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FULL PAPER

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Fruiting-body formation, cultivation properties, and host specificity of a fungicolous fungus, *Asterophora lycoperdoides*

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Abstract We report the fruiting-body formation and cultivation properties of *Asterophora lycoperdoides*, a fungicolous fungus. *Asterophora lycoperdoides* formed fruiting bodies on potato dextrose agar medium in approximately 1 week, although this fungus shows high host specificity to *Russula nigricans* in nature. Optimal temperature of mycelial growth and fruiting-body formation was 25°C. Mannitol or soluble starch was preferably used as a carbon source, and amino nitrogen was preferably used as the nitrogen source. For a better understanding of the relationship between *A. lycoperdoides* and *R. nigricans*, we cultivated *A. lycoperdoides* on media supplemented with freeze-dried fruiting bodies of various fungi. The germination rate was approximately 2.5 times higher on the medium containing freeze-dried *R. nigricans* than that on the PDA medium. The mycelia extended most rapidly in the presence of *R. nigricans*. Furthermore, the stipe length of its fruiting body was the longest on the medium containing *R. nigricans*. These results indicated that *A. lycoperdoides* can grow faster by utilizing certain substances that are abundantly contained in *R. nigricans*, such as mannitol, or by utilizing *R. nigricans* itself. It is considered that the constituents of *R. nigricans* might contribute to the host specificity of *A. lycoperdoides*.

Key words Basidiomycete · Cultivation study · Host–parasite specificity · Mycoparasite · *Russula nigricans*

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Introduction

Asterophora lycoperdoides (Bull.) Ditmar, a fungicolous fungus, mainly develops its fruiting bodies on the upper part of the old fruiting bodies of Russulaceae, especially *Russula nigricans* (Bull.) Fr., in nature. It is generally known that cultivation and fruiting-body formation under artificial culture conditions is difficult when fungi show high host specificity (host–parasite specificity). However, it was reported that *A. lycoperdoides* easily forms fruiting bodies on the Ebios–sucrose medium (Iwamoto 1963). It is common knowledge that some saprophytic fungi such as *Favolus arcularius* (Fr.) Ames and *Coprinus lagopus* (Fr.) Fr. easily fruit under artificial conditions in a short time (Kitamoto and Kasai 1968; Kitamoto et al. 1968; Madelin 1956). However, fungi of this type, i.e., those easily forming fruiting bodies, were rarely included in the group of parasitic fungi that show comparatively high host specificity. In the event that *A. lycoperdoides* grows easily under artificial conditions, it would be interesting to understand the reason for the generation of fruiting bodies mostly on *R. nigricans* in nature.

The host specificity of fungi has been extensively studied in phytopathology. For example, it is well known that phytoalexins, host-selective toxins, and cell wall-degrading enzymes influence host specificity in pathogenic fungi (Cruickshank and Perrin 1960; Oku 1977; Daly 1984). However, information on the factors influencing the host specificities of nonphytopathogenic fungi, particularly fungicolous fungi, is limited.

Few reports on *A. lycoperdoides* are currently available. Jahrmann and Prillinger (1983) and Koller and Jahrmann (1985) reported that this fungus exhibits dimorphism. However, this was later denied by Laaser et al. (1988). Iwamoto (1963) reported the fruiting-body formation of this fungus, but it was partial and the cultivation properties were mentioned only briefly. The host specificity of *A. lycoperdoides* has been rarely mentioned. In this study, we investigated the fruiting-body formation and cultivation properties of *A. lycoperdoides* in detail to understand its host specificity.

Materials and methods

Organisms

Asterophora lycoperdoides strains Ichikawa and Kashiwa were isolated from the chlamydospores on its fruiting bodies that were collected from two separate locations in Chiba Prefecture in July 1999 and October 2000, respectively. In this research, we mainly used the Ichikawa strain. Stock strains of *Aspergillus niger* van Tiegh. and *Penicillium expansum* Link ex Gray from our laboratory were used in the examination to investigate germination rate. These strains were maintained on potato dextrose agar (PDA) medium.

