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Cultivation characteristics of *Isaria japonica*

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Cultivation characteristics of fruit-body (synnema) formation of *Isaria japonica* were examined using liquid and solid media in order to produce fruit-bodies on a large scale. Mycelia grew well at 18–28°C on PDA medium with an initial pH of 7.0. The formation of fruit-bodies of *I. japonica* was induced by lowering temperature to below 20°C in PD liquid medium. In sawdust-rice bran basal medium mixed with pupal powder prepared from silkworms (*Bombyx mori*), the fresh weight of fruit-bodies increased with increasing content of pupal powder. The highest yields of fruit-bodies were obtained in carbon-rich barley grain medium supplemented with pupal powder. The fruit-bodies grown under CO_2 concentrations of 1,000 μl/L had coral-like, many-branched synnemata with numerous conidiospores, whereas those formed under high concentrations (9,000 μl/L) of CO_2 had unbranched and longer synnemata. High concentrations of CO_2 remarkably inhibited conidiospore formation on synnemata. Continuous high-intensity illumination at 2.93 $W \cdot m^{-2}$ inhibited the elongation of synnemata, and low-intensity illumination at 0.088 $W \cdot m^{-2}$ slightly inhibited the branching of synnemata. Fruit-bodies were produced on the pupa metamorphosed from living larvae of *Agrotis fucosa* placed on the surface of a culture of *I. japonica* incubated in sawdust-rice bran medium.

Key Words——conidiospore; cultivation; fruit-body formation; *Isaria japonica*; synnemata.

Pharmacological effects of several fungal species in *Cordyceps* have recently been discovered (Hobbs, 1995; Kiho and Ukai, 1995). The most common Clavicipitaceae species, *Cordyceps sinensis* (Berk.) Sacc., caterpillar fungus, has been used as a traditional medicinal fungus in China from ancient times (Hobbs, 1995). This fungus is known to possess diverse therapeutic effects (Tsunoo et al., 1995). Some polysaccharides isolated from *Cordyceps* species, e.g., *C. cicadae* Shing (Kiho et al., 1989, 1990; Kiho and Ukai, 1995) and *C. ophioglossoides* (Ehrenberg ex Link) Fr. (Yamada et al., 1984; Ohmori et al., 1986, 1989), are also known to have potent antitumor activity. Extracts from *Cordyceps militaris* (Vuill.) Fr. and *Isaria felina* (DC.) Fr. showed similarly strong negative inotropic effects to those from *C. sinensis* (Ikumoto et al., 1991). Recently, Kinjo et al. (1996) reported that extracts from cultured mycelia of *C. militaris* had several physiological activities.

The artificial induction of fruit-body formation of *C. sinensis* has rarely been successful, even though the cultivation of mycelia has recently been established (Lin and Yu, 1997). In *C. militaris*, fruit-bodies are artificially produced using rice or corn grain medium (Pen, 1995). Harada et al. (1995) reported the formation of mature fruit-bodies of *C. militaris* on the pupae of *Mamestra brassicae* L. inoculated with ascospores. They mentioned that this fungus, especially its ascospores, could be used as a microbiological insecticide (biological controller) to prevent harmful insects from attacking forest trees, fruit trees and vegetables. They also note that it is necessary to produce a large quantity of fruit-bodies of *C. militaris* in order to use the ascospores as a biological controlling agent. However, the large-scale production of fruit-bodies in Clavicipitaceae species has not yet been achieved. Mass culture of mycelia and mass production of fruit-bodies in these medicinal Clavicipitaceae fungi are, therefore, necessary in order to use them as medicines or medicinal health foods.

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. Although this fungus is expected to possess medicinal properties, fruit-body formation has rarely been successful in indoor production facilities. Mass production of fruit-bodies of this fungus is necessary if its medicinal properties are to be utilized or if the fruit-bodies are to be commercialized as a health food. We examined the cultural characteristics of mycelial growth and the cultivation conditions for fruit-body (synnema) formation in order to produce fruit-bodies of *I. japonica* on a large scale.

