Applied and Environmental Microbiology, June 2007, p. 3916–3923 Vol. 73, No. 12
0099-2240/07/$08.00+0 doi:10.1128/AEM.02770-06

Coprinus comatus Damages Nematode Cuticles Mechanically with Spiny Balls and Produces Potent Toxins To Immobilize Nematodes[▽]

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Received 28 November 2006/Accepted 11 April 2007

We reported recently a unique fungal structure, called the spiny ball, on the vegetative hyphae of *Coprinus comatus* (O. F. Müll.:Fr.) Pers. Although some observations regarding the role of this structure were presented, its function remained largely unknown. In this study, we showed that purified (isolated and washed) spiny balls could immobilize and kill the free-living nematode *Panagrellus redivivus* Goodey highly efficiently. Scanning electron microscopy studies illustrated that the spiny structure damaged the nematode cuticle, suggesting the presence of a mechanical force during the process of nematode immobilization. Severe injuries on nematode cuticles caused the leakage of inner materials of the nematodes. When these structures were ground in liquid nitrogen, their killing efficacy against nematodes was lost, indicating that the shape and the complete structure of the spiny balls are indispensable for their function. However, extraction with organic solvents never lowered their activity against *P. redivivus*, and the extracts showed no obvious effect on the nematode. We also investigated whether *C. comatus* was able to produce toxins which would aid in the immobilization of nematodes. In total, we identified seven toxins from *C. comatus* that showed activity to immobilize the nematodes *P. redivivus* and *Meloidogyne incognita* (Kofoid et White) Chitwood. The chemical structures of these toxins were identified with nuclear magnetic resonance, mass spectrometry, infrared, and UV spectrum analysis. Two compounds were found to be novel. The toxins found in *C. comatus* are O-containing heterocyclic compounds.

Nematophagous basidiomycetes refer to those fungi possessing clamp connections on hyphae with the capability of capturing, killing, and decomposing nematodes. These fungi are members of commonly known mushrooms, and they have developed several mechanisms to utilize nematodes as food sources, including that of nitrogen (19, 23, 24, 26). Most of these mushrooms use delicate and effective small weapons called appendages on either hyphae or conidia to attack nematodes. For example, *Nematoctonus* spp. produce hourglass-shaped adhesive knobs containing a secretory cell and the mucilage it produces (7, 8, 9, 10, 11). The knobs are enveloped in a thick mucus sheath and can capture nematodes by first sticking to the nematode cuticle. Subsequently, the knobs penetrate and kill the nematodes to start a new cycle of growth. Hourglass-shaped adhesive knobs are also found on *Hohenbuehelia*, which has been proved to be the teleomorph of the genus *Nematoctonus* (3). Stephanocysts are one- or two-celled structures on the hyphae of some species of *Hyphoderma* (5, 14). For many years, these structures were merely considered a curiosity, and their function was not known until Tzean and Liou made the exciting discovery that these specialized cells were coated with fibrous mucilages that can adhere to nematodes and eventually invade the nematode hosts (26). Tiny secretory cells on the vegetative hyphae of *Pleurotus* spp. can also attack nematodes in a remarkably different fashion. These appendages secrete droplets containing potent toxins which can paralyze nematodes in seconds but do not kill them (4). The specialized directional hyphae are then attracted by the paralyzed victims. The cuticle is penetrated and the contents digested by the hyphae. Secretory appendages similar to those of *Pleurotus* were found on the hyphae of the lawn mushroom *Conocybe lactea*, and its pattern of attacking nematodes is also similar to that of *Pleurotus* (15). The potent toxin droplets produced by the secretory cell can paralyze and kill the nematode victim. Finally, an acanthocyte is a spiky structure found in *Stropharia* species (12). It was recently discovered that this structure can damage the cuticle of nematodes and kill them very efficiently. These observations suggest that acanthocytes might be a nematode-attacking device (20).

Coprinus comatus, the shaggy mane mushroom, has dark red-brown spores and is commonly seen on newly disturbed grounds, grassy places, and road sides. This edible species is cultivable and has been commonly seen on the tables of Chinese people in recent years. We previously reported a novel fungal structure called spiny ball on vegetative hyphae of *C. comatus*. Typical spiny balls consist of a rod-shaped part in the core and quite a few peripheral projections with contracted ends (19). The spiny ball is quite different from other known

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▽ Published ahead of print on 20 April 2007.

fungal structures on other mushrooms in two aspects. First, its spiny shape is morphologically distinct and its size is much smaller than those of the other known appendages. Secondly, the spiny ball does not possess nuclei and seems to be an incomplete cell, while the others are obviously cells. Some observations showed that these structures are probably linked to the ability of *C. comatus* to attack nematodes (19). However, the function of spiny balls has not been elucidated.

The nematode-immobilizing effect of *C. comatus* is high. About half of the nematodes could be immobilized within 1 to 4 h by *C. comatus* cells grown on potato dextrose agar (PDA) or cornmeal agar plates. Within 8 h of incubation, about 90% of the worms were immobilized (19). The high efficiency of immobilization suggested that low-molecular-weight toxins produced by the fungus might be responsible. Although some earlier studies have isolated and identified nematotoxic compounds, the known number of such compounds is still quite limited. Until now, fewer than 100 metabolites from various fungi have been proven to possess nematicidal activity (6). Some of them are thought to be specifically produced to attack nematodes. These compounds included *trans*-2-decanedioic acid, which was isolated from *Pleurotus ostreatus* (18), and linoleic acid, *p*-anisoylalcohol, *p*-anisaldehyde, *S*-coriolic acid, 1-(4-methoxyphenyl)-1,2-propanediol, and *Z*-hydroxy-(4′-methoxy)-propiophenone, which were isolated from *Pleurotus pulmonarius* (22). The nematicidal abilities of *C. comatus* prompted us to investigate whether *C. comatus* produces nematotoxic compounds.