Culture medium and cultivation

The basal medium contained 20 g soluble starch, 3.0 g yeast extract, 0.10 g K_2HPO_4, 0.40 g $MgSO_4 \cdot 7H_2O$, 2.5 ml mineral salt solution, and 1000 ml distilled water. The mineral salt solution was composed of 0.5 g NaCl, 6.0 g $FeC_6H_5O_7 \cdot xH_2O$ (ferric citrate), 2.0 g $ZnSO_4 \cdot 7H_2O$, 1.0 g $MnCl_2 \cdot 4H_2O$, 0.10 g $CuSO_4 \cdot 5H_2O$, 0.10 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.10 g $CoCl_2 \cdot 6H_2O$, 0.20 g H_3BO_4, 4.0 g $CaCl_2 \cdot 2H_2O$, and 1000 ml distilled water. After autoclaving at 121°C for 20 min, the medium was poured into separate petri dishes or test tubes. When the basal medium was solidified, 20 g/l agar was added. To test the utilization of the carbon source, soluble starch was replaced with 20 g/l glucose, fructose, mannose, arabinose, galactose, xylose, maltose, trehalose, cellobiose, sucrose, lactose, sorbitol, mannitol, *N*-acetylglucosamine, soluble starch, peptone, polypeptone, casamino acid, chitin, chitosan, or olive oil, and the medium that removed soluble starch was defined as carbon free. The soluble carbon sources were sterilized separately by filtration through a membrane filter with a pore size of 0.22 µm (Millipore, Bedford, MA, USA). To test the utilization of the nitrogen source, yeast extract was replaced with 2.8 g/l $(NH_4)_2SO_4$, NH_4Cl, $CH_3COO(NH_4)$, NH_4NO_3, $NaNO_3$, $NaNO_2$, urea, sodium glutamate, sodium aspartate, glycine, or casamino acid, and the medium that removed yeast extract was defined as nitrogen free; however, in these cases, 0.2 g/l yeast extract was still added to the medium as a micronutrient. When the effect of pH on the growth of *A. lycoperdoides* was investigated, the initial pH of the basal medium was adjusted from 3.0 to 11.0 by adding hydrochloric acid or sodium hydroxide. Fruiting-body powder (FBP) solid medium was prepared by mixing distilled water and FBP (see following) at a ratio of 1:1 (w/w). The medium was autoclaved at 121°C for 20 min. For the preparation of FBP, freeze-dried fruiting bodies were milled in a mixer. Fruiting bodies used for FBP were collected by authors from various regions of Japan from April 1989 to July 1996. All chemicals were of the highest purity available. PDA, potato dextrose broth (PDB), and yeast extract were from Difco (Becton Dickinson, Franklin Lakes, NJ, USA), and other chemicals were from Wako (Osaka, Japan).

The cultures were usually incubated at 25°C under fluorescent light (approximately 800 lux). For the preparation of the inoculum, *A. lycoperdoides* was cultivated on the PDA medium for approximately 17 days, and its chlamydospores were inoculated into the culture media using a platinum loop.

Determination of mycelial vegetative growth and fruiting-body development

Utilization of the soluble carbon source and nitrogen source on mycelial vegetative growth was evaluated using mycelial dry weight. To measure mycelial dry weight, *A. lycoperdoides* was cultivated on the basal liquid media by shaking at 160 rpm for 7 days, and after filtration it was dried at 105°C for 24 h. The colony diameters on plate media were measured to examine the effect of temperature, the insoluble carbon source, and host fruiting-body components. In these examinations, *A. lycoperdoides* was cultivated under dark conditions to prevent fruiting-body formation. Fruiting-body formation was examined using the test tube culture. Development of fruiting bodies was assessed by stipe length because the stipe length and weight of the fruiting body showed a proportional relationship.

Enzyme assays

The activities of a polysaccharide-degrading enzyme were determined using soluble starch, pullulan, cellulose, pustulan, laminarin, xylan, or chitin as substrate. These substrates were reacted with the enzyme solution (see following) at 30°C for 120 min, and the amount of reducing sugar that was generated was measured by the Somogyi–Nelson method (Nelson 1944; Somogyi 1952). For the preparation of the enzyme solution, *A. lycoperdoides* was cultivated on basal liquid medium by shaking for 11 days. This culture solution was filtered to remove mycelia, and the low molecular components were subsequently removed from this solution by ultrafiltration using a 10000-MW membrane (Millipore).

Measurement of germination rate of chlamydospores

For preparation of the spore suspension, the chlamydospores of *A. lycoperdoides* were suspended in PDB medium containing 1 ml/l Tween 80 as a surfactant. After calculating the number of chlamydospores in the spore suspension by using a hemacytometer, the spore suspension was inoculated into basal plate media, and the rate of germination was calculated by counting the number of colonies that had formed within 4 days.