Materials and Methods

Strains The fruit-bodies (synnemata) of *I. japonica* grown on the pupa of Lepidoptera moth were collected at Iizuna Kogen Heights, Nagano Prefecture in the late summer, 1994 (Fig. 1). The fruit-bodies were microscopically examined and identified as *I. japonica*, which has characteristic comma-shaped, bead-like conidiospores (Shimizu, 1994). The isolate was obtained from conidiospores.

Mycelial growth Mycelial growth of *I. japonica* was examined on 9-cm plastic plates containing potato-dextrose

agar (PDA: Difco) medium. A mycelial agar disc 3 mm in diam precultured on PDA medium at 25°C for 7 d in the dark was inoculated onto the surface of the agar medium. The cultures were incubated at temperatures of 15–35°C for 8 d in the dark to determine the optimum temperature for mycelial growth. The cultures on PDA medium with initial pHs of 4.0–8.3 were also incubated at 25°C for 8 d in the dark to determine the optimum pH for mycelial growth. Mycelial growth on agar plate medium was expressed as the average colony diam of five plates in each experiment, and each test was replicated twice.

Optimum temperature and pH on the fruit-body formation To determine the optimum temperature and pH for induction of fruit-body formation, 100-ml Erlenmeyer flasks containing 20 ml of potato-dextrose broth (Difco) liquid medium were used. A mycelial agar disc 3 mm in diam precultured on PDA medium at 25°C for 7 d in the dark was inoculated into liquid medium in flasks. The cultures were incubated at 20, 25 and 30°C for 21 d in the dark to determine the optimum temperature for induction of fruit-body formation. Liquid media with initial pHs of 4.6 to 8.0 were used, and the cultures were incubated 25°C for 21 d to determine the optimum pH for fruiting. The cultures were then transferred to 20°C with a cycle of 15 min light and 45 min dark for 21 d. Fluorescent lamps (FLR40S·W/M white, Toshiba Electric, Tokyo) were used at about 2.93 $W \cdot m^{-2}$ for illumination. The average fresh weight of three flasks in each experiment was obtained, and two replications were examined.

Fig. 1. Fruit-bodies (synnemata) of *Isaria japonica* grown on a pupa of Lepidoptera moth. Bar = 1 cm.

Effects of additives to medium on the fruit-body formation To examine the effects of additives to the medium on the fruit-body formation, eight different media were tested (Table 1).

One-hundred-milliliter polypropylene jars containing 50 g (fresh weight) of medium were autoclaved for 30 min at 120°C, then inoculated with 5-g blocks of mycelia precultured on the sawdust-rice bran basal medium at 25°C for 21 d. The cultures were incubated at 25°C for 21 d. After mycelia had colonized the entire medium, the cultures were transferred to the fruiting conditions, at 18°C, 90% RH, 1,500 μl/L of CO_2 and a cycle of 15 min light at 2.93 $W \cdot m^{-2}$ and 45 min dark to induce the fruit-body formation. Average yields of fruit-bodies obtained from five jars in each test were expressed on a fresh weight basis. The experiment was replicated twice.

Effects of CO_2 concentration and light intensity on the fruit-body formation Glass Petri dishes 9 cm in diam containing 50 g of barley grain/pupal powder (pupa of silkworm, *Bombyx mori* L.) complex medium were autoclaved at 120°C for 30 min to examine the effects of CO_2 concentration and light intensity on the fruit-body formation. Barley grain/pupal powder complex medium was composed of 14.25 g of barley grain (dry weight, d.w.), 4.25 g of pupal powder (d.w.) and 31.5 g of distilled water. Moisture content was 63% on wet basis, and the medium was adjusted to pH 6.5 with 1 N KOH before autoclaving. A 5-g block of mycelia precultured

Table 1. Formulas for substrates and yield of fruit-bodies.

