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Original Ariticle

Cultural Characteristics for Inducing Fruit body of various Insect Mushrooms on Polyurethane Foams*

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Abstract

Cultural characteristics of various seven strains of *Isaria* and *Cordyceps* spp, by cultivating on four different kinds of polyurethane foams (PUF) were investigated. Significantly faster mycelial growth was recorded from *Cordyceps ophioglossoides* and *Isaria atypicola*. Highest whiteness was achieved from *I. sinclairii* on plant based with 36.5 % castor oil and plant based with 2 % wood and 0.9 % sucrose, but petroleum based and plant based with 1 % sucrose PUF were best for *C. sinensis*. Comparing to all PUF whiteness was comparatively much better on plant based with 1 % sucrose PUF for six species, while *I. farinose* was on plant based with 2 % wood and 0.9 % sucrose. The ergosterol contain was significantly high in *I. sinclairii*. Fruit bodies were successfully observed in five species in between 70 to 90 days.

Key words: insect mushrooms; polyurethane foam; fruit body

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* チャンドラ ポクレル・楊 柏松・澄川真也・前 賢生・大賀祥治：冬虫夏草菌類のポリウレタンフォーム培地での生育特性

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1. Introduction

Cordyceps and *Isaria* species, known as entomopathogenic fungi parasite on various kinds of larva, pupa and even those on fully grown insect host, kill their hosts and form fruit bodies from hosts. They are popularly known as insect mushroom. They have been used in traditional medicines in China, Japan and Korea (Yu *et al.*, 2001), currently being popular all over the world. The fruit bodies of insect mushrooms are highly value as medicinal herb, due to their various biological and pharmacological activities including immune stimulating and antitumor activities (Borchers *et al.*, 1999; Liu *et al.*, 1996; Lee *et al.*, 1996). However, they were very difficult to collect because of their size are very small and their growth is in restected to a specific area and season. They are also expected to be highly selective biological insecticide (Ito and Hirano, 1997). The glactosaminologlycan moiety (CO-N) has been obtained from an antitumor polysaccharides friction (SN-C) produced by *C. ophioglossoides* (Ohmori *et al.*, 1989). *I. sinclairii* showed inhibitory effect on T cell- dependent immuno responses (Fujita *et al.*, 1994). *C. sinensis* is used to replenish the kidney and soothe the lungs, for treatment of impotence, nocturnal emission, night sweats and chronic cough with hemoptysis (Chen *et al.*, 2004). Recently, isolated 4-acetyl-12, 13-epooxyl-9-trichothecene-3,15-diol (AETD) from the fruting body of *I. japonica* as a major active compound (Oh *et al.*, 2001). Recently, *Cordyceps* and *Isaria* species were reported to have high pharmacological activities (Miyashita and Yoneyama, 2002). Though, *Cordyceps* and *Isaria* have considered as a one of the most promising edible fungi in the global market, one of the unsolved problems may remain in the difficulty of its mass production capable of corresponding to the mounting demand of many users.

Regarding their potential medical and nutritional value, scare in nature or limited natural sources and rising public demands could be replaced by culture cultivation of these strains. Recently cultivation of these strains has been developed in various media. However, mass production of these strains will be needed for the fulfillment of present claim.

The aim of this study is the cultivation of various species of insect mushrooms (IM) (entomopathogenic fungi) using different kinds of polyurethane foams (PUF).

2. Materials and Methods

2.1. Organism

The followings seven strains of IM were studied, *C. sinensis* MH19911, *C. nutans* MH19914, *C. ophioglossoides* MH19945 and *Isaria atypicola* MH19947 were obatain from Hokuto Co. Ltd. *I. sinclairii* KS-78, *I. japonica* KS-15 and *I. farinose* KS-17

were from Kyushu University. The strains were cultured on potato dextrose agar (PDA) plates at 23 ℃ for 10 days than maintained at 5 ℃, and sub-culture every three months.

