in the honeybee's community is as a potential mate for a virgin queen and then they usually complete their span or they are expelled from the hive in the autumn.

The purpose of this study is to produce *C. sphecocephala* by using the drones, which are worthless for utility in apiculture and consequently promote their industrial value. Also, this study dealts with proliferation of *C. sphecocephala*. The study results can be utilized for mass production of *C. sphecocephala* in the future.

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