The aim of this paper is to provide some direct mechanistic evidences for the nematicidal activity by *C. comatus*. In an earlier study, a simple method to purify spiny balls was developed (19). The purified spiny balls showed limited efficacy against nematodes, which were much weaker than live hyphae. Such observations suggested to us that the nematicidal effects were due to a number of factors. In this paper, we investigated whether the ability to attack nematodes was due to the formation of spiny balls and, if so, what kind of role the spiny balls play during the interaction between the fungus and nematodes. In addition, to answer the question of whether *C. comatus* could secrete toxins to facilitate the infection process, we carried out a study on isolating and identifying nematotoxins. Since many nematodes are parasites on plants and can cause severe damage to world agriculture (1), the identification and structure elucidation of novel compounds with nematotoxic ability are of great interest for developing new nematicides and antihelminthic drugs.

MATERIALS AND METHODS

Strains and cultivation conditions. Two strains of *C. comatus* were used in this study: strain LHA-7, used in our previous study, and an isolate designated C-1 from a basidiocarp found in the field in Yunnan, China. The strains were deposited in the Laboratory for Conservation and Utilization of Bio-resources (Yunnan University, Kunming, Yunnan, China) as YMF1.00135 and YMF1.02579, respectively. The strains were maintained on PDA slants and transferred every 2 months. The free-living nematode *Panagrellus redivivus* was grown axenically in semiliquid oat medium (10 g of oat, 6 ml of distilled water) at 28°C for 4 to 6 days, stored at 4°C, and used within 15 days.

Regenerating PDA plates. The *C. comatus* strains LHA-7 and C-1 were cultured on PDA in petri dishes (diameter, 9 cm) for 8 to 10 days. When the agar surface was fully covered by fungal mycelia, these plates were carefully scraped with a scalpel to completely remove the aerial fungal hyphae under sterile conditions in a laminar flow cabinet. These plates were then incubated at 20°C for 18 to 28 h. During this time period, special aerial hyphae grew and produced abundant spiny balls. However, normal aerial hyphae formed only very sparsely (Fig. 1). These plates were designated regenerating PDA plates. The purpose of constructing the regenerating plate was to expose the spiny balls which would have been covered by dense aerial hyphae on normal culture plates of *C. comatus*. Bioassays with this regenerating plate were compared to those with the normal culture plate.

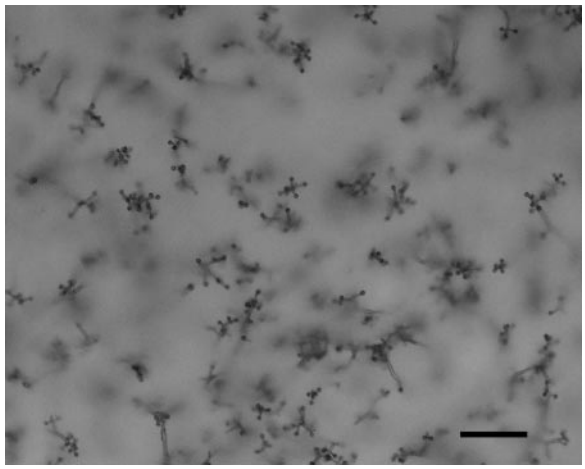

FIG. 1. A survey view (planform) of a regenerating PDA plate, showing abundantly produced spiny balls with sparse aerial hyphae. Bar, 100 μm.

Bioassay with the regenerating PDA plate. The regenerating PDA plates prepared as described above were inspected for the production of spiny balls before being used in bioassays. The *P. redivivus* nematode was thoroughly rinsed four times before use. The washed nematodes were picked with an inoculating loop, and 50 to 100 worms were transferred to a selected site on the regenerating PDA plate with abundant newly formed spiny balls. Three sites were selected to receive the nematodes on one plate. The plates were incubated at 24°C, and mobile and immobile nematodes were counted after 15 and 30 min at three random sites by using a light microscope (magnifications of ×40). Nematodes added to blank PDA plates served as a control. This experiment was carried out with three replicates and conducted twice.

Bioassay with purified spiny balls and smashed spiny balls. Purified spiny balls were obtained by following a previously described method (19). The spiny ball suspension was adjusted to ca. 10^{10} per ml. To smash the purified spiny balls, 200 μl of the spiny ball suspension was thoroughly grounded with liquid nitrogen by using mortars, and 5 μl of the suspension was inspected under a microscope to confirm that the vast majority of these spiny balls were completely smashed (like starch paste without or with few complete spiny balls). Then, blank water agar (WA) plates were prepared for the bioassay. On one plate, 20 μl of suspension of the purified spiny balls and 20 μl of the ground spiny balls were transferred to two close sites (ca. 1 cm between each other) in the middle of the plate and the suspension drops formed two circles with diameters of ca. 1 cm. About 15 *P. redivivus* worms prepared as described above were handpicked with a fine needle and added to each of the two circles. Immediate observations on the behavior of the added nematodes were made using a microscope at room temperature. Mobile and immobile nematodes were counted 5 and 10 min after their addition. The negative control contained a blank WA plate with about 15 nematodes added to the middle of the plate. This experiment was conducted twice, each with three replicates.

