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FULL PAPER

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Characterization of the nematocidal toxocyst in *Pleurotus* subgen. *Coremiopleurotus*

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Abstract Toxocysts of the genus *Pleurotus* are blastoconidia-like ovoid structures surrounded by a liquid droplet containing a toxin that paralyzes nematodes. This study investigated toxocyst development using a strain S396 of *Pleurotus cystidiosus* subsp. *abalonus* (subgen. *Coremiopleurotus*). The surface of the liquid droplet was found to be an elastic envelope. When a nematode touches the toxocyst, the envelope adheres to the worm and bursts. Toxocysts are induced simultaneously with coremia formation in the absence of nematodes and developed only from aerial hyphae in which nuclear division had ceased. In the early stage of toxocyst development, liquid springs repeatedly from the tip of the sterigma-like stipe before ovoid (blastoconidium-like structure) formation. A certain substance in the liquid might polymerize to form the envelope while the ovoid simultaneously budded in the droplet. The nucleus tends to locate near the toxocyst, especially in early stage of toxocyst development. Each dikaryotic cell predominantly formed one or two toxocyst(s) while each monokaryotic cell predominantly formed one. In rare cases, a nucleus existed in the toxocyst, suggesting the possibility that the toxocyst is a vestigial blastoconidium.

Key words Adhesive knob · Blastoconidium · *Coremiopleurotus* · Nematode · Toxocyst

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Introduction

The toxocyst is one of the nematocidal structures of nematophagous fungi. It is a blastoconidium-like ovoid knob surrounded by a liquid droplet containing a toxin that attacks nematodes. The use of nematode capture for nutrient supplementation has been previously reported for several species of fungi (Drechsler 1937; Pramer 1964; Barron 1977; Thorn and Barron 1984; Saikawa 1985; Saikawa and Wada 1986; Barron and Thorn 1987; Nakagiri and Ito 1996; Gomes et al. 2001; Nordbring-Hertz et al. 2002; Barron 2003). Among them, the anamorphic fungi *Arthrobotrys* spp. and *Nematoctonus* spp. are probably the most well known predators of nematodes (Barron 1977, 2003; Thorn and Barron 1984; Gomes et al. 2001). Nematophagous fungi form trapping structures, such as adhesive nets, rings, or adhesive knobs, at intervals along the length of the hyphae under nitrogen-deficient conditions. Certain substances released by the nematodes have been also implicated as inducers of these structures (Nordbring-Hertz 1973; Barron 1977; Thorn and Barron 1984; Barron 2003).

Clémençon (2004) proposed the term "toxocyst" for the nematocidal structure of *Pleurotus* (Fr.) P. Kumm. Toxocysts were initially described as adhesive knobs (Thorn and Barron 1984; Saikawa and Wada 1986; Barron and Thorn 1987). However, their adhesion to nematodes is not so tight as that of the adhesive knobs of *Nematoctonus* Drechsler. *Pleurotus ostreatus* (Jacq.) P. Kumm. structures trap only 30%–50% of the nematodes touching them (Saikawa and Wada 1986). Instead, they immobilize nematodes within

30 s with a toxin stored in the droplets (Thorn and Barron 1984; Saikawa and Wada 1986; Barron and Thorn 1987; Stalpers et al. 1991). The victim, paralyzed but not captured by the structure, is invaded by specialized directional hyphae into its body orifices and digested (Thorn and Barron 1984; Saikawa and Wada 1986; Barron and Thorn 1987). This mode of infection is also different from that of the adhesive knob, from which infectious hyphae protrude into the nematode through the adherent epidermis. The toxin attacks not only nematodes but also other microfauna such as mites, whereas the adhesive knobs are nematode specific (Barron 2003). The nematotoxic compound of *P. ostreatus* was identified as *trans*-2-decenedioic acid, which irreversibly paralyzes nematodes (Kwok et al. 1992). This dicarboxylic acid, as well as the corresponding monocarboxylic acid and some medium-chain fatty acids, is toxic not only to nematodes but also to insects and fungi, and is thought to destroy nerve and muscle function in nematodes by affecting membrane permeability (Kwok et al. 1992).