Fig. 1. Growth of *Asterophora lycoperdoides* Ichikawa strain on potato dextrose agar (PDA) medium 10 days after inoculation. *Bar* 1 cm

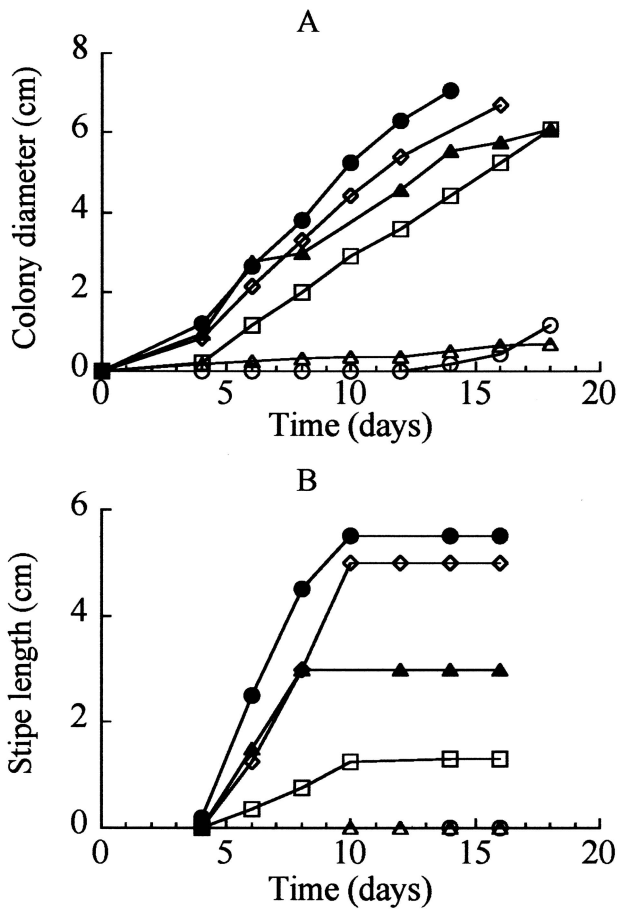

Fig. 2. Effect of temperature conditions on the growth of *A. lycoperdoides* on PDA medium. **A** Effect on mycelial extension. **B** Effect on fruiting-body formation. ○, 5°C; △, 10°C; □, 15°C; ◇, 20°C; ●, 25°C; ▲, 30°C

Results

Cultivation and fruiting-body formation on PDA medium

We observed the fruiting-body formation of *A. lycoperdoides* Ichikawa strain on PDA medium in test tubes. Germination was confirmed at approximately 3–4 days after inoculation, and the fruiting-body primordia were observed approximately 1 day after germination. The development of the fruiting body was completed by the 5th day of germination, and its final stipe length was approximately 6–7 cm. The yellow-brown chlamydospores were formed on the pileus approximately 7 days after germination (Fig. 1). We prepared 15 parallel samples, and fruiting-body formation was observed in all the samples. Fruiting bodies were not formed under dark conditions. We also observed fruiting-body formation of the Kashiwa strain, which was similar to that of the Ichikawa strain. Hence, we used only the Ichikawa strain in the following examination.

Effect of temperature conditions

The optimum temperature of mycelial vegetative growth and fruiting-body formation of *A. lycoperdoides* was determined on PDA medium. At 15° to 30°C, the colony diameter on the 10th day was greater than 3 cm, whereas it was less than 1 cm at 5°C and at 10°C (Fig. 2A). Optimal temperature of mycelial extension was 25°C. The fruiting body was formed at 15° to 30°C, and the largest fruiting body was formed at 25°C (Fig. 2B). Although a mature fruiting body was not formed at 5° and 10°C within the observation period, the formation of primordia at those temperatures was observed after 10 and 16 days, respectively. Thus, it was shown that the optimal temperature of both mycelial growth and fruiting-body formation of *A. lycoperdoides* is approximately 25°C.

Utilization of carbon source and nitrogen source

Utilization of the soluble carbon source was determined. Mycelial dry weights on glucose, fructose, trehalose, soluble starch, and mannitol were more than 0.05 g (Fig. 3A); on mannitol and soluble starch, particularly, the mycelial dry weights were greater than 0.13 g. We also investigated utilization of the insoluble carbon source by measuring the diameter of the colonies. Mycelial extension was faster when chitin was used when compared with the growth on PDA medium (Fig. 3B).