2.2. Mycelial growth examined

Mycelial growth was examined on PDA medium using plastic Petri dishs containing 20 ml. medium. A 5 mm diameter disc was isolated from the edge of actively growing mycelium from 7 days old culture on PDA medium and than used as inoculums to determine the mycelial growth of various strains, the culture ware incubated at 23 ℃ under dark condition for two weeks. The diameter of radial growth of mycelium was measured every two days of intervals for two weeks.

2.3. Preparation of polyurethane foams

Four various kinds of polyurethane foam are as, plant based with 1% sucrose (PBS), plant based with 2 % wood and 0.9 % sucrose (PBWS), plant based with 36.1% caster oil (PBCO) and petroleum based (PEB) were directly obtained from manufacture companies. Out of them three plant based were from Mitsui-Takeda Co. Ltd., other one petroleum based was from Bridgestone Co Ltd. Various PUF slices size in to 2.5 x 2.5 x 0.5 cm for the cultivation of various IM. The prepared PUF were dipped in to the prepared liquid medium of 2 % glucose and yeast dried, and sterilized at 121 ℃ for 15 min. Sterilized PUF containing 15 ml medium was set in to the Petri dishes (also in conical flask) and than cooled. A 5 mm diameter disc was isolated from the edge of actively growing mycelium from 7 days old culture from PDA medium and inoculated than incubated for 4 weeks at 23 ℃.

2.4. Whiteness

The whiteness of various strains was measured in one week intervals for four weeks. The surface whiteness of colonized slices of various PUF was measured using a Minolta Chroma Meter CR- 200. Data were indicated by three different values of x, y and Y and described by the following equation.

$$Z=\frac{(1-x-y)}{y}\times Y,\ Z/1.18=\text{whiteness}$$

2.5. Ergosterol

Ergosterol contain was determined from two weeks colonized PUF type PBS. One gram of dried PUF was assessed to determine the content of ergosterol in per gram

mycelium. The determination of ergosterol was according to Ohga and Wood (2000). One g dry sample was carried out with 20 ml methanol, 10 ml ethanol and 4 g KOH, incubated at 40 ℃ for 90 min. The solution was filtered and dilute with 20 ml distilled water and 20 ml hexane, mixed for five minutes. The hexane extract was evaporated under vacuum at 30 ℃. The dry residue was dissolve in 2 ml of methanol. Ergosterol was measured by high-performance liquid chromatography (HPLC) (Jasco, PU-1580) using a reversed-phase system consisting of a paked column (Inertsil ODS-3) and 99. 7 % HPLC grade methanol. Ergosterol was detected by absorption of 282 nm light (Tosoh, UV-8001). At a 1.0 ml per minute flow rate, ergosterol was eluted about at 9 min.

2.6. Fruit body formation

Fruit body formation was observed only in PUF foam PEB. After two weeks incubation, colonized PUF were transfer in to the mushroom house. The temperature of mushroom house was adjusted to 18 ±2 ℃, relative humidity was maintained 90 % and CO_2 concentration level was less than 1000 ppm, and a light intensity of 500 lux. for 8 h a day. A small amount of mycelium separate from PUF and moisture was determined by drying in hot oven at 60 ℃ for 24 h. Day to colonization and fruit body formation were recorded by simple observation.

3. Results and Discussion

3.1. Mycelial growth on PDA

Mycelial radial growth of various IM was studied for their growth ability of on PDA medium. The results of mycelial growth in relation to the days (two days interval) are shown in Fig.1. The mycelial diameter of various IM varied with the days of incubation.

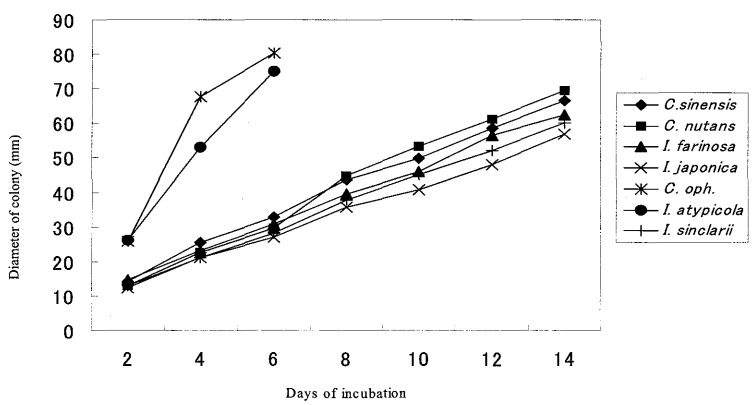

Fig. 1 Mycelial growth of various insect mushrooms on PDA medium. (Error bars showed the standard deviation)

