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Cultural Characteristics for Inducing Fruting-body of *Isaria japonica*

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눈꽃동충하초의 자실체 유도를 위한 배양조건

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ABSTRACT: To obtain basic data for mass production of *Isaria japonica*, cultural characteristics of *japonica* were investigated by using liquid, solid media and silkworms pupa. Mycelia grew favorably at the temperature of 23~28°C on MYG medium with pH 7.0. The fruiting-body of *I. japonica* was induced below 20°C in MYG liquid medium (Malt yeast glucose) under fluorescent light. In MYG basal medium mixed with pupal powder of silkworms, the fresh weight of fruiting-bodies was increased with increasing concentration of pupal powder. The highest yield of fruiting bodies was obtained in carbon-rich medium supplemented with pupal powder of silkworm. Also, fruiting-bodies of *I. japonica* were produced massively on the silkworm pupa placed on the stainless tray in the shortest time. The structure and shape of fruiting-bodies were coral-like, many-branched types with numerous conidiospores.

KEYWORDS: Cultivation, Fruiting-body, *Isaria japonica*, Pupal powder

Several *Cordyceps* species have recently been studied in different purposes (Sung *et al.*, 1995). Among these species, *Cordyceps sinnesis* (Berk.) Sacc., caterpillar fungus, is an entomogeneous fungus and has been used as a traditional medicine in Korea, China and Japan (Lee *et al.*, 1997). *C. sinensis* has been recorded to exhibit outstanding effects for curing cancer and other serious diseases (Li *et al.*, 1995).

Nowadays, beneficial mushrooms such as this fungus have been hardly found in the fields in accordance with a shortage of natural resources. The artificial production of fruiting-body of *C. sinensis* has been rarely tried successfully, even though the cultivation of its mycelia has recently been established (Lin and Yu, 1997). Therefore, many researches have so far been carried out in Europe and United States as well as in East Asian country.

However, some polysaccharides isolated from *Cordyceps* species such as *C. cicadae* Shing (Kiho *et al.*, 1989) and *C. ophiogloss-*

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oides (Ehrenberg ex Lind) Fr. (Yamada *et al.*, 1984) have been known to have potent anti-tumor activity. Recently, Sung *et al.* (1996) reported that Fruiting-bodies of *C. militaris* (Vuill.) Fr. and *Isaria felina* (DC.) Fr. were artificially produced by using rice or corn grain medium. The fruiting-body of *C. militaris* showed similarly strong negative inotropic effects to those from *C. sinensis* (Li *et al.*, 1997). However, the large-scale production of fruiting-bodies in Clavicipitaceae species has not yet been succeeded. Mass culture of mycelia and mass production of fruiting-bodies in these medicinal Clavicipitaceae fungi are, therefore, necessary in order to use them as medicines or health foods. They also mentioned that ascospores of fungus colud be used as a biological insecticide (biological control) to prevent harmful insects, which were attacking beneficial forest trees and vegetables (Li *et al.*, 1997).

In this study, *Isaria japonica* Yasuda, the anamorph of *Cordyceps takaomontana* Yakushiji et Kumazawa (Shimizu, 1994) and a parasite on pupae of Lepidoptera insects, was used to investigate cultural characteristics under different conditions. Previously, the basic information for mycelial growth of *I. japonica* was investigated by some researchers. (Kang, *et al.* and Institue of Silkthread Insect) Although this fungus is expected to possess medicinal properties, fruiting-body formation has rarely been obtained in artificial conditions. Mass production of *I. japonica* is urgent if its medicinal properties are to be used or if the fruiting-bodies are to be commercialized as a health food. Therefore, this study was carried out to find the possibility for an artificial cultivation of *I. japonica* and obtain basic data for its mass production.

Materials and Methods

Isolation and identification

The fruiting-bodies of *I. japonica* grown on the pupa of Lepidoptera moth were collected at Cheju island in July, 1997 (Fig. 1). The fruiting-bodies were microscopically examined and identified as *I. japonica*, which had characteristic comma-shaped, bead-like conidiospores (Sung, 1994). The isolate was obtained from conidiospores on potato dextrose agar (PDA) which was supplemented with streptomycin.

Cultural conditions

Mycelial growth of *I. japonica* was examined on Malt-yeast-glucose-agar (MYGA), which was selected a optimal medium in mycelial growth. A 3 mm diameter disc was isolated from PDA medium cultured under dark condition for 7 days at 25°C and then was used as a inoculum. To determine the optimum temperature for favourable mycelial growth, the cultures were incubated in the range of 15~35°C for 7 days under dark condition. To determine the optimum pH for favourable mycelial growth, the cultures were also incubated on MYGA medium with pH ranges of 4.0~8.3 for 8 days at 25°C under the dark condition. Mycelial growth on agar plate was measured on the basis of average colony diameter of three plates, and each test was replicated twice.