Medium[a]	Materials weight (g)[b]							Fruit-body yield (g)[c]
	sawdust	corncob meal	rice brain	soybean skin	barley grain	millet grain	pupal powder	
1[d]	10.00		8.50					2.25
2		4.60	9.30	4.60				2.81
3	9.55		8.10				0.85	2.81
4	9.10		7.70				1.70	3.04
5	8.20		6.90				3.40	3.71
6	6.40		5.30				6.80	3.94
7					14.25		4.25	9.55
8						14.25	4.25	7.65

a) Total weight was 50 g and moisture content was 63%.
b) Dry weight basis.
c) Fresh weight basis.
d) Sawdust-rice brain basal medium.

in sawdust-rice bran basal medium at 25°C was inoculated onto the surface of the medium in each dish. The cultures were incubated at 25°C for 30 d in the dark, then transferred into the plastic chambers maintained under the fruiting conditions described above, except that temperature was 18–20°C. The chambers had a volume of 24 L, and CO_2 concentration was regulated with ambient air mixed standard CO_2 gas. CO_2 concentrations were continuously monitored with a CO_2 meter (GM12B, Vaisala, Finland). The effect of CO_2 concentration was examined at 1,000 μl/L and 9,000 μl/L. To examine the effect of light intensity on the fruit-body formation, cultures were incubated at 25°C for 30 d in the dark, then placed at 18–20°C, 80–90% RH, under continuous light illumination at 0.088 $W \cdot m^{-2}$ and 2.93 $W \cdot m^{-2}$ with fluorescent lamps. Morphological characteristics of the fruit-bodies from three cultures in each experiment were examined.

Fruit-body formation from pupa of *Agrotis fucosa* To examine the formation of fruit-bodies from pupa, the fungus was incubated in a 300-ml Erlenmeyer flask containing 50 g of sawdust-rice bran basal medium at 25°C. A live larva of *Agrotis fucosa* Butler (a sort of Lepidoptera moth) was laid on the surface of the culture when the mycelia had colonized the entire medium at 14 d after inoculation. The culture was transferred to 20°C with the same light-dark cycle as above after the larva had metamorphosed into a pupa.

Results

Mycelial growth Mycelia of *I. japonica* grew well at 18–28°C, and the optimum temperature was about 24°C (Fig. 2). Mycelial growth rapidly decreased at temperatures above 27°C. The optimum initial pH of agar medium for mycelial growth was 7.0 (Fig. 3). Values lower than pH 6.0 were not suitable for mycelial growth.

Optimum temperature and pH on the fruit-body formation Fruit-bodies of *I. japonica* began to be formed on the surface of liquid medium in flasks incubated at 20°C within 19–23 d after inoculation, and the fruit-bodies grew and developed. However, no fruit-bodies were formed on the medium incubated at 25 or 30°C. This fungus also required the shift to lower temperature to induce the fruit-body formation. Fruit-bodies were formed on liquid media with initial pHs in the broad range of 4–8. Mycelia differentiated into fruit-bodies even when initial pH was as low as 4, in spite of the inhibited mycelial growth.

Effects of additives to medium on the fruit-body formation Table 1 shows the yield of fruit-bodies of *I. japonica* obtained with various solid media. In 100-ml jar cultures, the mycelia colonized the medium entirely within 21 d after inoculation. Primordia began to form in all tested media at 6–9 d after lowering the incubation temperature to 18°C and they developed into fruit-bodies of 2–4 cm in length within 44–50 d after inoculation.