After two weeks incubation the maximum mycelial diameter was observed in *C. ophioglossoides*, and *I. atypicola* 80.30 mm and 75.03 mm respectively, followed by *C. nutans, C. sinensis, I. farinosa I. sinclairii* and I. *Japonica* respectively. The mycelial growth was significantly high in *C. ophioglossoides* and I. atypicola (Figs. 1 and 2). Mycelial growth was sharply increased from days 6 to 8 in *C. nutans*. Mycelial growth enhancement in two of seven strains, However, others were also showed better growth of mycelium, whereas *I. Japonica* comparatively slower among the all. This date showed that mycelial growth is influence by species rather than nutrients.

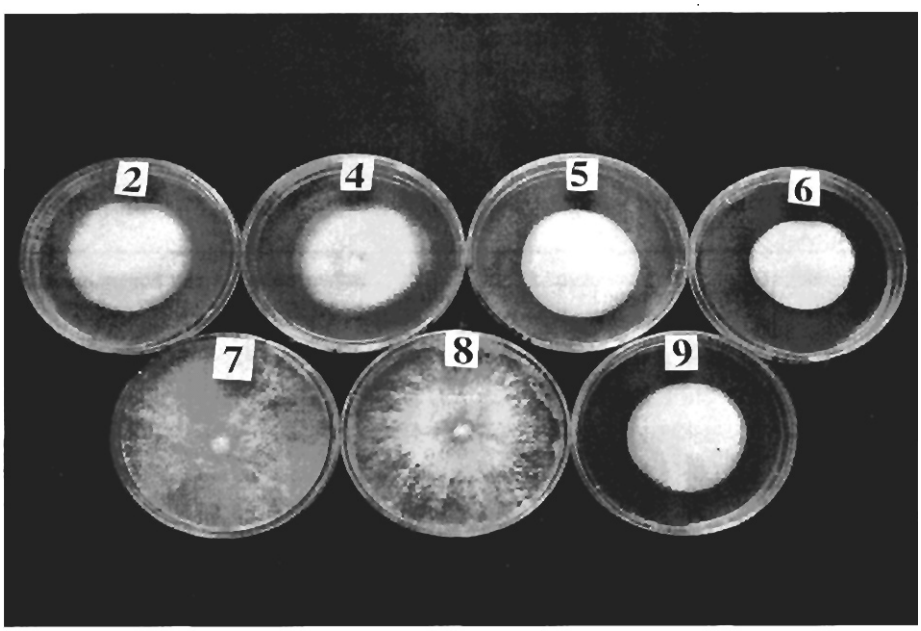

Fig. 2 Mycelial extension of various Insect mushrooms on PDA medium
2-C. sinensis, 4-C. nutans, 5- I. ferinosa, 6- I. japonica, 7- C.ophioglossoides, 8- I. atypicola and 9- I. sinclairii

3.2. Mycelial growth on PUF

The result of mycelial growth (whiteness value) of between four various kinds of polyurethane forms with in a species showed that all kinds of PUF were statistically significant ($P<0.05$) for whiteness in *I. siclairii, I. japonica, I. ferinosa, C. nutans* and *C. ophioglossoides*, but PEB and PBS were statistically high for *C. sinensis* and *I. atypicola*. This result indicated that all kinds of polyurethane foams were suitable for the cultivation five species and two types for *C. sinensis* and *I. atypicola*. Similar kind of tendency of whiteness value was observed on different polyurethane. However, highest whiteness was achieved on PBS type polyurethane foam in different six species, but only *I. ferinosa* was on PBWS. Comparatively PBS was much inflective on whiteness for six species and only one on PBWS. Maximum whiteness was

