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Coprinus comatus: A basidiomycete fungus forms novel spiny structures and infects nematode

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Abstract: Nematophagous basidiomycete fungi kill nematodes by trapping, endoparasitizing and producing toxin. In our studies *Coprinus comatus* (O.F.Müll. : Fr.) Pers. is found to be a nematode-destroying fungus; this fungus immobilizes, kills and uses free-living nematode *Panagrellus redivivus* Goodey and root-knot nematode *Meloidogyne arenaria* Neal. *C. comatus* produces an unusual structure designated spiny ball. Set on a sporophore-like branch, the spiny ball is a burr-like structure assembled with a large number of tiny tubes. Purified spiny balls exhibit moderate nematicidal activity. Experiments show that spiny balls are not chlamydospores because of the absence of nuclei in the structures and quick formation within 3 d in a young colony. Nematodes added to *C. comatus* cultures on potato-dextrose agar (PDA) and cornmeal agar (CMA) become inactive in hours. Infection of nematodes by the fungus occurs only after the nematodes are immobilized (feeble or dead), probably by a toxin. Electron micrographs illustrate that *C. comatus* infect *P. redivivus* by producing penetration pegs with which hyphae colonize nematode bodies. An infected nematode is digested and consumed within days and hyphae grow out of the nematode.

Key words: *Coprinus comatus,* Nematophagous fungi, spiny ball

INTRODUCTION

Nematophagous fungi draw much attention with their biological control potential of capturing, killing and digesting nematodes. Nematophagous basidiomycete fungi were investigated about 63 y ago and many of them are wood-decay mushrooms. Our knowledge in this area begins with the discovery of *Nematoctonus* (Drechsler 1941), with its teleomorph in the genus *Hohenbuehelia* (Barron and Dierkes 1977). This is an unfamiliar aspect of the biology of these saprophytic fungi. It was suggested that the wood-decay fungi obtain nitrogen supplement from prey, including nematodes, to survive in such nitrogen-restricted habitat as rotting wood and forest soil (Thorn and Barron 1984, 1986). Along the hyphae of these nematophagous basidiomycete fungi, some appendages were found to be attack or defense weapons. *Nematoctonus* is characterized by the ability to capture nematodes by sticking to the nematode cuticle with hourglass-shaped adhesive knobs that are enveloped in a thick mucus sheath (Drechsler 1943, 1946, 1949, 1954). *Pleurotus* paralyzes nematodes with toxin droplets produced by tiny secretory cells on vegetative hyphae (Barron and Thorn 1987). Similar secretory appendages were found on lawn mushroom *Conocybe lacteal,* in which they are more likely to be defense apparatus. After paralyzing and killing nematodes, *C. lacteal* does not use them as food (Hutchison et al 1995). Some species of *Hyphoderma* produce stephanocysts that once were considered asexual spores, dispersal propagules (Burdsall 1969), chlamydospores (Kendrick 1979) or beneficial nutritional structures (Hallenberg 1990), but Tzean and Liou showed that they were trapping devices (Tzean and Liou 1993). In this article, a basidiomycete fungus, *Coprinus comatus,* is shown to be a nematode-destroying fungus, producing a new structure designated spiny ball. The infection process of *P. redivivus* by the fungus is studied with SEM and TEM. A simple method to purify spiny balls is suggested. The connection between the spiny balls and killing of nematode is discussed here.

MATERIALS AND METHODS

Culture of C. comatus.—One isolate designated LHA-7 was obtained by tissue isolation. This strain was grown on PDA in 9 cm diam Petri dishes. When the colonies reached 2 cm diam, 5 mm agar plugs from the leading edge of the individual colonies were transferred to new PDA and CMA. The colonies occupied the whole agar surface in 9–12 d at 24 C, and these first-transferred Petri plates were used in bioassays.