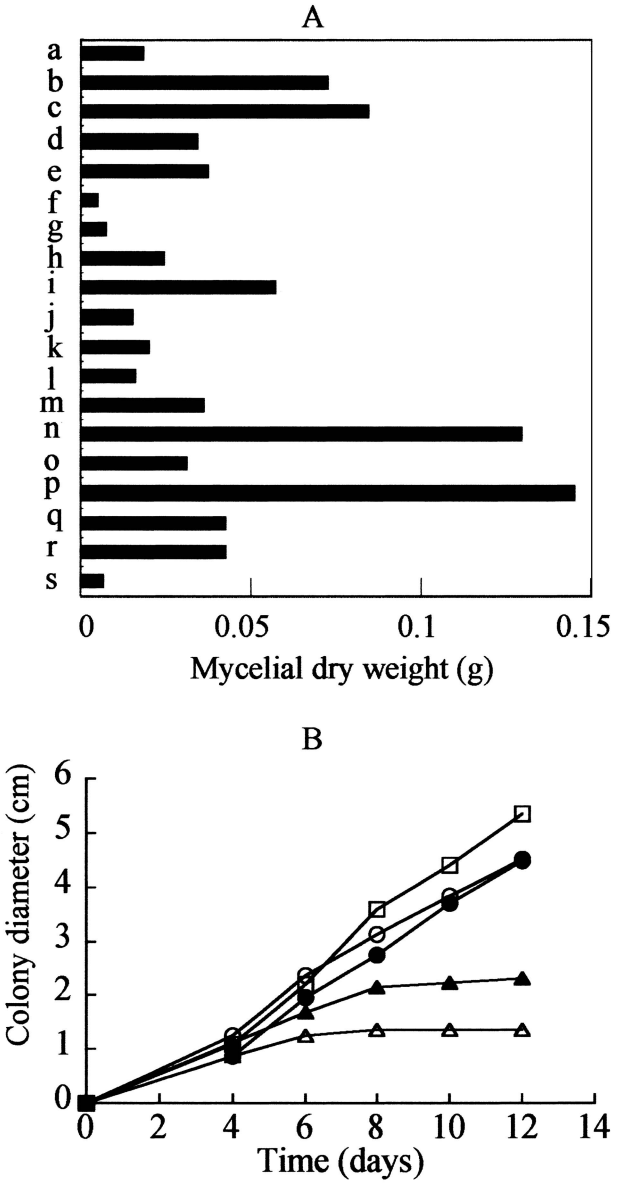

Fig. 3. Effect of carbon source on mycelial growth. **A** Effect of soluble carbon source. *a*, carbon free; *b*, glucose; *c*, fructose; *d*, mannose; *e*, arabinose; *f*, galactose; *g*, xylose; *h*, maltose; *i*, trehalose; *j*, cellobiose; *k*, sucrose; *l*, lactose; *m*, sorbitol; *n*, mannitol; *o*, *N*-acetylglucosamine; *p*, soluble starch; *q*, peptone; *r*, polypeptone; *s*, casamino acid. **B** Effect of insoluble carbon source. ○, PDA; △, carbon free; □, chitin; ●, chitosan; ▲, olive oil

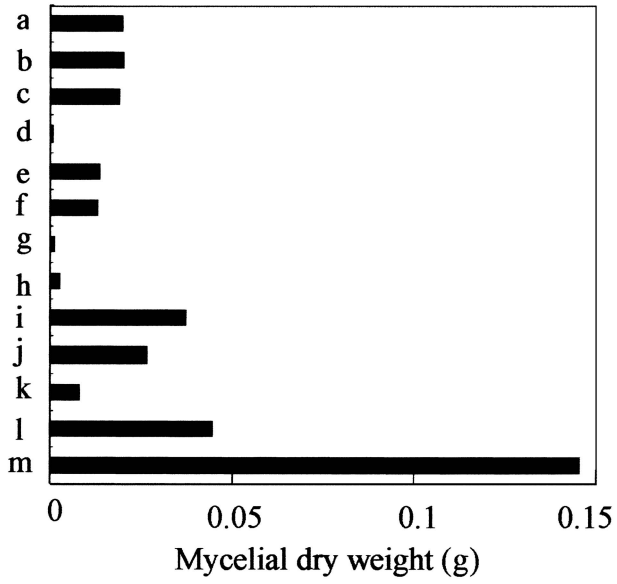

Fig. 4. Effect of nitrogen source on mycelial growth. *a*, nitrogen free; *b*, ammonium sulfate; *c*, ammonium chloride; *d*, ammonium acetate; *e*, ammonium nitrate; *f*, sodium nitrate; *g*, sodium nitrite; *h*, urea; *i*, sodium glutamate; *j*, sodium aspartate; *k*, glycine; *l*, casamino acid; *m*, yeast extract