observed in *C. sinensis* (87.36) and *I. sinclairii* (86.70) followed by *I. japonica* (81.54), *I. farinosa* (70.04), *I. atypicola* (69.52), *C. nutans* (66.00) and *C. ophioglossoides* (54.08). Comparing to different species with in same polyurethane type, whiteness value for *C. sinensis, I.sinclarii* and *I. ferinosa* was statistically significant ($P<0.05$) on PBS, while higher value was achieved from *C. sinensis*, similarly whiteness of three species were statistically significantly from other three kinds of PUF, whiteness was significantly lowest in *C. ophioglossoides*. However, PEB and PBS were best for *C. sinensis* and PBWS and PBCO for *I. sinclarii*. The specific activities of whiteness depend on strain type than PUF because all the species are significantly alike on different kinds of polyurethane foams. The whiteness value during two weeks incubation ranges from 46.90-79.23 on PEB, 47.34-77.37 on PBCO, 49.08-81.88 and 54.08-87.36 on PBCO and PBS, respectively. The whiteness range was lowest in PEB and highest on PBS. The whiteness value of various IM on colonized polyurethane

Table 1 Mycelial whiteness of various Insect mushrooms on different kinds of polyurethane foams.

Species/ PUF	Petroleum based	Plant based with 2 % wood and 0.9 % sucrose	Plant based with 36.1% castor oil	Plant based with 1% sucrose
I. sinclairii	77. 37ab ±1. 01	77. 37a ±1. 59	82. 54a ±3. 90	86. 70a ±0. 48
C. sinensis	79. 21a ±25. 01	71. 77ab ±1. 54	77. 77ab ±1. 53	87. 36a ±1. 42
I. japonica	74. 63ab ±1. 21	75. 02ab ±0. 34	78. 03ab ±1. 10	81. 54a ±3. 17
I. atypicola.	68. 93bc ±1. 24	54. 24c ±0. 34	59. 39c ±2. 55	69. 51b ±0. 76
C. nutans	62. 95c ±2. 48	49. 17cd ±0. 26	57. 12c ±3. 96	66. 00b ±2. 07
I. ferinosa	61. 76c ±2. 57	70. 04b ±2. 20	69. 25bc ±0. 99	63. 58bc ±2. 23
C.ophioglossoides	46. 90d ±2. 42	46. 90d ±1. 20	49. 07d ±2. 123	54. 08c ±3. 24

Small alphabet letters indicate the same letters in the same column are not statistically significance different (Values are the mean ± SE of whiteness)

foams during second week incubation is shown in Table1.

The results showed that the metabolic activities of mycelial growth is considerably maximized by plant based with 1% sucrose (PBS) PUF in various six species of IM, other one was on PBWS. However, all kinds of polyurethane were better for whiteness for five species and other two species better for two kinds of PUF. The study result came to know that polyurethane was not suitable for cultivation of *C. ophioglossoides*. Comparing to various species with different PUF *I. sinclairii, C. sinensis* and *I. japonica* were comparatively better for the cultivation of on all kinds of PUF, but PBS is much better for them.. However, except *C. ophioglossoides* all species showed the relatively positive result for whiteness. Increasing and decreasing of whiteness is a specific metabolic activity of fungal mycelium and mushroom. The loss of whiteness of mushroom results for the most part from the activity of o-

diphenolloxidase on phenolic compounds (Pai and Sastry, 1989). However, there might be various causes of whiteness value change such as duration of cultivation or ageing, temperature, moisture, cultivation substrates and some other physiological changes etc. A decrease of mushroom whiteness was observed (Beaulieu *et al.*, 2002), the loss of whiteness of pileus (cap) and stipes (stem) separately (Minamide *et al.*, 1980). Whiteness is an indicator of quality decisive factor. There are several indicators that determine the quality of mushrooms such as whiteness, cap development, stripe elongation etc. (Gormley, 1975; Lopez-Briones *et al.*, 1992). In this study we assumed that whiteness is the identifying tools to maximum mycelial growth (density) during colonization and quality achievement of mycelium as well as best way of screening the PUF for synthetic cultivation of various IM. The increasing and decreasing trends of whiteness were recorded in the experiments (data are not shown). The whiteness was increased from week 1 on all PUF type, and rapidly decreased from weeks 3, in six strains. Though, whiteness value was increased from weeks 2 to 3, and decreased from week 4 in *I. farinosa*. But highest whiteness value was achieved in *C. nutans* in week 4, on PUF PBWS type and *C. sinensis* on week 3 on PBCO. Majority of species showed the similar kind of tendency in increasing and decreasing whiteness value in order to cultivation period and PUF types. However, some mixed results were obtained. The whiteness value was highly varied in relation to various strains even with in same PUF type but same species with different PUF types showed significantly alike.