Nematode culture.—The free-living nematode *P. redivivus* was grown axenically in a semiliquid oat medium (10 g of

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TABLE I. Immobilization of nematodes by the isolate LHA-7

Plates	Nematodes	Incubation time	No. of nematodes		Immobilized nematodes (%)	Control		Statistics[a]	
			Mobile	Immobile		Mobile	Immobile	χ^2	P
PDA	*P. redivivus*	4 h	129	122	48.6	318	6	176.0	<0.005
PDA	*P. redivivus*	8 h	12	218	94.8	355	11	499.0	<0.005
CMA	*P. redivivus*	4 h	132	109	45.2	135	2	78.6	<0.005
CMA	*P. redivivus*	8 h	74	194	72.4	187	2	227.3	<0.005
PDA	*M. arenaria*	4 h	51	204	80.0	213	66	167.0	<0.005
PDA	*M. arenaria*	8 h	11	252	95.8	170	84	220.8	<0.005
CMA	*M. arenaria*	4 h	73	115	61.2	201	35	96.3	<0.005
CMA	*M. arenaria*	8 h	17	146	89.6	185	24	221.9	<0.005

[a] χ^2 tests (df = 1) comparing frequencies of mobile and immobile nematodes in treated versus control samples.

oat, 6 mL of distilled water) at 28 C for 4–6 d, stored at 4 C and used within 15 d. The root-knot nematode *M. arenaria* was cultured on tomatoes in greenhouse conditions and second-stage juveniles were extracted and stored according to Kerry's methods (Kerry and Bourne 2002).

Purification of spiny balls.—LHA-7 was inoculated on PDA in 9 cm diam Petri dishes and incubated at 24 C for 9 d. Lens paper in 12 layers was folded to fit a glass funnel, moistened in the funnel, and the assembly was sterilized at 121 C for 20 min. Seven mL of sterile water were added to each culture plate. The surface of the culture was scraped gently for 2 min with a glass sterile spreader. The suspension was transferred to the sterile funnel and the filtrate was collected in sterile 1.5 mL Eppendorf tubes and centrifuged at 12 000 rpm for 5 min. After transferring the supernatant to other Eppendorf tubes, the precipitate was washed by resuspending in sterile distilled water, and the suspension was centrifuged at 10 000 rpm for 3 min. The precipitate was stored at 4 C.

Bioassays.—Bioassay with fungal cultures were conducted on first-transferred PDA and CMA Petri plates. The nematodes were washed thoroughly with sterile distilled water and the concentration was adjusted to 10 000 nematodes per mL. Each Petri plate received 0.5 mL of the nematode suspension that then was scattered with a glass spreader. The plates were incubated at 24 C and mobile and immobile nematodes in five random visual fields were counted after 4 and 8 h. This experiment was carried out with three replicates and conducted twice. Nematode-inoculated PDA and CMA plates without fungal cultures served as controls.

The bioassay with purified spiny balls of the isolate LHA-7 was conducted with 96 well cell culture clusters (Costar). Each test well received 30 μL of purified ball precipitate and ca 100 nematodes in 20 μL of suspension. Treatment control wells contained 30 μL of the supernatant obtained during the purification of spiny balls and 20 μL of nematode suspension. To maintain humidity, 100 μL per well of sterile water was added to the remaining wells. The plates

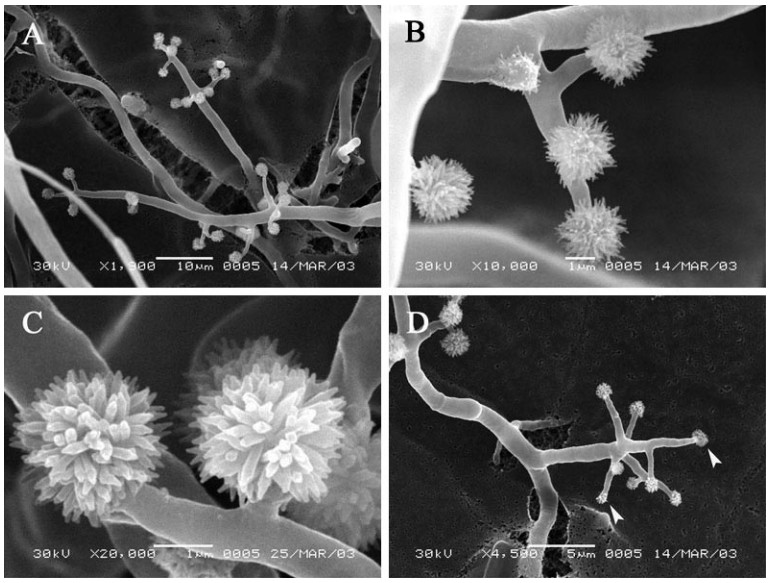

FIG. 1. Scanning electron micrograph of the spiny balls. A. The spiny balls on specific aerial hyphae. B. High-magnification view of 5 d old spiny balls on a furcated branch. C. High-magnification view of 15 d old spiny balls. D. The nascent spiny balls with less fuzz (arrows) compared with the mature ones. Bars: A = 10 µm, B, C = 1 µm, D = 5 µm.