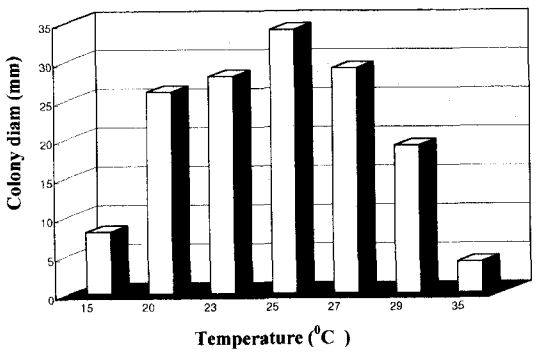

Fig. 1. Influence of temperature on mycelial growth of *Isaria japonica*.

Effect of temperature and pH for the fruiting-body formation

To determine the optimum temperature and pH for induction of fruiting-body formation, 100 m*l* Erlenmeyer flasks containing 20 m*l* of MYG liquid medium were used. A 3 mm diameter mycelial agar disc was isolated from PDA medium cultured for 7 days at 25°C under the dark condition and then was inoculated into liquid medium in flasks. To determine the optimum temperature for inducing fruting-body of *I. japonica*, the cultures were incubated at 20, 25 and 30°C for 21 days in the dark. Liquid media with initial pHs of 4.6 to 8.0 were used, and the cultures were incubated for 21 days at 25°C to determine the optimum pH for inducing fruiting-body. The cultures were then transferred to another condition and then incubated at 18°C under condition with a cycle of 12 hour light and 12 hour dark, when a full growth of mycelium of *I. japonica* was checked on the medium. Fluorescent lamps were used for suitable illumination to induce the fruiting-body formation. The average fresh weight of three flasks in each experiment was obtained, and two replications were examined.

Effect of additives for the fruiting-body formation

To investigate the effects of additives for inducing the fruiting-body, different additives were added to MYGA medium. (Table 1).

Eight hundred m*l* polypropylene bottle containing 400g of medium was supplemented with tap water capable of adjusting to 100% moisture content, autoclaved for 50 min at 120°C, and then inoculated with 1 m*l* suspension of mycelia subcultured on the PD broth. The cultures were incubated at 25°C without light until full growth of mycellium was checked on the medium. After mycelia had colonized the entire medium, the cultures were transferred to the conditions with a cycle of 12 hour light and 12 hour dark at 18°C to induce the fruiting-body formation. Average yields of fruiting-bodies obtained from five bottles in each test were

Table 1. The yield of Fruting-bodies of *Isaria japonica* on various additives

Medium[a]	Material weight (g)[b]							Fruiting-body yield(g)[c]
	MYGA	rice	rice brain	Dried silkworm	Dried silkworm powder	Chinese medicine waste	silkworm pupal powder	
1	200	200						16.9ef[e]
2	200		200					16.6f
3[d]	200	100		80			20	17.1e
4	200	100			80		20	16.8ef
5	200	100				80	20	18.5b
6	200	100	60				40	17.2de
7	200	100	40				60	17.6d
8	200	100	20				80	18.1c
9	200	100					100	18.9a

[a]Total weight was 400g in the treatment of each medium.
[b]Dry weight basis.
[c]Fresh weight basis.
[d]MYGA-rice basal medium.
[e]The different letters are significantly different at p=0.05 according to Duncan's multiple range test.

determined on a fresh weight basis. The experiment was replicated twice.

Effect of silkworm pupa for fruiting-body formation

To examine the formation of fruiting-bodies from silkworm pupa, the fungus was inoculated in a 300 m*l* Erlenmeyer flask containing 150 m*l* of PD broth medium and incubated in dark for 15 days at 25°C. A silkworm pupa was dipped into the solution which was mixed with 20% gelatin, and incubated in the suspension of this fungi. After that, it was taken out from the suspension, laid on the sterlized stainless tray (30×20×5 cm) and incubated at 25°C. When the mycelia of *I. japonica* had colonized the entire surface of pupa, the pupa was transferred to the condition with a cycle of 12 hour light and 12 hour dark at 18°C to induce the fruiting-bodies of *I. japonica*.

Result

Optimum temperature and pH

Mycelia of *I. japonica* grew favorably at 23~27°C, and the optimum temperature was about 25°C. (Fig. 1) Mycelial growth of *I. japonica* was decreased rapidly above 27°C. The optimum initial pH of solid medium for mycelial growth was 7.0 values lower than pH 6.0 (Fig. 2).