Bioassay with organic solvent-extracted spiny balls. According to our previous hypothesis, the spiny ball could be a reservoir for some potent toxins against nematodes. To test the hypothesis, 100 μl of the purified spiny ball suspension (ca. 10^{10} per ml) was first extracted in 1.0 ml of ethanol at 28°C for 24 h in a 1.5-ml Eppendorf tube. After centrifugation at 13,000 × *g* for 5 min, the supernatant was transferred to another Eppendorf tube and then dried in Eppendorf concentrator 5301. Then, 1.0 ml of acetone was added to the tube and held at 28°C for 24 h. After another centrifugation, the supernatant was transferred and dried as described above. Finally, 1.0 ml petroleum ether was added to the tube and was kept at 28°C for another 24 h. After the supernatant (treated as described above) was removed, the precipitate of the purified spiny balls was washed with 1.0 ml of sterile water for five times, followed by centrifugation at

TABLE 1. Immobilization of *P. redivivus* on regenerating PDA plates by *C. comatus* strains

Strain	Incubation time (min)	No. of nematodes		% Immobilized nematodes	No. of controls		χ^2 value[a]	*P* value
		Immobile	Mobile		Immobile	Mobile		
LHA-7	15	76	27	73.7	3	96	103.2	<0.005
	30	116	2	98.3	3	102	199.6	<0.005
C-1	15	56	18	75.7	1	88	95.5	<0.005
	30	92	1	98.9	2	76	155.3	<0.005

[a] Results of χ^2 tests (1 df) comparing the frequencies of mobilized and immobilized nematodes in treated versus control samples.

13,000 × *g* for 5 min to remove (discard) the supernatant. Then, 20 μl of the spiny ball suspension was transferred to a blank WA plate at the central location of the plate and the suspension drop formed a circle with a diameter of ca. 1 cm. The plate was held at room temperature for 15 min to allow the excessive water to be absorbed by the agar. *P. redivivus* was prepared as described above, and about 15 nematodes were picked with a fine needle and placed in the circle containing spiny balls on the WA plate. The immediate interaction between the spiny balls and the nematodes was studied by microscopy at room temperature. After 5 and 10 min, mobile and immobile nematodes were counted. This experiment was carried out with three replicates and repeated twice. To construct the supernatant control, 100 μl of distilled water was added to each of the dried supernatant-containing tubes and the mixture was vortexed for 1 min to dissolve the extracts. The resulting solutions were pooled and 20 μl of the mixture was transferred to the same WA plate containing the suspension of spiny balls to produce another circle (ca. 1 cm away from the suspension circle). About 15 nematodes were added to the area containing the solution mixtures on the WA plate to serve as the negative control.

Scanning electron microscopy study of immobilized nematodes. Some immobilized nematodes in the bioassay with regenerating PDA as well as some nematodes in the bioassay with purified spiny balls as described above were handpicked and immediately immersed in a 2.5% glutaraldehyde solution at 4°C for 4 h in a 1.5-ml Eppendorf tube. A brief centrifugation was adopted to precipitate the nematodes, and the glutaraldehyde solution was carefully removed and discarded. The fixed nematodes were then rinsed three times in 1 ml of 0.2 M phosphate buffer (pH 7.2), followed by a brief centrifugation to remove the buffer with a pipette. Then, 300 μl of 1% OsO_4 was added to the tube and these nematodes were postfixed in the same buffer for 1 h at room temperature. A graded acetone series was used to dehydrate the nematodes, and each step was followed by a brief centrifugation to remove the liquid. Then, the nematodes were exchanged in an isoamyl acetate series, dried with an HCP-2 critical point dryer (Hitachi) for 7 h, mounted on aluminum stubs, coated with a gold-palladium mixture by an IB-3 ion coater (Eiko), and viewed with a scanning electron microscope (Philips XL30 ESEM) operating at 20 to 30 kV.

Toxin extraction. One of the two strains, C-1, was used to further examine whether there is toxin production for the fungus. Strain C-1 was cultured on PDA (20 liters) at 25°C for 15 days. The cultures were extracted twice with 15 liters MeOH at room temperature. The crude extracts (160 g) were resuspended in 500 ml distilled water and extracted twice with 500 ml ethyl acetate. The organic phase and residues were separately reduced to solid extracts. The extracts were tested for nematicidal activity, and only the ethyl acetate extract showed such a property. The extracts (10 g) containing active compounds were subjected to liquid chromatography on Silica Gel G (200 to 300 μm; column, 55 by 450 mm; Meijing Co. Ltd.). Stepwise elutions with chloroform-methanol (20:1 [vol/vol], 2.5 liters, and 9:1 [vol/vol], 2 liters) were performed. The eluant was collected with 50-ml flasks and combined for thin-layer chromatography (TLC). TLC was carried out using precoated Silica Gel G plates (0.2 mm thick; Meijing Co. Ltd.). Each 20-μl eluant was developed with chloroform-methanol (9:1; vol/vol) under saturated conditions. Chromatograms were detected by the color reaction with 5% sulfuric acid-ethanol spraying solution after heating at 100°C. Subsequently, the combined eluants were tested for nematicidal activity. Active fractions were further separated and purified by a silica column and eluted stepwisely with petroleum ether-acetone (3:1 and 1:1, vol/vol). The eluants were collected with 20-ml flasks. Each eluant was developed with petroleum ether-acetone (3:1, vol/vol) on TLC and was tested for nematicidal activity. We obtained seven compounds with nematicidal activity from *C. comatus*. All purified compounds were subjected to nuclear magnetic resonance (NMR), mass spectrometry (MS), infrared (IR), and UV spectrum analyses for identification and structure elucidation.

Spectrum analysis. IR spectra were measured on a Bio-Rad FTS-135 spectrometer with KBr pellets. UV spectra were obtained on a Shimadzu double-beam 210A spectrometer. Mass spectra were recorded on a VG Autospec-3000 spectrometer. One-dimensional and two-dimensional NMR spectra were run on a Bruker AM-400 and DRX-500 instrument, respectively, with trimethylsilane as an internal standard. TLC was performed on plates precoated with Silica Gel G (Qingdao Marine Chemical Ltd., China).