The highest yield of fruit-bodies was 9.55 g per jar containing 50 g of barley grain-silkworm pupal powder mixed medium (No. 7) and followed by 7.65 g per jar containing millet grain-pupal powder mixed medium (No. 8). In sawdust-rice bran basal medium, the yield of fruit-bodies gradually increased with increasing content of pupal powder in the medium. Moreover, carbon-rich grain media remarkably enhanced the fruit-body yield, compared with sawdust-rice bran media, when silkworm pupal powder was present. Therefore, grain media containing pupal powder are the most suitable for large-scale production of the fruit-bodies of *I. japonica*. Figure 4 shows the fruit-body formation of *I. japonica* in barley grain/pupal powder complex medium.

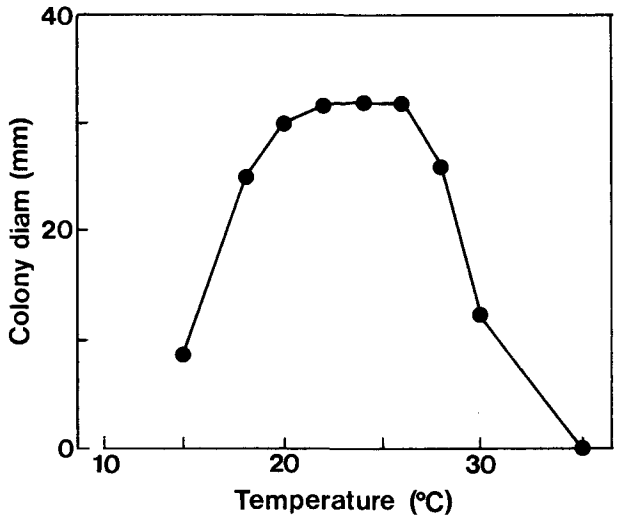

Fig. 2. Influence of temperature on mycelial growth of *I. japonica*.

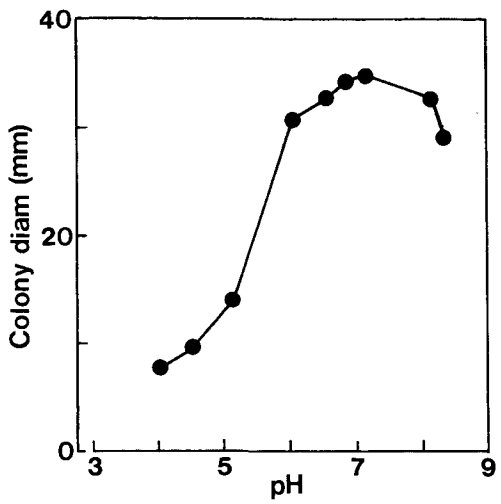

Fig. 3. Influence of initial pH of PDA medium on mycelial growth of *I. japonica*.

Effects of CO_2 concentration and light intensity on the fruit-body formation Mycelia colonized sawdust-rice bran/pupal powder complex media entirely at 25°C within 14 d after inoculation. Cultivation of these cultures was continued at 25°C for 16 d to allow mycelial maturation. Then the solid inoculum was scraped from the sur-