Fruiting-body of *I. japonica* was initiated to be formed on the surface of liquid medium in flasks within 16~18 days after inoculation, and has been matured gradually. However, none of fruiting-body were formed above 27°C. This fungus also appers to require low temperatures to induce the fruiting-body formation. The fruiting-body was formed on liquid media with initial pHs in the broad range of 4~8. Mycelia were differentiated into fruting body even when initial pH was as low as 4, though this pH value inhibited mycelial growth.

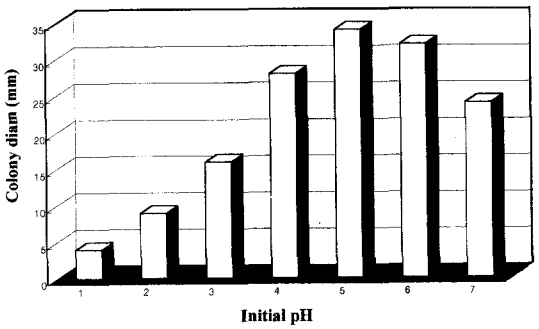

Fig. 2. Influence of initial pH on mycelial growth of *Isaria japonica*.

Effect of additives

The yield of fruiting bodies of *I. japonica* obtained in various solid media were shown in Table 1. In 800 m*l* bottle cultures, the mycelia entirely colonized the medium within 15 days after inoculation. Primordia began to be formed on all the media within 5~7 days at 18°C and developed into fruiting-bodies of 2~4 cm in length within 13~15 days after transfer to condition for inducing fruiting bodies.

The highest yield of fruiting-bodies recorded 18.9g in the bottle containing 200 m*l* of MYGA, 100g of rice and 100g of silk worm pupal powder (No. 9) and was followed by 18.5g in the bottle containing basal medium (MYGA and rice) and Chinese medicine waste (No. 5). In basal medium, the yield of fruiting-bodies was increased gradually with increasing content of pupal powder in the medium (Fig. 3).

Therefore, the media which contained pupal powder seem to be suitable additives for the production of *I. japonica*. Also, Chinese medicine waste seems to have one of the merits to produce fruiting-bodies of *I. japonica* because of its cheap price. It seems to be likely that carbon-rich media enhanced the fruiting-body formation compared with other media.

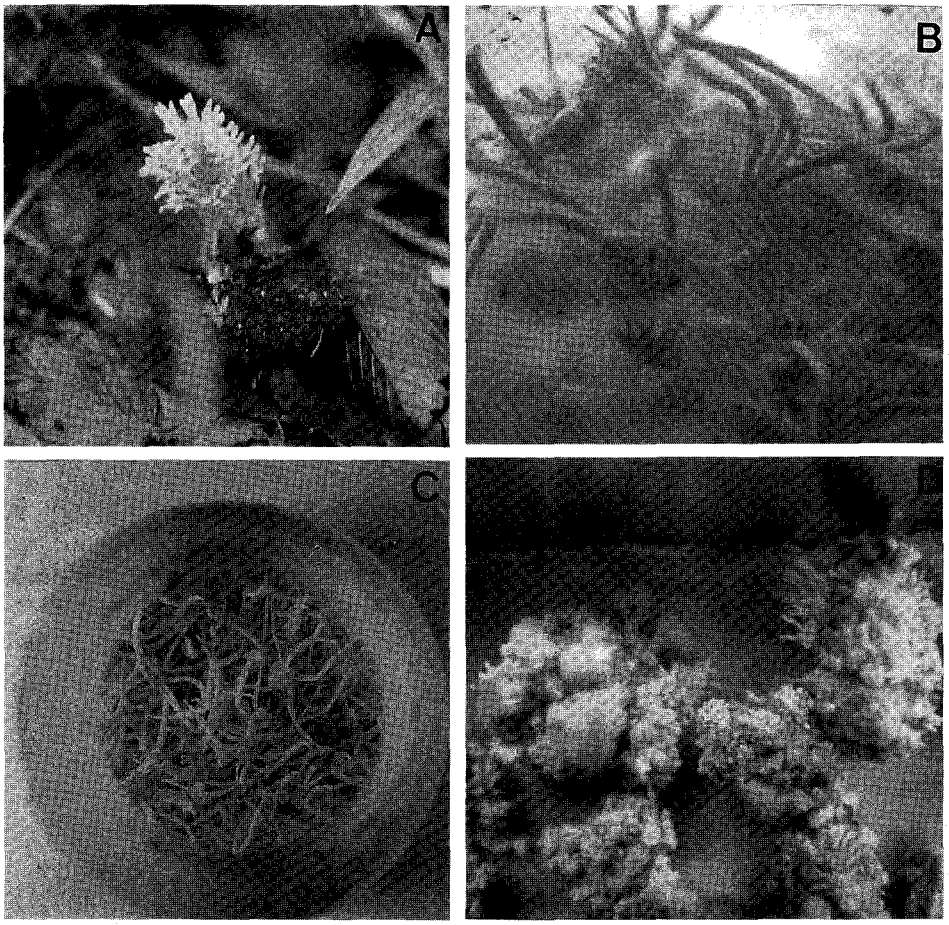

Fig. 3. Fruting-body of *Isaria japonica*.
A. *Isaria japonica* formed on a pupa of Lepidoptera moth.
B. *Isaria japonica* formed in rice medium.
C. *Isaria japonica* in complex media which composed of chinese medicine waster.
D. *Isaria japonica* formed in silkworm pupa on tray.