Whiteness could be used as a mycelial density and quality indicator, it decrease indicated that the maximum mycelial growth (density and quality) was achieved. The present study clearly indicated that majority of species showed maximum mycelial growth in two weeks cultivation, only one species showed differ results from others. The present study reveals that various kind of PUF could be used as supporting materials in the cultivation of IM. The whiteness values of *C. ophioglossoides* did not corresponds with the value of mycelial growth on PDA.

3.3. Ergosterol

Ergosterol is often used as a measure of living fungal biomass (Montgomery, 2000). The fungal membrane lipid ergosterol has been increasingly used as a indicator of fungal biomass (Cecilia *et al.*, 2004), ergosterol and ATP may correlated strongly (Suberkropp *et al,*. 1993). Ergosterol contain of mycelium of various IM after 15 days cultivation on PUF (PBS) is shown in Fig. 4. The results showed that highest ergosterol content was observed from *I. sinclairii* followed by *I. japonica, C. sinensis, I. farinosa, C. ophioglossoides C. nutans* and *I. atypicola*. Ergosterol amount was monitored as an indicator of culture maturity (Ohga, 2000). The ergosterol is much lower in *I. atypicola, C. nutans* and *C. ophioglossoides* The fully grown substrates in

the ergosterol content was more than 2000 $\mu g/g$ (Ohga and Donoghue, 1998 ; Ohga and Wood, 2000). These three species were showed indication of insufficient maturity. However, other four species showed the result of fully grown on PUF. The ergosterol content in *I. sinclairii* was significantly high among the all. The whiteness value was considerable comparable with the results of ergosterol contain in various species. The ergosterol method is a major approach to estimation of fungal biomass or measure of fungal growth, so we decided to test the ergosterol as a biomarker for living fungi. PBS is more inflective in the accumulation of fungal biomass for *I. sinclairii.*

3.4. Colonization, moisture and fruit body formation

The day to colonization, mycelial moisture and fruit body observation on PUF PBS are shown in Table 2. Two species colonized on day 7, three on day 8 and remaining other two on day 9 and 10, respectively. Various IM colonized on PUF PBS shown in Fig. 3. Relatively faster colonized was observed from *I. japonica* and *I.* atypicola,

Table 2 Colonization day, mycelium moisture and fruiting.

Species	Colonization day	Mycelial moisture (%)	Fruit body formation
I. sinclairii	8	84.86 ± 1.57	Observed
C. sinensis	9	92.77 ± 0.76	Not detected
I. japonica	7	85.64 ± 0.33	Observed
I. atypicola.	7	89.01 ± 0.54	Observed
C. nutans	8	85.96 ± 0.50	Observed
I. ferinosa	8	91.00 ± 0.28	Observed
C. ophioglossoides	10	Not determine	Not detected

while *C.* ophioglossoides was slower among the all. The mycelial moisture content was highest in *C. sinensis* (92.77) lowest in *I. sinclarii* (84.86).

Fruit body was observed in various five species in between 70 to 90 days. Fruit bodies were appeared in *I. japonica* and *I. sinclairii* on day 70 and 90, respectively, but *I. farinosa* and *I. atypicola* and *C. nutans* in between day 80- 90. Fruit bodies of various IM are shown in Fig. 5. The appearance of fruit bodies of *I. japonica* was rather faster and larger in size and among the all.