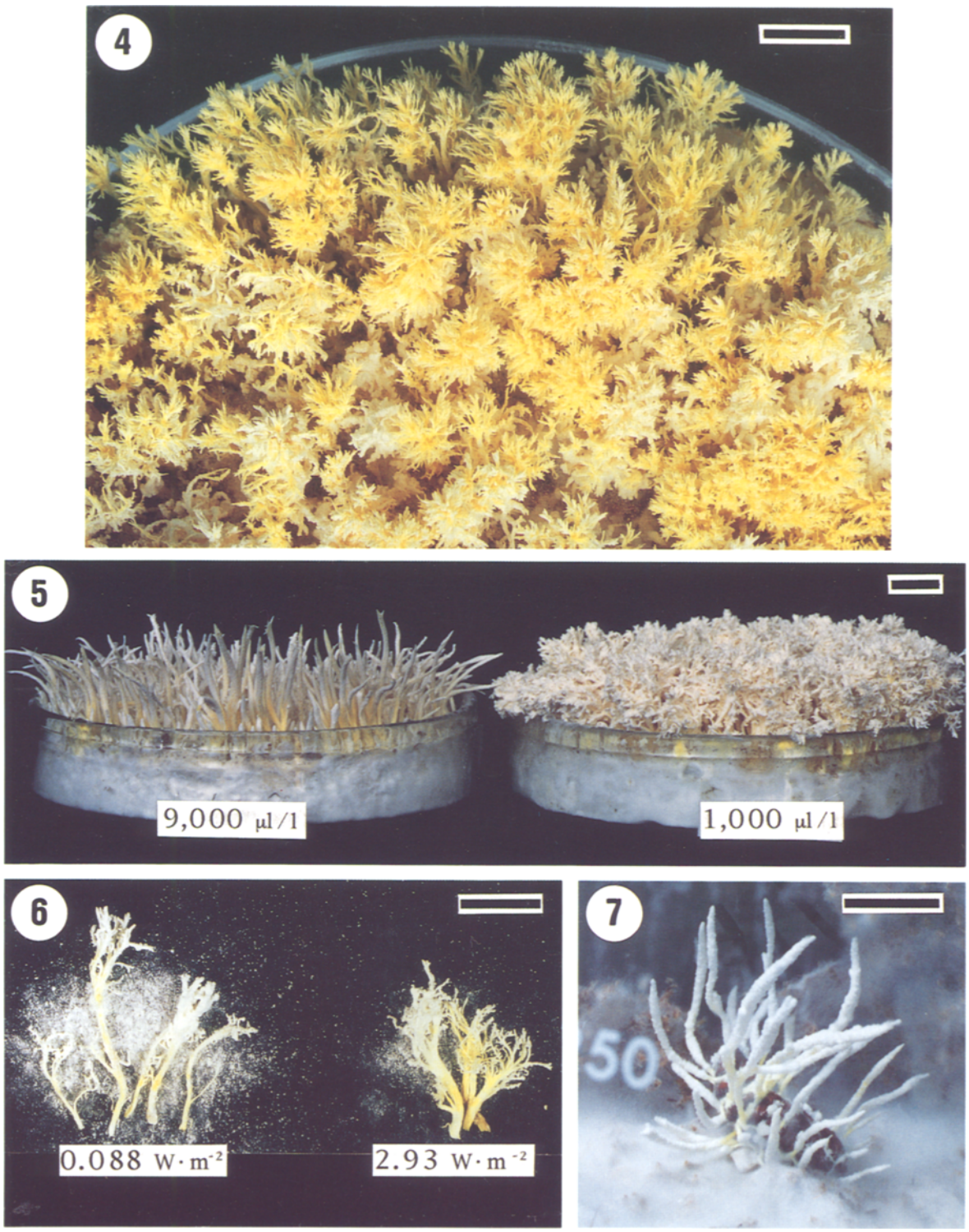

Fig. 4. The fruit-bodies of *I. japonica* formed in barley/pupal powder complex medium.
Fig. 5. Influence of CO_2 concentration on the fruit-body formation of *I. japonica*. Fruit-bodies grown under 9,000 μl/L (left) and 1,000 μl/L (right) of CO_2.
Fig. 6. Influence of light intensity on the fruit-body formation of *I. japonica*. Fruit-bodies grown under illumination at 0.088 $W \cdot m^{-2}$ (left) and 2.93 $W \cdot m^{-2}$ (right).
Fig. 7. Fruit-bodies of *I. japonica* formed on a pupa of Lepidoptera moth, *Agrotis fucosa*. Bars = 1 cm.

face of the matured culture and the incubation temperature was shifted down to 20°C, and within 6-8 d primordia formed on the culture surface. The primordia developed into mature fruit-bodies at 16–18 d after fruiting induction.

