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Production of the Wild Entomopathogenic Fungi, *Cordyceps militaris*, in the Silkworm, *Bombyx mori*

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The wild entomopathogenic fungi, *Cordyceps militaris*, were collected at the Whawang mountain, Korea. The pupae of the silkworm, *Bombyx mori*, were used as infecting hosts for the production of the *silkworm-militaris dongchunhacho*, silkworm vegetable wasps and plant worms with *C. militaris*. Three inoculation methods in terms of injection, spray and immersion were tested against the silkworm pupae. The three inoculation methods revealed 100% infectivity to the silkworm pupae tested. Of the three inoculation methods, the injection method was highly effective in the reduction of the period required for the endosclerotium and the completion of fruiting body formation. These results indicate that the silkworm pupae are very effective host insects for the production of *C. militaris*.

Key words : Entomopatohgenic fungi, *Cordyceps militaris, Bombyx mori, Silkworm-militaris dongchunghacho*, Injection inoculation

Introduction

A great variety of entomopathogenic fungi have been collected and a few of them are being investigated for their pharmaceutical use (Shimizu, 1994, 1997; Samson *et al.*, 1988; Sung *et al.*, 1997, 1998; Lin, 1999; Shin, 1999; Carlile and Watkinson, 1996; Choi, 1999; Jianzhe *et al.*, 1989; Montefiori *et al.*, 1989; Yamada, 1984). Ascomycetous fungi of the genus *Cordyceps* are an interesting subject. *Cordyceps* is placed in the family Clavicipitaceae of the order Clavicipitales in the class Pyrenomycetes, whose members are known to be exclusively endoparasitic to insects (Samson *et al.*, 1988; Spatafora and Blackwell, 1993; Shimizu, 1994).

In general, vegetable wasp and plant worm with *Cordyceps* indicates the fruiting body formed on the larval integument of its insect host, which is insect-born mushroom showing special appearance produced from the larvae attacked by entomopathogenic fungi, *C. sinensis* (Lin, 1999). Therefore, a mass-production of insect-born mushrooms is very important for industrial use of the *Cordyceps* spp. A method for artificial massproduction of the silkworm vegetable wasp and plant worm with *Paecilomyces japonica* called silkworm-dongchunghacho was already established in Korea, and its pharmacological activity was also reported in recent years (Cho, 1999; Choi, 1999; Shin, 1999; Lee *et al.*, 1999a; Lee *et al.*, 1999b; Lee and Park, 1998; Lee *et al.*, 1998). An artificial *in vivo* mass-production technology of *C. sinensis* and *C. militaris* was not currently established in the living insect as a host.

We have produced the silkworm vegetable wasp and plant worm with *C. militaris* using silkworm pupae. In this paper, the artificial *in vivo* mass production methods of *C. militaris* in the silkworm pupae as a living host insect are described.

Materials and Methods

Insects

The variety of the silkworm, *Bombyx mori* (Baegokjam,

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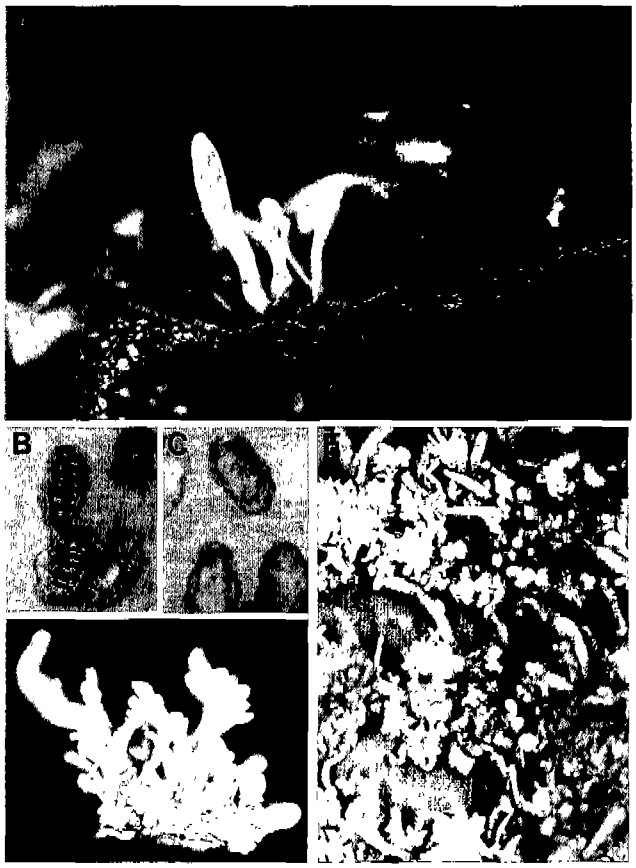

Fig. 1. The production of the silkworm vegetable wasps and plant worm with *C. militaris*. A, wild entomopathogenic fungi, *C. militaris*, collected at the Whawang mountain, Changryung, Korea; B, silkworm pupae mummified (endosclerotium); C, silkworm pupae covered with mycelium; D, a *silkworm-militaris dongchunghacho* formed completely fruiting body; E, mass-produced *silkworm-militaris dongchunghacho*.

Jam 123 × Jam 124) was obtained from the Department of Sericulture and Entomology, National Institute of Agicultural Science and Technology, Korea. Larvae were reared on mulberry leaves.

Entomopathogenic fungi

The wild entomopathogenic fungi, *Cordyceps militaris*, were collected at the Whawang mountain, Changryung, Korea (Fig. 1A). The spores were isolated on the PDA (potato dextrose agar) medium from the fruiting body of *C. militaris*, and the hyphal body on the PDA was subcultured in PD medium in shaking incubator of 120 rpm for 7 days at 25±1°C. The liquid cultured hyphal body was stored at 4°C until use.