The results showed that highest ergosterol content was observed from *I. sinclarii* followed by *I. japonica, C. sinensis, I. farinosa, C. ophioglossoides C. nutans and I. atypicola.* Ergosterol contain was monitored as an indicator of culture maturity (Ohga, 2000). The ergosterol is much lower in *I. atypicola.*

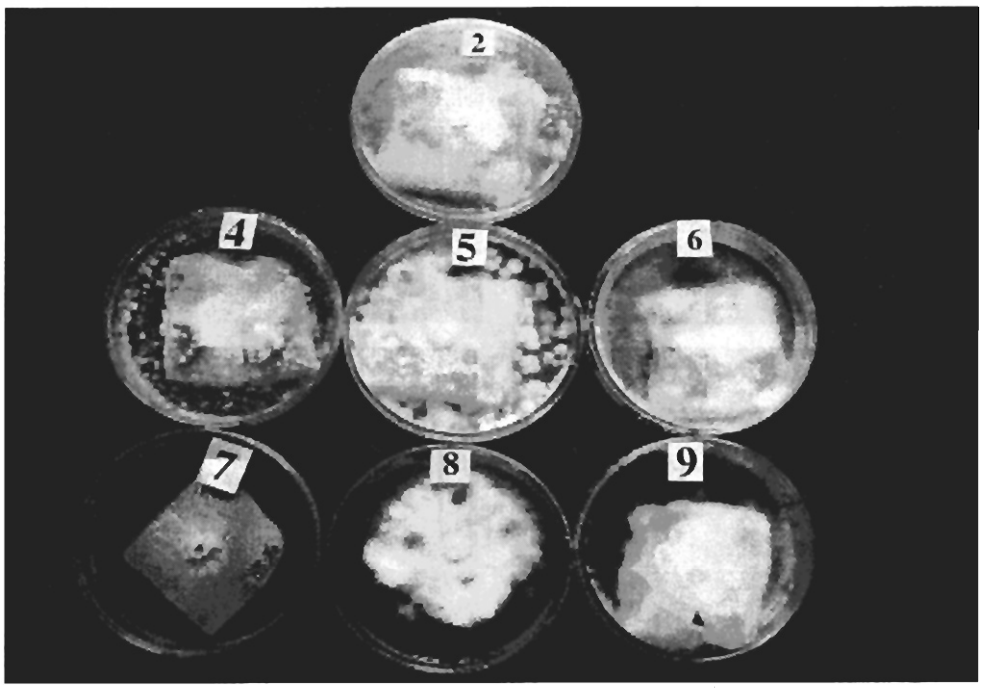

Fig. 3 Colonization condition after15 days cultivated of various IM on PUF PBS
(Species numbers are same as those in Fig. 2)