Nematicidal assay of compounds. Worms of the root-knot nematode *Meloidogyne incognita* were cultivated on tomato plants in the greenhouse at 25°C, and second-stage juveniles were extracted and stored according to the method of Kelly and Bourne (16). The nematode *P. redivivus* was cultured as described above. The nematodes were separated from the culture medium by the Baermann funnel technique (2, 13). The assay for nematicidal activity was carried out as described by Stadler et al. (21).

RESULTS

Immobilization effect on regenerating PDA plate. Regenerating PDA plates allowed for the exposure of spiny balls with sparse hyphae (Fig. 1). We compared the amount of spiny balls yielded on a normal culture plate with that on a regenerating PDA plate by purifying the spiny structures. The results showed that the number of spiny balls from a regenerating PDA plate is less than that from a normal plate (data not shown). However, nematodes were immobilized much faster on a regenerating PDA plate than on a normal culture plate (19). As shown in Table 1, 73.7 and 98.3% of the *P. redivivus* nematodes added were immobilized by the *C. comatus* strain LHA-7 and 75.7 and 98.9% of the nematodes were immobilized by strain C-1 after 15 and 30 min of contact with the strains, respectively. Highly significant differences were observed between the samples treated and the controls ($P < 0.005$). Most of the immobilized nematodes looked complete under an optical microscope.

Immobilization of nematodes by purified spiny balls. Intact purified spiny balls on WA plates exhibited an outstanding capacity to immobilize *P. redivivus*. The nematodes added were restricted to a small area and immobilized the nematodes very quickly (Fig. 2A). For *C. comatus* strain LHA-7, 75.0 and 93.8% of the nematodes added were immobilized by the nonsmashed spiny balls 5 and 10 min after being added, respectively. For strain C-1, 76.9 and 92.3% of the nematodes added were immobilized (Table 2). Highly significant differences existed between the samples treated and the negative controls ($P < 0.005$). Grinding spiny balls with liquid nitrogen can finely destroy the structure of the spiny balls, but no immobilization effect was detected with the smashed spiny balls (data not shown).

Immobilization of nematodes by organic solvent-extracted purified spiny balls. When the spiny balls were in turn ex-

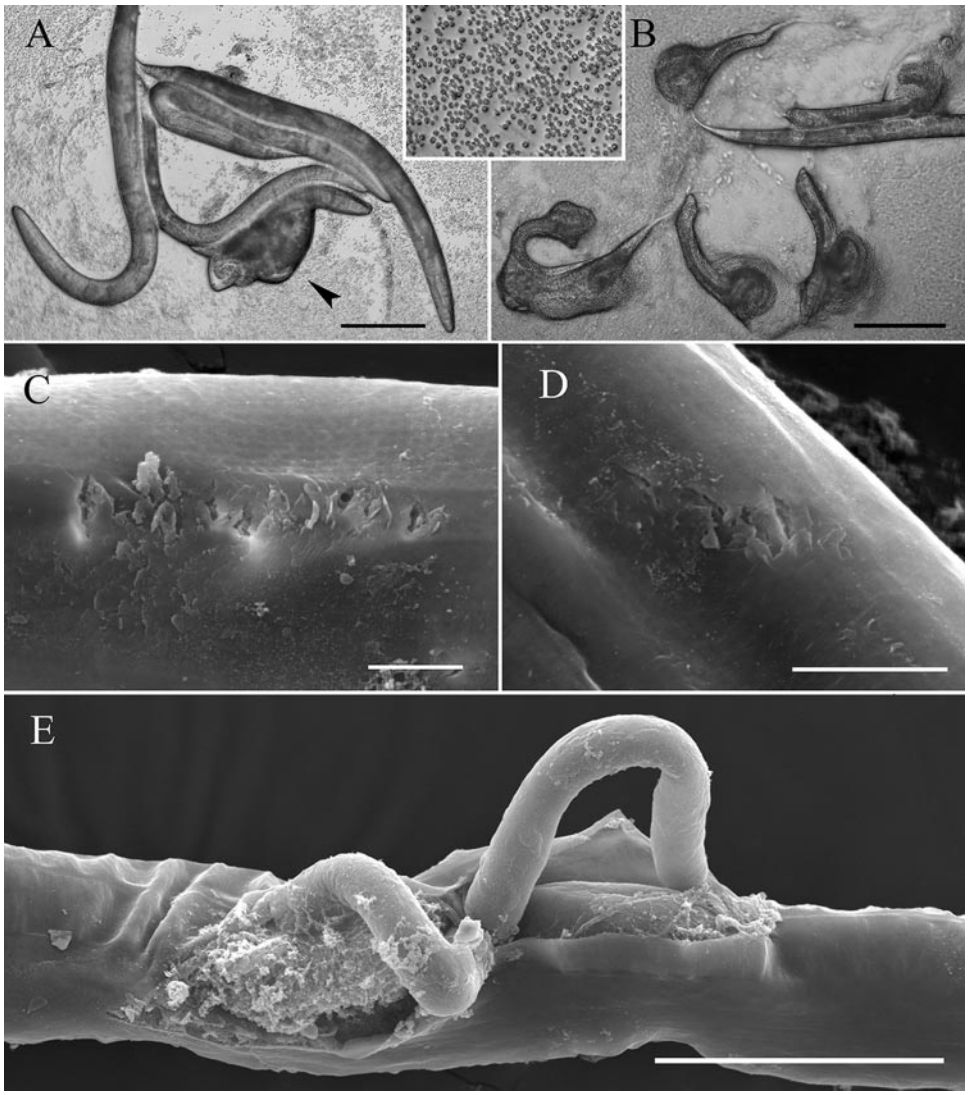

FIG. 2. Immobilization and mechanical injury of *P. redivivus* by purified spiny balls of LHA-7. (A) Nematodes were immobilized and killed by purified spiny balls 5 min after being added on a WA plate, and the inner materials of one nematode leaked out (arrowhead). Bar, 100 μm. (B) Twenty minutes after being added, most of the immobilized nematodes had their inner materials leak out. A closer view of purified spiny balls on the WA plate is shown in the insert. Bar, 100 μm. (C) Mechanical injury was found on the ventral side of a nematode immobilized by purified spiny balls on a WA plate. Bar, 10 μm. (D) Mechanical injury was found on the ventral side of a nematode immobilized by purified spiny balls on a regenerating PDA plate. Bar, 10 μm. (E) Severe breach on a nematode caused by purified spiny balls on a WA plate, with leakage of inner materials and two unborn nematodes. Bar, 50 μm.