The effect of the nitrogen source on mycelial vegetative growth is shown in Fig. 4. Mycelial dry weights on medium containing sodium glutamate, sodium aspartate, casamino acid, and yeast extract were higher than those under nitrogen-free conditions.

Productivity of polysaccharide-degrading enzyme

It is known that the utilization of polysaccharides is influenced by the productivity of polysaccharide-degrading enzymes. On the other hand, it is also known that the productivity of polysaccharide-degrading enzymes is one of the important factors in host specificity (Daly 1984; Oku 1977). Thus, the productivity of the polysaccharide-degrading enzyme of *A. lycoperdoides* was investigated. Activities of amylase, pullulanase, cellulase, pustulanase, laminarinase, xylanase, and chitinase were detected (Table 1). In particular, amylase activity was 1.9 μmol/min and chitinase activity was 1.2 μmol/min. Pullulanase and laminarinase also showed activities greater than 0.5 μmol/min.

Effect of pH

The effect of initial pH on fruiting-body formation was determined by measuring the stipe length of the fruiting body 14 days after inoculation. Mycelial growth and fruiting-body formation were observed from pH 4.0 to 11.0, and the largest fruiting body was formed at pH 7.0 (Fig. 5).

Effect of FBP on germination

Asterophora lycoperdoides generates fruiting bodies almost always on *R. nigricans*, although there are many fungi in the natural environment. Thus, we added FBP of various fungi to the medium, and the influence of *R. nigricans* on each growth stage of *A. lycoperdoides* was compared with that of other fungi. First, FBP of various fungi were added to the basal media instead of soluble starch, and the germination rates of chlamydospores on these media were measured. In the medium containing *R. nigricans*, the germination rate of *A. lycoperdoides* was approximately 2.5 times higher than

Table 1. Productivity of polysaccharide-degrading enzyme

Polysaccharide	Composition sugar	Main linkage	Enzyme activity[a] (μmol/min)
Soluble starch	Glucose	α-1,4	1.9
Pullulan	Glucose	α-1,4 and α-1,6	0.64
Cellulose	Glucose	β-1,4	0.098
Pustulan	Glucose	β-1,6	0.16
Laminarin	Glucose	β-1,3	0.79
Xylan	Xylose	β-1,4	0.22
Chitin	*N*-Acetylglucosamine	β-1,4	1.2

[a] Activity is expressed as per flask (50-ml medium in 100-ml Erlenmeyer flask)

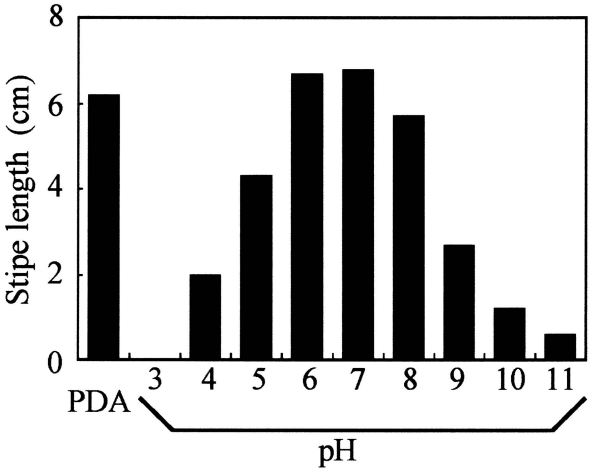

Fig. 5. Effect of initial pH on fruiting-body formation of *A. lycoperdoides* on basal medium