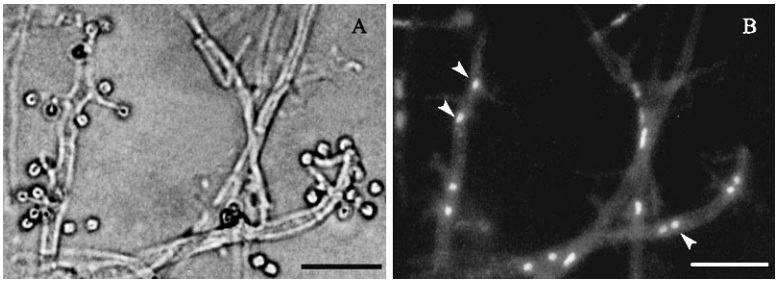

FIG. 2. Nuclear staining of spiny balls. A. A light microscopic picture of the spiny balls. B. The fluorescence micrograph of the same balls. The white spots (arrows) in the hyphae were nuclei. Bars: A, B = 10 μm.

were incubated at 24 C and examined after 24 and 48 h. Mobile and immobile nematodes were counted with an inverted light microscope. This experiment was performed with three replicates and conducted twice.

Nuclear staining and germination assays.—Fluorescent dye Hoechst 33258 (Sigma) was used as the stain. To keep delicate structures intact, the slide culture technique was adopted (Riddell 1950). Mycelia of LHA-7 with spiny balls expanded across the cover slips in 5 d, when the cover slips were used for fluorescence staining. Stock stain solution was prepared by dissolving 5 mg of Hoechst 33258 in 5 mL of distilled water and diluting the solution to 100 mL; working solution contained 10% stock solution, 90% 0.2 M phosphate-citric acid buffer (pH 7.0). Fifteen percent glycerol was added to the working solution before use. The cover slips with mycelia downward were laid on drops of working stain solution placed on slides beforehand. After 1 min the slides were observed in a Nikon Eclipse 800 microscope and photos were taken with Kodak Gold 400 film.

The germination experiment was conducted with purified spiny balls. Approximately 10 μL of spiny-ball precipitate was added to 1 mL of sterile water, and the suspension was spread on PDA and CMA plates with a glass spreader, 200 μL of the suspension each. These plates then were incubated at 24 C and examined with a light microscope every 24 h for 9 d.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM).—The isolate LHA-7 was grown on PDA in 9 cm diam Petri dishes at 24 C for 5 d. Mycelia did not cover all the agar surface and contained less dense aerial mycelia, which could aid TEM and SEM processes. Infected nematodes used in electron microscopic observation were prepared by adding ca 5000 nematodes (*P. redivivus*) in 0.5 mL of suspension at each center of the mycelia. The plates then were incubated 12 h, 24 h, 48 h, 72 h, 5 d, 7 d or 9 d. Dense aerial hyphae covering the infected nematodes were removed with adhesive tape in SEM study. Samples for both TEM and SEM studies underwent the same initial steps: small blocks of agar (7 × 7 mm for SEM, 2 × 2 mm for TEM) containing spiny balls or infected nematodes were cut out and fixed in 2.5% glutaraldehyde solution at 4 C for 24 h. The agar pieces were rinsed in 0.2 M phosphate buffer (pH 7.2) three times and postfixed in 1% OsO_4 made up in the same buffer for 1 h at room temperature. A graded acetone series was used in dehydration. For SEM the agar blocks were exchanged in an isoamyl acetate series, dried with a HCP-2 Critical Point Dryer (Hitachi) for 7 h, mounted on copper stubs, coated with a gold/palladium mixture by an IB-3 ion coater (EIKO) and viewed with a JSM-5600LV scanning electron microscope (JEOL). For TEM samples were embedded in epoxy resin (Epon 812) after fixation and dehydration and cut with glass knives. Ultrathin sections were mounted on formvar film on 100 mesh copper grids and stained with uranyl acetate and lead citrate. Observation was taken with a JEM-100 CX transmission electronic microscope (JEOL) operating at 60 kV.