tracted with ethanol, acetone, and petroleum ether, the structures maintained their spiny shape with no obvious changes. To determine whether these organic solvent-extracted spiny balls have an immobilization effect on nematodes, we performed a bioassay with WA. As shown in Table 3, the strain LHA-7 immobilized 82.4 and 94.1% of the nematodes and C-1 immobilized 71.4 and 92.9% of the nematodes 5 and 10 min after the nematodes were added, respectively. There are highly signifi-

TABLE 2. Immobilization of *P. redivivus* by purified spiny balls of *C. comatus* strains on WA plates

Strain	Incubation time (min)	No. of nematodes		% Immobilized nematodes	No. of controls		χ^2 value[a]	*P* value
		Immobile	Mobile		Immobile	Mobile		
LHA-7	5	12	4	75.0	0	14	14.5	<0.005
	10	15	1	93.8	0	14	22.6	<0.005
C-1	5	10	3	76.9	1	16	13.1	<0.005
	10	12	1	92.3	1	16	19.0	<0.005

[a] Results of χ^2 tests (1 df) comparing the frequencies of mobilized and immobilized nematodes in treated versus control samples.

TABLE 3. Immobilization of *P. redivivus* by purified spiny balls extracted by organic solvents

Strain	Incubation time (min)	No. of nematodes		% Immobilized nematodes	No. of controls		χ^2 value[a]	*P* value
		Immobile	Mobile		Immobile	Mobile		
LHA-7	5	14	3	82.4	0	16	19.6	<0.005
	10	16	1	94.1	0	16	25.6	<0.005
C-1	5	10	4	71.4	0	15	13.3	<0.005
	10	13	1	92.9	0	15	21.6	<0.005

[a] Results of χ^2 tests (1 df) comparing the frequencies of mobilized and immobilized nematodes in treated versus control samples.

cant differences between the samples treated and the controls ($P < 0.005$). These results were similar to the immobilization efficiency produced by normal purified spiny balls (Table 2). Nevertheless, none of the extracts obtained showed any obvious effects on the tested nematodes.

Mechanical injury of nematodes by spiny balls. Under an optical microscope, most of the immobilized nematodes showed no obvious changes just after they were immobilized. However, within two to four more minutes, a few of them had their inner materials leaked (Fig. 2A). Fifteen to 30 minutes after the nematodes were added, the vast majority of the added nematodes burst, accompanied by the leakage of the inner materials (Fig. 2B). The burst of nematodes happened frequently in the bioassay with purified spiny balls. On regenerating PDA plates, this burst phenomenon could be observed but at a much lower frequency (<10%). In the bioassays with normal culture plates of *C. comatus*, this phenomenon was seldom observed. We found only a few cases during extensive searches.

Similar results were found with scanning electron microscopy studies of the nematodes immobilized on regenerating PDA plates and those immobilized by purified spiny balls. Figure 2C shows that a nematode was immobilized by purified spiny balls and mechanically injured on its ventral side. Nematodes immobilized on regenerating PDA plates also illustrated the presence of mechanical injury on the ventral side of the nematode body (Fig. 2D). Because only purified spiny balls came into contact with the nematodes in the bioassay, these physical damages on nematode cuticles must have been caused by those spiny structures. We previously reported that the projections on the spiny balls have a contracted end (19), and the contraction made the projection very sharp, enabling it to cut into the nematode cuticle. Severe damage of nematode cuticles caused the nematodes to breach and their inner materials to leak out (Fig. 2E).

Spectrum data and structure elucidation of nematotoxins from *C. comatus*. We successfully isolated and identified seven potent compounds against nematodes from strain C-1. The seven compounds are shown in Fig. 1. The structures of these compounds were identified with NMR, IR, UV spectrum, and MS analyses. Among these, compounds 3 and 7 were identified as novel natural products never reported previously.

High-resolution MS experiments of compound 3 suggested that its molecular formula is $C_6H_8O_4$, which was confirmed by ^1H and ^{13}C NMR spectra. The ^{13}C NMR and distortionless enhancement by polarization transfer spectra of compound 3 showed six signals, including one methyl, one methylene, one methine, and three quaternary carbon atoms (one carbonyl carbon). In the ^1H NMR spectrum, 7.12, 4.27, and 1.59 ppm corresponded to methine at C-4, methylene at C-6, and methyl protons at C-7, respectively. The methylene signal at 56.77 ppm was downfield shifted, but it should have been connected to hydroxyl. The IR spectrum exhibited absorptions at 3,381 and 1,765 cm^{-1} due to the hydroxyls and to the unsaturated carbonyl group. Absorptions at 1,631, 1,553, and 1,450 cm^{-1} indicated the presence of a furan ring. Based on the above-described analysis, the structure of compound 3 was identified as 5-hydroxy-3-(hydroxymethyl)-5-methylfuran-2(5*H*)-one.