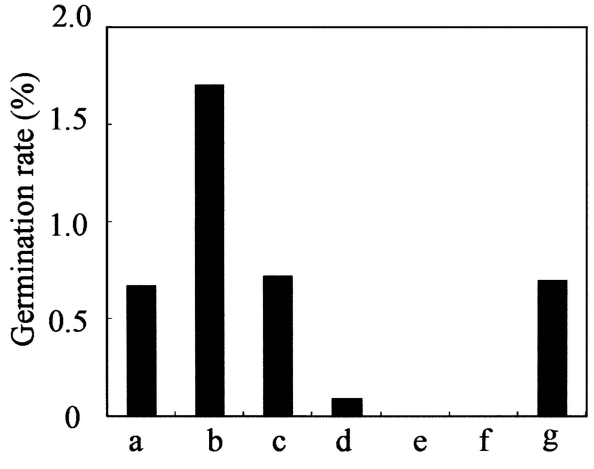

Fig. 6. Effect of host fruiting-body constituents on the germination rate of *A. lycoperdoides* on basal medium that contained fruiting-body powder. *a*, PDA; *b*, *Russula nigricans*; *c*, *Russula delica*; *d*, *Coprinus atramentarius*; *e*, *Russula densifolia*; *f*, *Russula japonica*; *g*, carbon free

that on the PDA medium (Fig. 6). On the other hand, the germination rates of *A. lycoperdoides* were less than 0.1% on the media containing *Russula densifolia* Secr. ex Gillet, *Russula japonica* Hongo, and *Coprinus atramentarius* (Bull.) Fr.

The effect of components of *R. nigricans* on the germination of fungi other than *A. lycoperdoides* was also examined using *A. niger* and *P. expansum*. Germination of *A. niger* and *P. expansum* was not promoted by *R. nigricans* (data not shown). Thus, it was indicated that germination of every fungus is not necessarily promoted by constituents of *R. nigricans.*

Table 2. Effect of fruiting-body ingredients on the time required for chlamydospore germination of *Asterophora lycoperdoides*

Fruiting-body powder	Time required for germination (days)[a]
PDA	4
Russula nigricans	4
Russula densifolia	4
Russula delica	4
Russula japonica	11
Russula senecis	4
Tricholoma giganteum	18
Amanita pantherina	20
Macrolepiota procera	10
Coprinus atramentarius	4
Agrocybe cylindracea	4
Suillus bovinus	4
Ganoderma lucidum	10
Calvatia craniiformis	4
Gyromitra infula	17

PDA, potato dextrose agar

[a] Days required for germination is the average of five replications, observed once every 2 days

The effect of host fruiting-body constituents on the time required for chlamydospore germination of *A. lycoperdoides* was investigated. *Asterophora lycoperdoides* germinated by the 4th day after inoculation on the PDA medium, and it also germinated by the 4th day on the medium containing *R. nigricans*. However, germination was delayed on the media containing *R. japonica*, *Tricholoma giganteum* Massee, *Amanita pantherina* (DC.) Krombh., *Macrolepiota procera* (Scop.) Singer, *Ganoderma lucidum* (Curtis) P. Karst., and *Gyromitra infula* (Schaef.) Quél. (Table 2).

Effect of FBP on mycelial extension

Asterophora lycoperdoides was cultivated on basal medium containing the FBP of various fungi instead of soluble starch, and mycelial extension was measured by using colony diameter. The colony diameter was more than 4 cm on the 10th day on the media containing *R. nigricans*, *R. densifolia*, *Agrocybe cylindracea* (DC.) Gillet, and *C.*