In the SEM study for purified spiny balls, 10 μL of spiny-ball precipitate was added to 1 mL of 2.5% glutaraldehyde solution at 4 C for 24 h. After centrifugation the precipitate was transferred to a cover slip and spread evenly with an inoculating loop. The spiny balls on the cover slip were coated and viewed as described above without being postfixed and dehydrated.

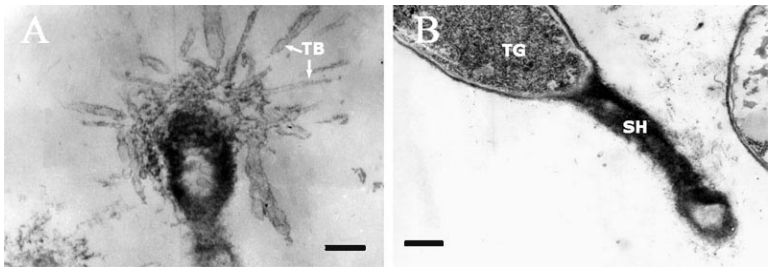

FIG. 3. Transmission electron micrograph of spiny balls. A. Longitudinal section of a spiny ball. The ball was composed of an electron-dense shaft and many irregular tubes (TB). B. Longitudinal section of a spiny ball on a branch (TG). The shaft (SH) was electron-dense except the slightly inflated end. Bars: A, B = 0.5 μm.

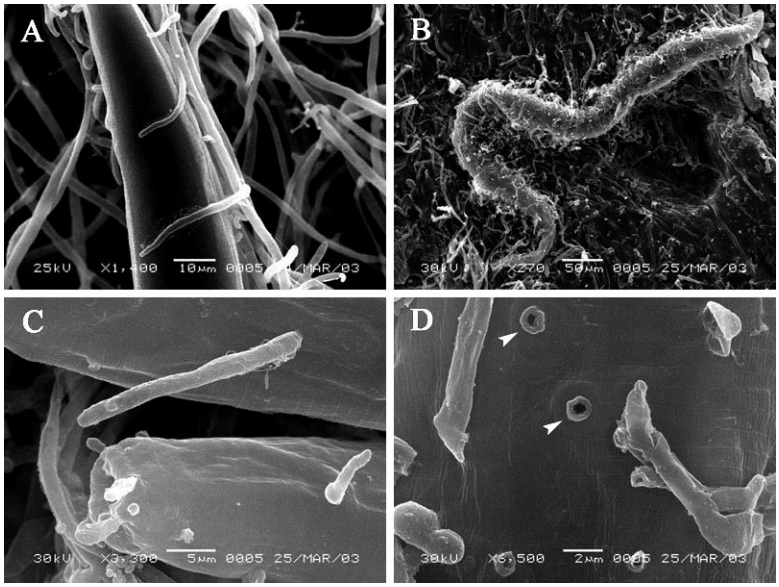

FIG. 4. Scanning electron micrograph of immobilized and infected nematodes. A. An inactive nematode (treated 12 h). Two hyphae wound the nematode. B. A nematode treated 7 d was wrapped in hyphae. C. Hyphae protruded from infected nematodes (treated 5 d). D. Pores (arrows) in nematode cuticle left by pulling out hyphae with adhesive tape. Bars: A = 10 μm, B = 50 μm, C = 5 μm, D = 2 μm.