High-resolution MS experiments with compound 7 suggested that its molecular formula is $C_{10}H_{10}O_5$ (high-resolution electrospray ionization [HRESI]-MS, *m/z*: 233.0432 $[M + Na]^+$, calculated: 233.0425). This structure contains six unsaturated degrees. Four of these could be assigned to a benzene ring, and the NMR data suggested the presence of carbonyl and formyl carbon. The methylene signals downfield shifted at 62.79 ppm should be connected to an oxygen atom. Heteronuclear multiple bond correlation experiments showed that methylene correlated to methyl and carbonyl and that formyl correlated to C-3, C-5, and C-6. Also, the single signals of protons suggested that two methines should be at the *meta* position. The IR spectrum exhibited absorption at 3,440 cm^{-1}, which confirmed the existence of hydroxyls. The structure of compound 7 was identified as 3-formyl-2,5-dihydroxybenzyl acetate.

The spectrum data of the seven isolated compounds from *C. comatus* are as follows. 5-Methylfuran-3-carboxylic acid (compound 1) $C_6H_6O_3$, colorless crystal; UV (MeOH)λ_{max} (ε): 204 nm (8,945); IR (KBr): 3,448, 2,919, 2,850, 1,752, 1,710, 1,631, 1,552, 1,462, 1,339, 1,111, 1,041 cm^{-1}. FAB^- MS *m/z*: 125 $[M - H]^-$; ^1H NMR (500 MHz, $CDCl_3$)δ: 7.93 (s, H-2), 6.33 (s, H-4), 2.29 (s, H-7); ^{13}C NMR (125 MHz, $CDCl_3$)δ: 147.56 (d, C-2), 119.72 (s, C-3), 105.55 (d, C-4), 154.06 (s, C-5), 168.41 (s, C-6), 13.35 (q, C-7).

5-Hydroxy-3,5-dimethylfuran-2(5*H*)-one (compound 2) $C_6H_8O_3$, colorless crystal; $[\alpha]_D^{28}$: −16.67° (c 0.06, MeOH); UV (MeOH)λ_{max} (ε): 204 nm (8,317); IR (KBr): 3,440, 2,929, 1,766, 1,747, 1,629, 1,447, 1,404, 1,374, 1,188, 1,051, 929, 875, 762 cm^{-1}; FAB^- MS (*m/z*: 127 $[M - H]^-$); ^1H NMR (500 MHz, $CDCl_3$)δ: 6.79 (s, H-4), 1.85 (s, H-6), 1.62 (s, H-7); ^{13}C NMR (125 MHz, $CDCl_3$)δ: 171.74 (s, C-2), 131.71 (s, C-3), 147.64 (d, C-4), 104.19 (s, C-5), 10.34 (q, C-6), 24.71 (q, C-7).

5-Hydroxy-3-(hydroxymethyl)-5-methylfuran-2(5*H*)-one (compound 3) $C_6H_8O_4$, colorless crystal; $[\alpha]_D^{28}$: −0.75° (0.00669 g/ml); UV (MeOH)λ_{max} (ε): 226 nm (11,594); IR (KBr): 3,381, 2,995, 2,939, 1,765, 1,631, 1,553, 1,450, 1,407, 1,378, 1,348, 1,271, 1,223, 1,180, 1,113, 1,066, 1,022, 927, 874, 797, 759,cm^{-1};

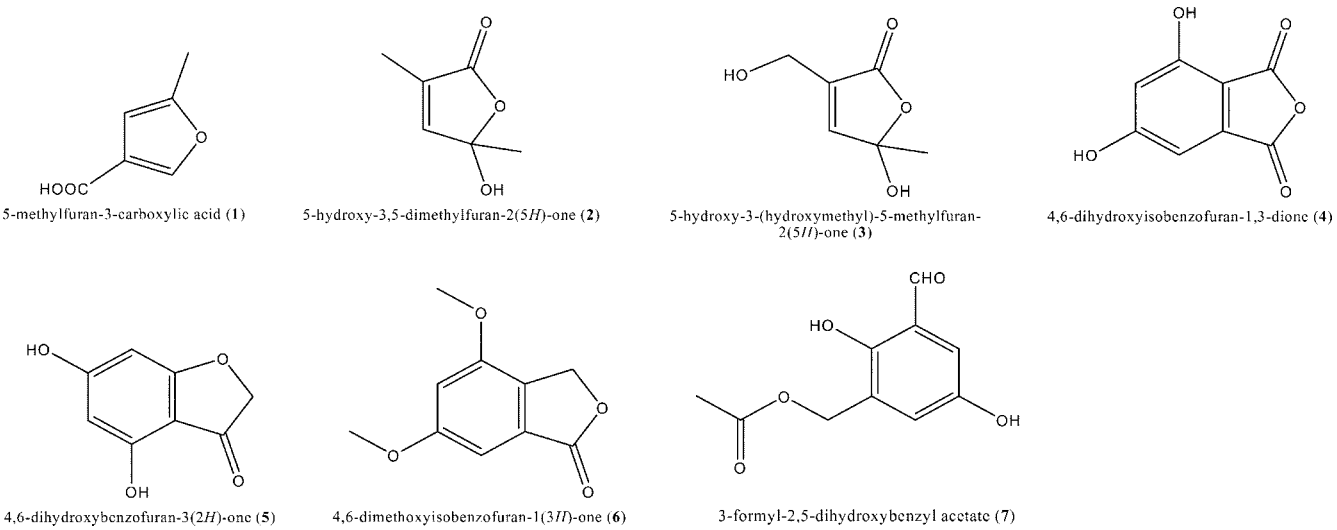

FIG. 3. Nematicidal compounds isolated from *C. comatus*.

HRESI-MS (*m/z*: 143.0344 $[M - H]^-$, calculated: 143.0344); FAB$^-$ MS (*m/z*: 143 $[M - H]^-$); ^{1}H NMR (500 MHz, $CDCl_3$)δ: 7.12 (s, H-4), 4.27 (s, H-6), 1.59 (s, H-7); ^{13}C NMR (125 MHz, $CDCl_3$)δ: 172.07 (s, C-2), 136.42 (s, C-3), 149.96 (d, C-4), 106.89 (s, C-5), 56.77 (t, C-6), 24.86 (q, C-7).