The fruit-bodies developed under 1,000 μl/L of CO_2 were corally branched on the head of synnema, whereas those formed under the high concentration of 9,000 μl/L had rarely branched, long synnemata (Fig. 5). The high concentration of CO_2 remarkably inhibited conidiospore formation on the apex of synnemata. The rarely branched, long synnemata were not identified as fruit-bodies of *C. takaomontana*, teleomorph stage of *I. japonica* because they lacked asci and ascospores, showing only synnemata and characteristic conidiospores.

The effects of illumination on the fruit-body formation are shown in Fig. 6. Low-intensity illumination at 0.088 $W \cdot m^{-2}$ accelerated the elongation and slightly inhibited the branching of synnemata. Continuous, high-intensity illumination at 2.93 $W \cdot m^{-2}$ inhibited the elongation of synnemata and accelerated the branching at the head of synnemata.

Fruit-body formation from pupa of *Agrotis fucosa* The living larva of *A. fucosa* put on the surface of the culture colonized with mycelia of *I. japonica* in 300-ml Erlenmeyer flask metamorphosed into a pupa after 5 d. Fruit-bodies had grown only on the pupa at 25 d after the beginning of fruiting treatment. Unbranched synnemata were formed on the pupa because of cultivation under a high concentration of CO_2 in the flask (Fig. 7). The formation of fruit-bodies only on the pupa suggests that certain chemical components of the pupa are effective in inducing fruit-body formation of *I. japonica*.

Discussion

Wild fruit-bodies of *I. japonica* are composed of synnemata 1–2 mm in diam, 1–4 cm in length and branched at the apex. In general, the total weight of fruit-bodies of this fungus on a pupa of Lepidoptera moth is extremely small. In fact, fruit-bodies are rarely found, and it is therefore difficult to collect fruit-bodies of *I. japonica* in large quantities for use as a medicinal fungus. For this reason, we examined the cultivation conditions for mycelial growth and mass production of the fruit-bodies. No fruit-bodies were formed at 25 and 30°C, although the mycelia grew in the broad temperature range of 10–30°C and the best at around 24–25°C. In this fungus, it is essential the temperature to 20°C or below from 24–25°C in order to induce the primordia and to grow the fruit-body.

The supplement of silkworm pupal powder to several media had a significant effect on the induction of fruit-body formation compared with pupal powder-free media. Moreover, the fruit-bodies were formed only on the pupa of *A. fucosa* in flask culture within 39 d after inoculation of mycelia of *I. japonica*. These results suggest that common chemical components in the pupae of Lepidoptera moths may function to induce the fruit-body formation of *I. japonica*. On the other hand, grain media, barley or millet, supplemented with pupal powder were also obviously effective to increase the amount of fruit-bodies. Therefore, starch-rich barley grain medium supplemented with pupal powder is recommended for large-scale indoor production of fruit-bodies.

Morphology of the fruit-bodies varied remarkably depending on the CO_2 concentration at the fruiting stage. The fruit-bodies formed under high concentration of CO_2 (9,000 μl/L) had unbranched and elongated synnemata. They scarcely produced conidiospores on the apical parts of synnemata. It is interesting that high concentration of CO_2 promotes synnema elongation and inhibits conidiospore formation and synnema branching in *I. japonica*. In Basidiomycetes fungi, high concentration of CO_2 promoted stipe elongation and inhibited pileus development (Kinugawa et al., 1986, 1994). On the contrary, high-intensity illumination inhibited stipe elongation and promoted pileus development in some species of Basidiomycetes, for instance, *Flammulina velutipes* (Curtis: Fr.) Singer (Inatomi and Yamanaka, 1994) and *Pholiota nameko* (Ito) 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 fundamentally the same as those in Basidiomycetes 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.

Fruit-bodies formed under different environmental conditions can be used for different purposes making full use of the morphological characteristics. For instance, the long, thick synnemata with few conidiospores are available for health food or medicinal use, although biological activity has not been sufficiently researched in this fungus. Fruit-bodies producing abundant conidiospores may be effective as biological controllers for use against harmful insects.

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