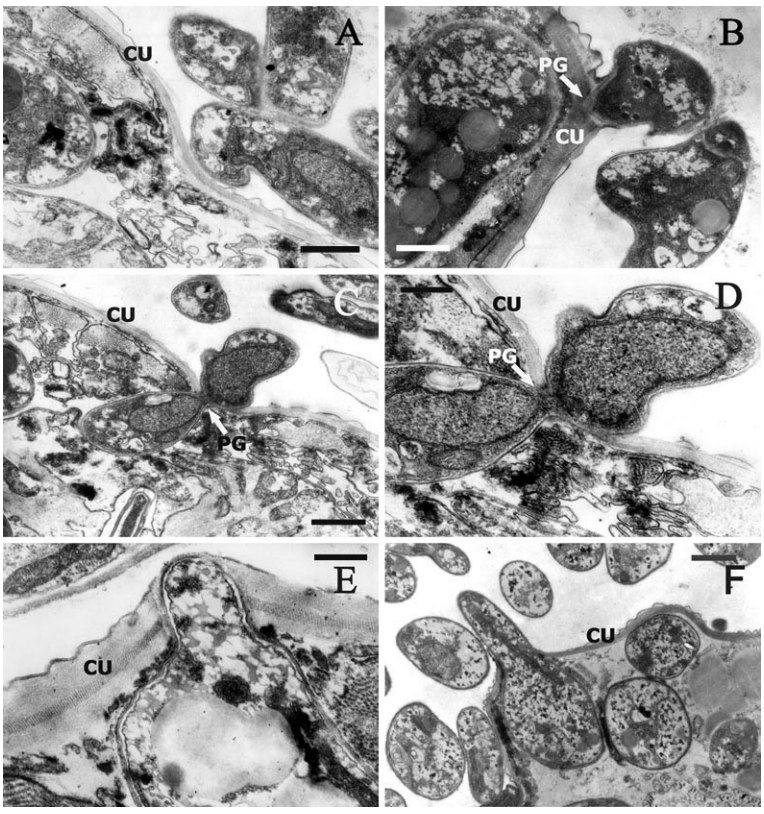

FIG. 5. Transmission electron micrograph of infected nematodes. A. A hypha grew toward and pressed the cuticle (CU) of a nematode. B. A penetration peg (PG) was forming. C. A hypha penetrated nematode cuticle (CU) with a penetration peg (PG). D. High-magnification view of the penetration peg (PG). E. A hypha was about to grow out from an infected nematode. F. A hypha protruded from the nematode. Bars: A, B, C = 1 μm, D = 0.5 μm, E = 0.25 μm, F = 2 μm.

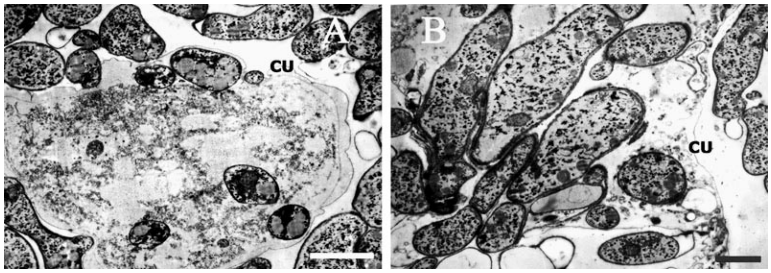

FIG. 6. Colonization and digestion of *P. redivivus* by LHA-7. A. A nematode was digested partially 3 d after adding nematodes. B. An infected nematode was digested fully and occupied by the hyphal body 7 d after adding nematodes. Bars: A = 5 μm, B = 1 μm.

Culture of basidiocarps.—To verify the identity of the cultures we isolated, solid-state cultivation was done to obtain basidiocarps. In 250 mL flasks, mycelia of LHA-7 were grown in potato dextrose broth (PDB) medium at 24 C for 7 d by shaking at 150 rpm. Mushroom culture bags were prepared with the medium containing 90% cottonseed hull, 4.5% bran, 4.5% cornmeal and 1% lime. The bags were sterilized at 121 C for 4 h and inoculated with the liquid culture. The bags were incubated at 24 C in a greenhouse for 40 d until all bags were colonized fully. The cores were transferred to barrels and covered with forest soil containing abundant organic debris. Relative humidity was adjusted to 85% and the containers were placed in the greenhouse for induction of basidiocarps at 20 C.

RESULTS

Immobilization of nematodes.—On PDA and CMA plates, LHA-7 immobilized nematodes with high efficiency (TABLE I). At same intervals, LHA-7 immobilized more nematodes on PDA than that on CMA.