4,6-Dihydroxyisobenzofuran-1,3-dione (compound 4) $C_8H_4O_5$, yellow powder; UV (MeOH)λ_{max} (ε): 206 nm (14,424); IR (KBr): 3,441, 2,925, 2,854, 1,751, 1,717, 1,622, 1,470, 1,421, 1,342, 1,169, 1,062, 1,012, 860, 735 cm^{-1}; high-resolution atmospheric-pressure photo ionization-MS (*m/z*: 181.0132 $[M + H]^+$, calculated: 181.0136); impact ionization-MS (70 eV) (*m/z*: 180 $[M]^+$); ^{1}H NMR (500 MHz, CD_3OD)δ: 6.47 (s, H-5), 6.69 (s, H-7); ^{13}C NMR (125 MHz, CD_3OD)δ: 170.54 (s, C-1, C-3), 166.45 (s, C-6), 159.36 (s, C-4), 137.91 (s, C-3a, C-7a), 108.54 (d, C-7), 104.30 (d, C-5).

4,6-Dihydroxybenzofuran-3(2*H*)-one (compound 5) $C_8H_6O_4$, yellow powder; UV (MeOH)λ_{max} (ε): 216 nm (5,193); IR (KBr): 3,359, 3,217, 2,926, 1,719, 1,616, 1,486, 1,455, 1,368, 1,351, 1,214, 1,166, 1,052, 1,013, 977, 861, 780 cm^{-1}; HRESI-MS$^-$ (*m/z*: 165.0185$[M - H]^-$, calculated: 165.0187); FAB$^-$ MS (*m/z*: 165 $[M - H]^-$); ^{1}H NMR (500 MHz, CD_3OD)δ: 6.86 (s, H-7), 6.68 (s, H-5), 5.16 (s, H-2); ^{13}C NMR (125 MHz, CD_3OD)δ: 69.05 (t, C-2), 170.36 (s, C-3), 104.60 (s, C-3a), 151.72 (s, C-4), 104.06 (d, C-5), 159.85 (s, C-6), 101.35 (d, C-7), 166.75 (s, C-7a).

4,6-Dimethoxyisobenzofuran-1(3*H*)-one (compound 6) $C_{10}H_{10}O_4$, white crystal; UV (MeOH)λ_{max} (ε): 207 nm (5,178); IR (KBr): 3,435, 3,088, 3,008, 2,969, 2,883, 2,843, 1,753, 1,614, 1,506, 1,470, 1,338, 1,244, 1,215, 1,110, 1,040, 997, 848 cm^{-1}; impact ionization-MS (70 eV) (*m/z*: 194 $[M]^+$) (36), 166 (10), 165 (100), 137 (24), 122 (14), 107 (6), 77 (3); ^{1}H NMR (500 MHz, $CDCl_3$)δ: 6.91 (d, J = 1.69 Hz, H-7), 6.66 (d, J = 1.68 Hz, H-5), 5.18 (s, H-3), 3.84 (s, H_3-8), 3.85 (s, H_3-9); ^{13}C NMR (125 MHz, $CDCl_3$)δ: 171.25 (s, C-1), 68.04 (t, C-3), 154.91 (s, C-4), 104.81 (d, C-5), 162.57 (s, C-6), 98.65 (d, C-7), 128.27 (s, C-3a), 128.01 (s, C-7a), 55.95 (q, C-8), 55.64 (q, C-9).

3-Formyl-2,5-dihydroxybenzyl acetate (compound 7) $C_{10}H_{10}O_5$, colorless crystal; UV (MeOH)λ_{max} (ε): 209 nm (3,299); IR (KBr): 3,440, 2,926, 2,857, 1,739, 1,629, 1,478, 1,456, 1,337, 1,345, 1,304, 1,235, 1,167, 1,069, 1,024, 857, 840, 524 cm^{-1}; HRESI-MS$^+$ (*m/z*: 233.0432 $[M + Na]^+$, calculated: 233.0425); FAB$^-$ MS (*m/z*: 209 $[M - H]^-$); ^{1}H NMR (500 MHz, $CDCl_3$)δ: 10.05 (s, CHO), 6.47 (s, H-6), 6.36 (s, H-4), 5.29 (s, CH_2), 2.10 (s, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$)δ: 192.24 (d, CHO), 170.68 (s, C═O), 165.28 (s, C-5), 166.08 (s, C-2), 140.73 (s, C-1), 111.71 (s, C-3), 111.16 (d, C-6), 103.05 (d, C-4), 62.79 (t, CH_2), 20.77 (q, CH_3).

The structures of all the isolated metabolites are shown in Fig. 3.

Nematicidal properties of the isolated metabolites from *C. comatus*. The nematicidal assay showed that compounds 1 and 2 were the most nematotoxic of the seven, with 90% lethal dose (LD_{90}) values of 200 μg/ml against both *M. incognita* and *P. redivivus* (Table 4). The other compounds isolated from *C. comatus* also displayed nematicidal activity but at slightly higher doses (400 to 800 μg/ml) (Table 4).

DISCUSSION

Wood decay mushrooms help to recycle organic components of dead trees, and these fungi are crucial to a forest's survival. The development of the ability to take advantage of microfauna can aid some mushrooms (including wood decay mushrooms) in obtaining a nutritional supplement, especially nitrogen, which is usually the limiting factor for growth in their natural habitats (19, 22, 23, 24). Some mushrooms have even

TABLE 4. Nematicidal activities of isolated compounds[a]

Compound	*M. incognita*		*P. redivivus*	
	LD_{50}	LD_{90}	LD_{50}	LD_{90}
1	100	200	100	200
2	100	200	100	200
3	200	400	200	400
4	>200	400	>200	>400
5	>200	>400	>200	400
6	>200	>400	>200	400
7	>200	>400	400	800

[a] Values are reported in μg/ml.

developed the ability to destroy bacteria in order to obtain such nutrients (25).