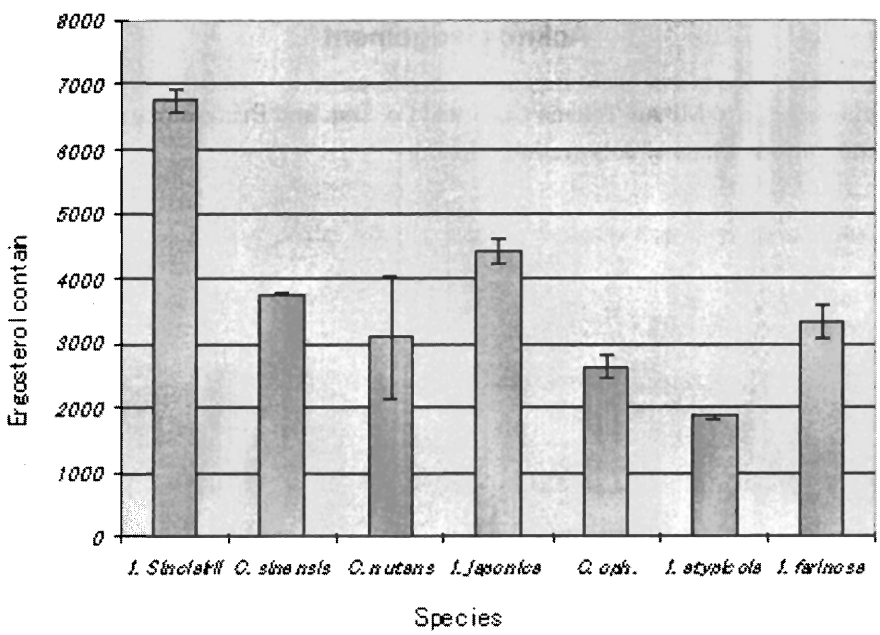

Fig. 4 Ergosterol contain (μ g/g dry mycelium of various *Cordyceps* and *Isaria* species after 15 days incubation on pulyurathane foam PBS type. (Error bars showed standard deviation)

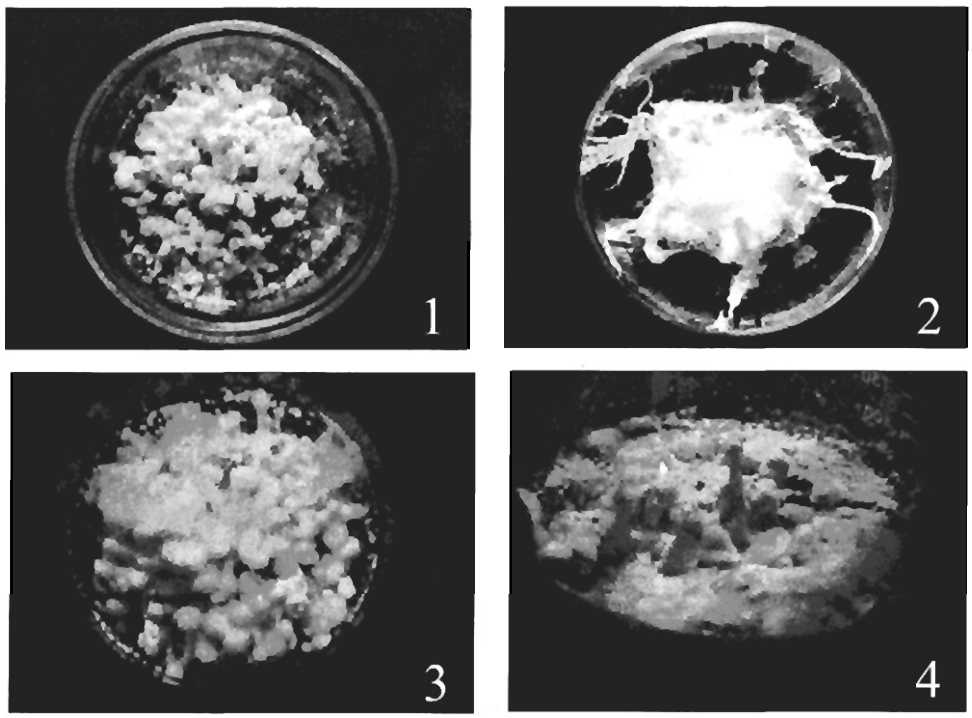

Fig. 5 Fruit bodies of various insect mushrooms on PUF type PBS, fruit bodies were appears between 70- 90 days (1-*I. atypicola*, 2- *I. japonica*, 3-*C. nutans* and 4- *I. sinclairii*)

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冬虫夏草菌類のポリウレタンフォーム培地での生育特性

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大賀　祥治

要　約

冬虫夏草菌類の生育についてポリウレタンフォームを基材として検討した．冬虫夏草菌類の寒天培地上での生育は，ハナヤスリタケとクモタケが良好であった．菌糸密度を評価するために，培地表面の白色度と培地のエルゴステロール含有量を測定した．冬虫夏草とツクツクボウシタケが高い白色度を示し，エルゴステロール含有量はツクツクボウシタケの培地で多かった．ポリウレタンフォームの材質が大きな因子となり，植物由来でスクロースを含有しているもので，良好な生育が認められた．ポリウレタンフォーム上で，冬虫夏草菌類の子実体形成を70－90日培養すると確認することができた．

キーワード：冬虫夏草菌類，ポリウレタンフォーム，子実体