Effect of silkworm pupa

The silkworm pupa was inoculated by the mixture of 20% gelatin solution and mycelial suspensions and incubated in the same conditions as those mentioned above. The fruiting bodies of *I. japonica* were developed within 15 days and matured without special treatment such as low temperature. (Fig. 3) It took the shortest incubation time to produce fruiting-bodies in contrast to other medium and was considered to be the best way for the large scale production of *I. japonica*.

Presumably, certain chemical components of pupa seem to exhibit effects to stimulate fruting-bodies of *I. japonica*.

Discussion

Wild fruiting-bodies of *I. japonica* are composed of synnemata 1~2 mm in diam, 1~4 cm length and branched at the apex. In general, the total weight of fruiting-bodies of the fungus on a pupa of Lepidoptera moth is extremely small. In fact, fruiting-bodies are hardly found in the nature, and it is difficult to collect fruiting-bodies of *I. japonica* in

large amounts for use as a medicinal purposes. For this reason, we examined the cultural conditions and technique for mycelial growth and mass production of the fruiting-bodies. No fruiting-bodies were formed over 27°C, although the mycelia grew in the broad temperature range of 10~30°C and the optimum temperature was around 24~25°C. It is essential in this fungus to adjust culture temperature to 20°C or lower optimal temperature in order to induce the primordia and to produce the fruiting-body.

The supplement of silkworm pupal powder to several media was significantly effective in inducing fruiting-body formation as compared with pupal powder-free media. Moreover, the fruiting-bodies were formed on the silkworm pupa in tray culture within 15 days after inoculation of mycelia of *I. japonica*. These results suggest that some unknown chemical components in the pupae of Lepidoptera moths may be effective to induce the fruiting-body formation of *I. japonica*, respectively. On the other hand, basal media supplemented with Chinese medicine waste were obviously effective to increase the amount of fruiting-bodies. Therefore, starch-rich grain medium supplemented with pupal powder, Chinese medicine waste and silkworm pupa by using special inoculation technique is recommended for large-scale indoor production of fruiting-bodies because of the reduction of time spent to produce fruting-bodies of *I. japonica*.

It seems to be likely that morphology of the fruiting-bodies varied remarkably depending on the CO_2 concentration at the fruiting stage. The fruiting-bodies formed under high concentrations of CO_2 like flask culture had unbranched and elongated synnemata. They scarcely produced conidiospores on the apical parts of synnemata. It is interesting that high concentration of CO_2 promote synnema elongation and inhibit conidiospore formation and synnema branching in *I. japonica*. On the contrary, high-intensity illumininations inhibited stipe elongtion and promoted pileus development in some species of Basidiomycetes, for instance, *Flammulina velutipes* Singer and *Pholiota nameko* Ito et Imai apud Imai (Inatomi and Yamanaka, 1996). High-intensity illumination also inhibited the stipe elongation and accelerated the branching of the synnema in *I. japonica*. The effects of CO_2 concentration and light intensity on the morphogenesis in *I. japonica* seem to be basically the same as those in Basidiomycete fungi with pileus and stipe, although the mechanism of promotion or inhibition by CO_2 and illumination in fruit-body formation has not been sufficiently elucidated, especially in the branching of synnema.

적 요

눈꽃동충하초의 대량 인공재배를 위한 배양적 특징을 조사하기 위하여 기본실험을 수행하였다. 공시배지로는 액체, 고체의 MYG(Malt yeast glucose) 배지를 이용하였고 균사생장은 23~27°C의 범위의 온도에서 비교적 좋은 생장률을 나타내었으며 최적 온도는 25°C, pH는 7.0에서 가장 좋은 생장을 나타내었다. 자실체 형성을 위한 부산물로서 번데기가루, 한약찌꺼기 등이 적합하였고, 눈꽃동충하초의 대량생산을 위한 간편한 방법으로서 일반 누에번데기를 균현탁액과 gelatin을 혼합한 용액을 접종원으로 이용하여 접종한 후, 배양하였을 때 우수한 자실체 형성을 보여 주었다.

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