C. comatus has been shown to have the ability to destroy nematodes (19). In this paper, we showed that *C. comatus* can immobilize *P. redivivus* by two strategies. First, with the spiny ball structures, *C. comatus* can damage the cuticle of the nematode with the mechanical force provided by its sharp projections. Second, this fungus can also secret potent toxins to immobilize and kill nematodes within hours. The combined mechanisms are different from the typical patterns of attacking nematodes with sticking materials and killing them with potent toxins. However, the mechanical part is somewhat similar to another nematode-attacking device, the acanthocyte, in *Stropharia* species. Both spiny balls and acanthocytes can immobilize and kill nematodes with mechanical forces. However, with long projections (acanthae), acanthocytes can pierce nematode bodies and cause more-serious damage than spiny balls. The spiny ball on *C. comatus* is a much smaller structure, and it is unlikely to be capable of penetrating nematode cuticles only by its projections. However, with numerous contracted projections on many spiny balls, *C. comatus* can cause many small cuts on the cuticle, and an accumulation of the small cuts can lead to severe damage of the cuticle, which could then cause the leakage of the inner materials of the nematode. Nematodes are organisms with high internal turgor pressures. Such pressures could be a driving force for nematode burst and the leakage of inner materials.

The bioassay with regenerating PDA plates showed a much higher killing efficacy against nematodes than that on a normal culture plate of *C. comatus*. The regenerating PDA plate method provides a satisfying production of spiny balls with sparse aerial hyphae. Once the nematodes touched a few of these spiny structures, they could be quickly immobilized. Initially, they may struggle and draw back when touching the structures. The situation became dramatic when the bioassay was conducted with purified spiny balls on WA plates. The nematodes added were severely hurt and immobilized very quickly by the purified structure, and most of the immobilized nematodes burst with serious leakage of their inner contents. These results strongly support the spiny ball as a nematode-attacking device.

We recently found that the presence of excessive water in all the bioassays we performed with the culture and purified spiny balls of *C. comatus* could significantly reduce the immobilization effect on nematodes. If added water allowed the nematodes to float and swim, they could survive for a very long time (more than 7 days) (our unpublished data). This phenomenon puzzled us for some time because it cannot be the characteristic of a toxin. We once considered the spiny ball a toxin reservoir, but the burst of nematodes and the leakage of the inner material made us reevaluate the hypothesis. Interestingly, we ground some purified spiny balls and found that their ability to immobilize nematodes dropped dramatically. Therefore, we further designed a bioassay with spiny balls ground with liquid nitrogen and a complete loss of activity was observed. This result indicated that the shape and the structure of spiny balls were indispensable for their function as a nematode-attacking device. Moreover, we extracted purified spiny balls with organic solvents, and the extracts showed no immobilization effects on nematodes whereas no reduction in killing activity was detected with the organic solvent-extracted spiny balls. Taken together, the results suggest that the spiny ball is not a toxin reservoir.

Most of the mechanical injury was found on the ventral side and at the middle part of the nematodes. Coincidentally, severe leakage of the inner materials also happened at the middle part of the immobilized nematodes. Our explanation for these observations is that the middle part of a nematode bears the major weight of the body. Thus, the middle part comes into tighter contact with the spiny balls than the other parts of the nematode and therefore is more easily injured.

We reported in our previous article that the immobilized nematodes, when put into a drop of water, showed a response similar to that of nematodes attacked by a toxin produced by *Pleurotus* (19). We therefore thought that a toxin(s) might be involved in the process. But, with further studies, we found that these immobilized nematodes cannot recover in water probably because of the mechanical injury on their cuticle. The nematode body has a high internal turgor pressure, a pressure (16 to 225 mm Hg) generally higher than what is seen in most other invertebrates. When the nematode cuticle is ruptured, the internal body contents could be ejected through the wound due to this high internal pressure. According to our unpublished data, the immobilized nematodes did recover on PDA or some other nutrient media or in hypertonic solutions as long as no obvious leakage had occurred.

On the one hand, we demonstrated that spiny balls could immobilize nematodes through mechanical forces and therefore were considered to be a nematode-attacking device. On the other hand, the remarkable efficiency displayed by *C. comatus* in immobilizing nematodes may suggest the involvement of low-molecular-weight toxins produced by the fungus. We succeeded in isolating and characterizing seven nematotoxic metabolites from *C. comatus*. The structures of these toxins were identified with NMR, MS, IR, and UV spectrum analyses, and structures 3 and 7 were identified as novel compounds. These compounds suggest that the immobilization process could be more complex than we originally expected. These compounds probably contribute to the rapid immobilization of nematodes. The combination of the mechanical force from spiny balls and the toxins produced by the fungus could be more efficient in immobilizing nematodes than mechanical force or toxins alone. When nematodes are injured by projections of spiny balls, potent toxins may enter into the nematode body directly through the wounds and act more efficiently, which could speed up the immobilization process.

The compounds isolated from *C. comatus* are O-containing heterocyclic compounds, analogs of furanone and its derivates. Compounds 1 and 2 isolated from *C. comatus* were the most effective, with LD_{90}s of 200 ppm against both *M. incognita* and *P. redivivus*. In previous works that aimed to identify nematicidal metabolites, 13 furan and furanone derivates have been found to have nematicidal activity from fungi, and the best activity was 60 ppm (LD_{90}) (6, 17). The identification of novel nematotoxic compounds could be of interest for controlling the harmful effects of nematodes on crops and animals.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (no. 30470067 and 30230020) and the Science and Technology Department of Yunnan Province (2005NG05).

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