Spiny balls.—LHA-7 produced abundant spiny balls on PDA, and spiny balls were formed quickly in culture, usually within 3 d of inoculation. On an aerial hypha, a spiny ball was a ''fluffy'' structure having a diameter of about 2.5 μm. The spiny balls and the support branches, respectively, resembled spores and sporophores and the sporophore-like branches arranged in a sympodial pattern (FIG. 1A). The branches were measured 0.5–8 μm in length, the lower parts were somewhat wider (0.5–1.3 μm), and the upper parts were slender (0.3–0.6 μm). In high-magnification TEM, the spiny balls were assembled by many tiny tubes (ca 0.2 μm wide) with slightly narrowed apices (FIG. 1B, C). The development of the spiny balls differed from that of spores: Specific aerial hyphae branched to form sporophore-like branches, many fibers grew out from tips of the branches and the fibers elongated themselves and surrounded the ends of the branches to form spiny balls (FIG. 1D). Nuclear staining illustrated the absence of nuclei in the structures and in the sporophore-like branches and that the aerial hyphae bearing these balls were dikaryotic (FIG. 2). Purified spiny balls did not germinate on PDA and CMA. After 9 d no obvious change of the balls was found on either medium. In TEM the core of a spiny ball was an electron-dense shaft that had a slightly inflated end with some saccular organelles and the shaft was surrounded by many tubes (FIG. 3A). The shaft sprouted from the top of a sporophore-like branch and was the base of a spiny ball (FIG. 3B). Consistent with the result of nuclear staining study, no nuclei were found by using TEM technique.

Infection of nematodes.—Most of the *P. redivivus* added to the cultures on PDA and CMA were immobi-

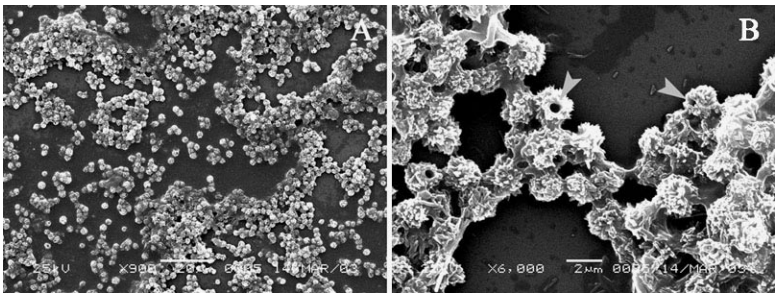

FIG. 7. Scanning electron micrograph of purified spiny balls. A. Low-magnification view of abundant purified spiny balls. B. High-magnification view of purified balls. The pores (arrows) were the sites where shafts fixed in. Bars: A = 20 μm, B = 2 μm.

TABLE II. Immobilization of nematodes by the purified spiny balls

Nematode	Incubation time	No. of nematodes		Immobilized nematodes (%)	Control		Statistics[a]	
		Mobile	Immobile		Mobile	Immobile	χ^2	*P*
P. redivivus	24 h	108	77	41.6	174	37	26.7	<0.005
P. redivivus	48 h	40	149	78.8	155	51	113.2	<0.005

[a] χ^2 tests (df = 1) comparing frequencies of mobile and immobile nematodes in treated versus control samples.

lized within 8 h. Many of the nematodes transferred to a drop of water by hand moved feebly, but one hardly could recover or survive. When the nematodes were immobilized (i.e. they were very weak or dead) the hyphae of LHA-7 grew toward and wounded the nematodes (FIG. 4A). An infected nematode enveloped by a layer of hyphae still could be recognized easily 15 d after adding nematodes (FIG. 4B). The nematode body was colonized fully by hyphal body, and from the third day on hyphae protruded from the nematode (FIG. 4C). Adhesive tape was used to prepare samples for SEM study so that pores perforated by hyphae were exposed because some hyphae were pulled out (FIG. 4D). Transmission electron micrographs revealed a detailed infection process, concerning how a nematode was colonized by *C. comatus* and how hyphae grew from an infected nematode. In the first stage hyphae grew toward and pressed onto the cuticle of the nematode (FIG. 5A); then a penetration peg was formed (FIG. 5B); in the third stage the hyphae penetrated nematode cuticle with the peg and rapidly colonized the body (FIG. 5C, D); finally, hyphae protruded from the infected nematode (FIG. 5E, F). The inward and outward deformation of cuticle at the sites where hyphae invaded and emerged from nematodes indicated that mechanical force was involved in penetration and outgrowth. After penetration of hyphae, the content of a nematode was digested in days and the nematode was occupied fully by the hyphal body (FIG. 6).

Immobilization of nematodes by excised spiny balls.—The SEM micrograph showed a great number of purified spiny balls could be obtained in a simple way (FIG. 7A). A pore in each ball showed the site where a shaft fixed in (FIG. 7B). The purified balls showed a moderate activity in immobilizing nematodes (TABLE II). The same situation was found when spiny balls were ground with liquid nitrogen.