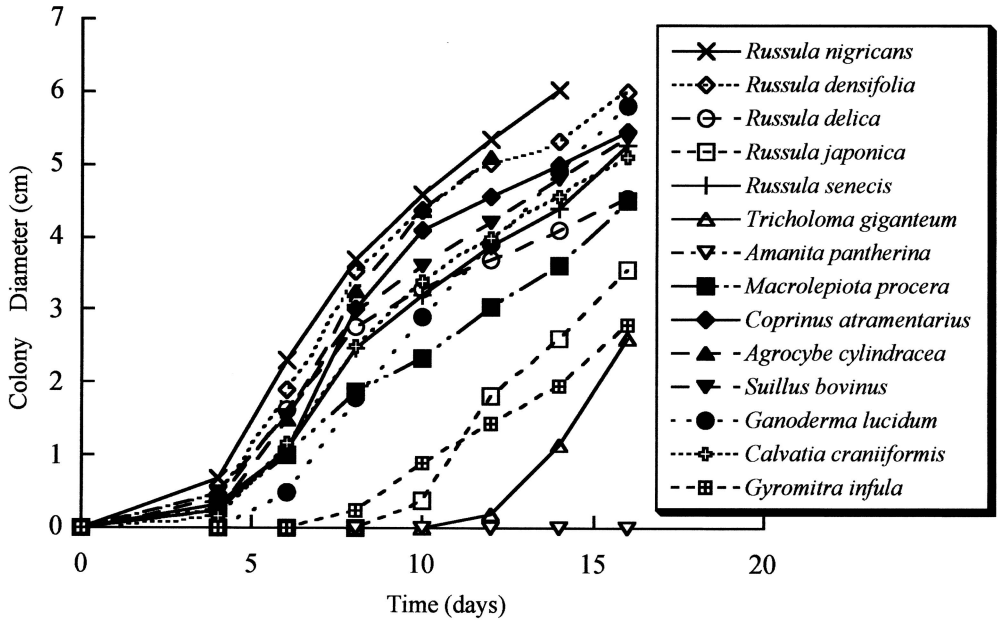

Fig. 7. Effect of host fruiting-body constituents on mycelial extension of *A. lycoperdoides* on basal medium that contained fruiting-body powder

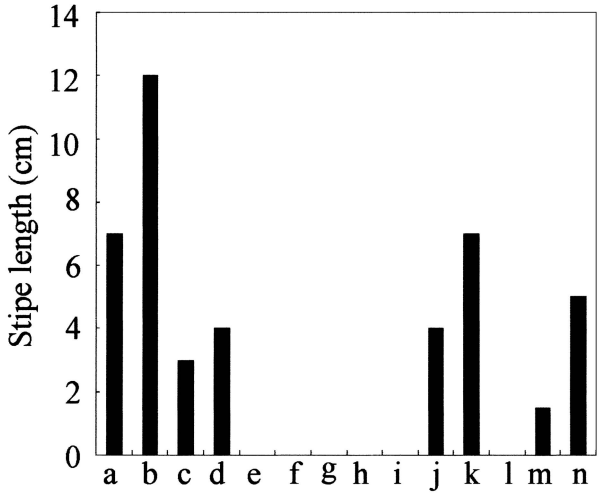

Fig. 8. Effect of host fruiting-body constituents on fruiting-body formation of *A. lycoperdoides* on fruiting-body powder solid medium. *a*, PDA; *b*, *Russula nigricans*; *c*, *Russula densifolia*; *d*, *Russula delica*; *e*, *Russula japonica*; *f*, *Russula senecis*; *g*, *Tricholoma giganteum*; *h*, *Amanita pantherina*; *i*, *Macrolepiota procera*; *j*, *Coprinus atramentarius*; *k*, *Suillus bovinus*; *l*, *Ganoderma lucidum*; *m*, *Calvatia craniiformis*; *n*, *Gyromitra infula*

atramentarius, and extension of the mycelia was fastest on the medium containing *R. nigricans* (Fig. 7).

Effect of FBP on fruiting-body formation

Asterophora lycoperdoides was cultivated on the FBP solid media of various fungi for 18 days, and the stipe length of the fruiting bodies was measured. The largest fruiting body was formed on the *R. nigricans* medium, and it was approximately 1.7 times larger than the fruiting body on the PDA medium (Fig. 8). The time required for fruiting-body formation was shorter in the *R. nigricans* medium than in the other media.

Discussion

It is known that certain fungicolous fungi form their fruiting bodies on the fruiting bodies of other fungi, and only a few species such as *A. lycoperdoides*, *Xerocomus astraeicola* Imaz., and *Cordyceps ophioglossoides* (Ehrh.) Link are categorized under this group. Among these fungi, *C. ophioglossoides*, which grows from the fruiting bodies of *Elaphomyces granulatus* Fr., has been extensively studied (Yamada et al. 1984; Kawaguchi 1995). However, these authors mainly focused on its pharmacodynamic effects. Thus, the cultivation properties and host specificities of these fungi are still unclear. In this study, we determined the cultivation properties of *A. lycoperdoides* to elucidate its host specificity.