Inoculation of *C. militaris*

The fourth day-old silkworm pupae were immersed for about 2 min in 70% ethanol solution for disinfection of the pupal surface in the clean bench, and then the pupae were taken out from the fluid and dried in the same clean bench (Lee *et al.*, 2001). A pupa was injected with 100 μl of the cultured hyphal body of *C. militaris* by percutaneous inoculation. The amount equivalent to the above percutaneous inoculation was, alternatively, sprayed and immersed on the pupae of the corresponding experimental plots, respectively. The inoculation concentration of *C. militaris* was 10^8 hyphal bodies/ml. The experimental plots were designed according to the ways of inoculation to the silkworm pupae such as injection, spray and immersion. The total tested numbers of each experimental plot including three replications were ninety silkworm pupae (30 pupae per replication).

Induction of endosclerotium and fruiting body formation

To induce the formation of the endosclerotium, the pupae that *C. militaris* was inoculated by injection, spray and immersion were incubated at 28°C, 95% R.H. for five to eight days according to the experimental plots. After the wild entomopathogenic fungi completed the endosclerotium in the silkworm pupae, they were continuously cultivated at 19-20°C, 90% R.H. under the photoperiod of 12L:12D until the formation of fruiting bodies.

The time for the completion of fruiting body formation indicates that the fruiting body becomes 2.5 - 3 cm in length with a yellowish red color. The silkworm vegetable wasps and plant worm with *C. militaris*, *silkworm-militaris dongchunghacho*, was harvested for further investigation.

Source of illumination

The fluorescent light being used in the current farm house was applied in the present study. The pupae inoculated with *C. militaris* were continuously cultivated in the incubator equipped with illuminating apparatus of fluorescent light under the photoperiods of 12L:12D.

Results and Discussion

The wild entomopathogenic fungi, *C. militaris*, were collected at the Whawang mountain located in Changryung, Korea (Fig. 1A). The hyphal body of *C. militaris* collected from the wild conditions was cultured and used as an inoculum in this study. The 4 days-old silkworm pupae were infected with the inoculum of *C. militaris*. The method of inoculation in this study was applied to three treatments of injection, spray and immersion.

The endosclerotium was induced at 28°C with 95%

Table 1. The effect of the inoculation method on the production of the silkworm vegetable wasps and plant worm with *C. militaris*

Inoculation methods	Period required for endosclerotium (days)	Period required for the completion of fruiting body formation (days)	Percentage of the pupae showing the complete fruiting body formation (%)
Injection	5	35	100
Spray	8	40	100
Immersion	8	40	98

R.H. The periods required for the completion of endosclerotium (Fig. 1B) were respectively determined in three treatments (Table 1). The periods required for the completion of endosclerotium were 5 days in injection treatment and 8 days in both spray and immersion treatments. These results showed that injection treatment is the best among the three inoculation methods for the early completion of endosclerotium.

After completion of endosclerotium, the pupae were covered with a mycelium (Fig. 1C). The periods required for the completion of fruiting body formation were 35 days in injection treatment and 40 days in both spray and immersion treatments (Table 1 and Fig. 1D). These results apparently showed that the period required for the completion of fruiting body formation in injection treatment is the shortest among the three inoculation methods tested in this study.

The percentage of the pupae showing the complete fruiting body formation among the inoculation methods tested was 100% in both injection and spray treatments, and 98% in immersion treatment (Table 1). The result indicates that silkworm pupae are an effective insect host for the production of *C. militaris*.

In conclusion, these results reveal that it will take about 40 - 48 days to harvest the silkworm vegetable wasps and plant worms with *C. militaris*, *silkworm-militaris dongchunghacho*. Also, silkworm pupae were highly effective insect host for the *in vivo* mass production of *C. militaris* (Fig. 1E).

Among the three inoculation methods, even though the injection of the fungi to the early pupae is the best in the light of production periods and stability of infection rate, it has a minor disadvantage, which means that the injection needs more labor and time for the processing compared with the other two inoculation methods tested. So it suggests that some further improvement at this point should be done.

At the maturity of the *Cordyceps* fungi, fruiting bodies of a spectacular shape grow out of the host insect. In this study, the shape of fruiting bodies between the wild collected sample and the mass-reared silkworm vegetable wasps and plant worms with *C. militaris* was slightly different from the morphological characters (Fig. 1A and D). In their shape, size, and coloration, in combination with the host species, the morphology of fruiting bodies can be varied according to the occasions performed in the tests, although morphological characters are generally stable in a particular species (Samson *et al.*, 1988; Shimizu, 1994).

In general, the species of the wild entomopathogenic fungi have their own host specificity. Some species have a wide host range, others are restricted to a single species or a closely related group of species (Shimizu, 1994). For the *in vivo* mass production of fungi of diverse interest, therefore, identification of the insect host specific to the corresponding fungi, easy infection processing of the fungi to the host and the systematic mass rearing of the host insect are essential.

In the present study, the wild fungi whose host insects were not identified exactly in wild habitat were able to attack the silkworm, *B. mori*, at the metamorphic form of larvae (data not shown) or pupae. The systematic mass rearing of silkworm have been already well-established in many countries. Therefore, the selection of the easy infection methods of *C. militaris* to the silkworm is a key factor for the mass production of silkworm vegetable wasps and plant worm with *C. militaris*, *silkworm-militaris dongchunghacho*. Mass-production technology of the silkworm-dongchunghacho, using *P. japonica* as an infecting agent to silkworm, have been already developed in Korea (Cho, 1999). In this study, our results revealed that the newly developed mass-production technology of *C. miltaris* using silkworm as an insect host will be a good foundation and information for further insect industry using the *silkworm-militaris dongchunghacho*.

Acknowledgments

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