Basidiocarps of C. comatus.—Basidiocarp primordia formed after 15 d and mushrooms emerged 30 d after placing the cores into soil (FIG. 8). Identification with well developed basidiocarps reconfirmed the identity of the cultures as *C. comatus*. Furthermore, the isolates obtained from second-time tissue isolation also possessed spiny balls and nematicidal activity.

DISCUSSION

C. comatus was considered a saprophytic fungus before, but in this report it is shown to infect and kill the free-living nematode *P. redivivus* and the plant-parasitic nematode *M. arenaria*. Our results support the view that some mushrooms develop the function of feeding on microfauna, enabling them to survive or thrive in nitrogen-restricted niches (Thorn and Barron 1984, 1986).

Many gilled fungi produce chlamydospores in culture, and some of them are ornamented strongly.

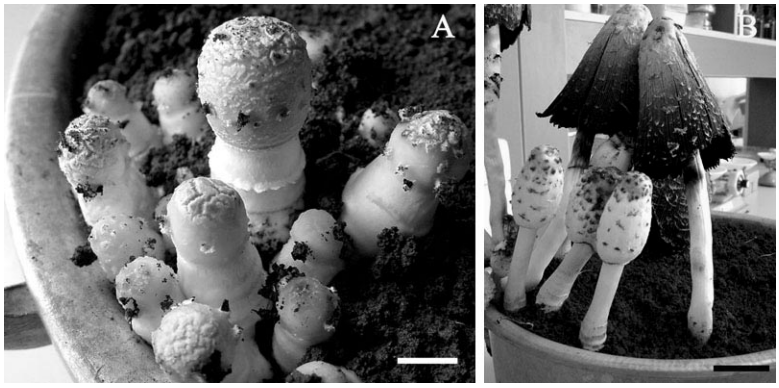

FIG. 8. Basidiocarps of the isolate LHA-7. A. Newly formed basidiocarps were emerging from soil. B. Mature basidiocarps. Bars: A = 1 cm, B = 4 cm.

The spiny balls first were thought to be terminal chlamydospores, but the evidence—lack of nuclei and quick formation—contradicted this conclusion. Considering that the purified spiny balls showed moderate nematicidal activity, these unusual structures might be devices or appendages relevant to killing nematode. Purified spiny balls showed a lesser ability to immobilize nematodes than the cultures, possibly because they need proper physiological conditions. So far the devices and the appendages found on basidiomycetes are specialized cells producing toxins or adhesive substances. On *Pleurotus* and *C. lacteal* toxin droplets flow from secretory cells (Barron and Thorn 1987, Hutchison et al 1995). Stephanosysts and hourglass-shaped adhesive knobs produce infection tubes or pegs (Drechsler 1946, 1949, 1954; Tzean and Liou 1993). The spiny balls seem to be incomplete cells and do not resemble any of them. The assumption that the spiny ball might be a nematode-killing appendage needs further investigation.

Although we cannot draw a conclusion about what the spiny ball is, experiments showed some evidence relevant to the nematode-killing capability of the fungus. We obtained 31 isolates after tissue isolation, and they all produced the spiny balls and immobilized nematodes with varying efficiency when grown on PDA and CMA. Among them isolates with higher nematode-killing capability produced more spiny balls than those with lower nematode-killing capability. In the bioassays nematodes remained alive and active at the margin area of a colony where the spiny balls were rare or not well developed. As mentioned above, purified spiny balls showed moderate nematicidal activity. It is probable that the spiny balls play some role in the infection of nematodes. On the other hand, the production of the spiny balls was not induced by the presence of nematodes, and no notable increase in the number of these balls was found after adding nematodes. This should be explained through further studies.

When immobilized nematodes were immersed in a drop of water, many of them made feeble movements suggesting they were alive. A similar occurrence was found in the interaction between nematodes and a toxin-producing nematophagous fungus *Pleurotus ostreatus* (Barron and Thorn 1987). Here a toxin identified as trans-2-decenediotic acid immobilized the nematode *P. redivivus* (Kwok et al 1992). It was probable that the inactive nematodes in *C. comatus* cultures were immobilized by a toxin.

Organisms are energy-efficient systems and tend not to produce unnecessary structures. The actual function of the spiny balls remains to be determined, yet we are continuing to study these structures.

ACKNOWLEDGMENTS

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