As shown in Fig. 3A, *A. lycoperdoides* prefers mannitol as the carbon source. Yoshida et al. (1986) reported that *R. nigricans* contained 24.5% (w/w) of mannitol within the dried fruiting body, and mannitol was the main low molecular component of *R. nigricans*. They also reported on the mannitol content of other fungi. For example, the mannitol content of *Russula delica* Fr. was 22.9% (w/w) and that of *Russula vesca* Fr. was 18.6% (w/w) (Yoshida et al. 1986). The mannitol content in *A. pantherina*, *Lentinula edodes* (Berk.) Pegler, and *M. procera* was 5.9% (w/w), 4.6% (w/w), and 1.7% (w/w), respectively (Yoshida et al. 1979, 1982, 1986). Thus, mannitol was abundant in fruiting bodies of the Russulaceae; however, it was present in small amounts in fungi other than those of the Russulaceae. On the other hand, it was reported that the mycelial growth of *Naematoloma sublaterium* (Schaeff.) P. Karst., an edible mushroom, utilizing mannitol was 34 times slower than that when glucose was utilized (Yoshida et al. 1990). Similarly, mycelial growth of *A. cylindracea* and *Lyophyllum shimeji* (Kawam.) Hongo by utilizing mannitol was approximately 15 times slower than that when glucose was utilized

(Yoshida et al. 1992, 1994). Thus, it was reported that many other basidiomycetes did not prefer mannitol as the carbon source. Based on these reports, it can be concluded that the utilization of mannitol is an important factor of the host specificity of *A. lycoperdoides*.

It is generally accepted that the production of the extracellular enzymes, particularly the cell wall-degrading enzymes, is an important factor for host specificity in phytopathogens (Oku 1977; Daly 1984). In this study, it was found that *A. lycoperdoides* produced extracellular enzymes such as chitinase, laminarinase, and pustulanase. The main components of fungal cell walls are chitin and β-glucan, which has both β-(1, 3) and β-(1, 6) linkages (Wessels et al. 1972). Laminarin is a β-(1, 3)-linked glucan, and pustulan is a β-(1, 6)-linked glucan. Therefore, the extracellular enzymes produced by *A. lycoperdoides* may be involved in the degradation of the host cell wall.

The time required for fruiting-body formation also appeared to be a factor that influenced the host specificity of *A. lycoperdoides*. In this study, it was shown that *A. lycoperdoides* formed fruiting bodies on the PDA medium by approximately the 8th–9th day after inoculation. It is well known that certain fungi such as *F. arcularius* and *C. lagopus* easily fructify under artificial conditions in a short time. Among these, *F. arcularius* required 8 days for fruiting-body formation (Kitamoto and Kasai 1968; Kitamoto et al. 1968), and *C. lagopus* showed fruiting-body formation by the 6th–7th day after inoculation (Madelin 1956). Thus, it appeared that *A. lycoperdoides* is also one of the fungi that shows fruiting-body formation in a short time period. In addition, the optimal temperature of both mycelial growth and fruiting-body formation of *A. lycoperdoides* was approximately 25°C, whereas it was reported that in many fungi the optimal temperature for fruiting-body formation was lower than that for mycelial growth (Kinugawa and Furukawa 1965; Suzuki 1979). Thus, it appears that *A. lycoperdoides* forms fruiting bodies as soon as possible, even if it grows under the optimal temperature of vegetative growth. It is reasonable to consider that *A. lycoperdoides* immediately forms fruiting bodies to complete its life cycle because fruiting bodies of almost all fungi (excepting polypore fungi) are immediately decomposed in the natural environment by autolysis and microbial degradation. Decomposition of fruiting bodies required less than 1 week in most fungi. On the other hand, decomposition of *R. nigricans* is comparatively slower than other Agaricales. When the authors observed it, *R. nigricans* required approximately 10–14 days for complete decomposition (data not shown). Therefore, it appears that there is sufficient time for *A. lycoperdoides* to form fruiting bodies on *R. nigricans*. Conversely, formation of fruiting bodies on other fungi may be difficult because *A. lycoperdoides* required at least 8–9 days to form fruiting bodies.

It was also shown that *R. nigricans* promoted all stages of *A. lycoperdoides* growth. Germination rates were the highest and mycelial growth was fastest on the medium containing *R. nigricans*. Further, the size of the fruiting bodies that were formed on the *R. nigricans* medium was the largest. *Russula nigricans* may contain constituents that promote the growth of *A. lycoperdoides*. On the other hand, some fungi including the Russulaceae (such as *R. densifolia* and *R. japonica*) inhibited the germination of *A. lycoperdoides*. From these results, it is considered that *R. nigricans* is particularly suitable as a host for *A. lycoperdoides*. Additionally, it is probable that some parameters such as utilization of the carbon source, extracellular enzymes, time required for fruiting-body formation, and the constituents in the fruiting body contribute to the host specificity of *A. lycoperdoides.*

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