Among the genus *Pleurotus*, the subgenus *Coremiopleurotus* shows a unique asexual proliferation by producing a tremendous amount of dikaryotic arthroconidia through the formation of coremia (Truong et al. 2006). The toxocysts of *Coremiopleurotus* or its anamorph *Antromycopsis* Pat. & Trab. have been described as slime-covered structures or adhesive knobs (Thorn and Barron 1984; Barron and Thorn 1987; Barron 2003), or as blastoconidia, simply based on their morphology (Han et al. 1974; Moore 1977; Nair and Kaul 1980; Hilber 1982; Stalpers et al. 1991; Reid et al. 1998; Capelari 1999). All the blastoconidia-like structures observed in the *Coremiopleurotus* are toxocysts. Clémençon (2004) also described the toxocyst as a cell. The possibility has not been excluded that the toxocysts are also blastoconidia. Although many studies have demonstrated the nematocidal activities of fungi, development of the toxocyst (e.g., how it is surrounded by the toxin-containing droplet) is poorly understood. This article reports on the action, structure, and developmental process of toxocysts in the *Coremiopleurotus*, *Pleurotus cystidiosus* subsp. *abalonus* (Y.H. Han, K.M. Chen & S. Cheng) O. Hilber.

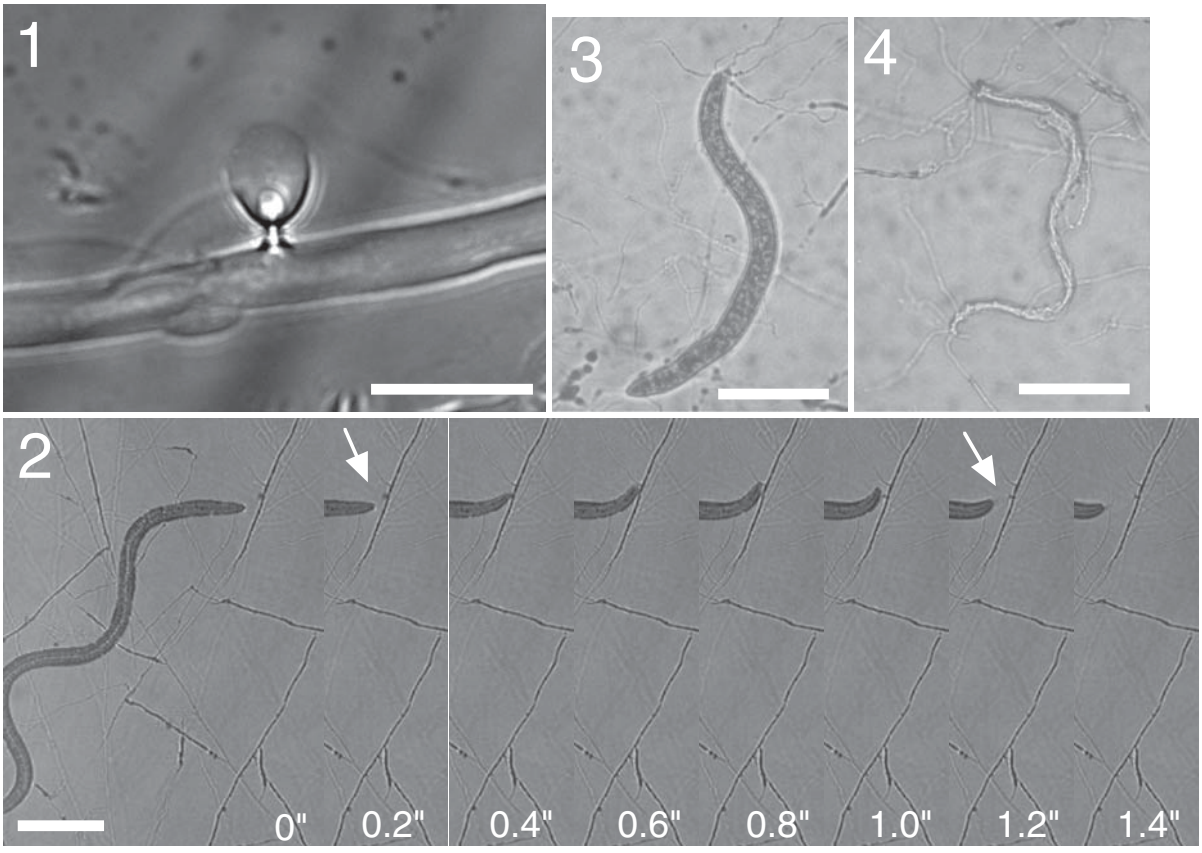
Materials and methods

Pleurotus cystidiosus subsp. *abalonus* (S396) and its monokaryotic derivatives have been described previously (Truong et al. 2006). Mycelia were cultivated on potato dextrose agar (PDA) (Difco), water agar (WA), and yeast extract peptone dextrose agar (YPDA). The pinewood nematode (*Bursaphelenchus xylophilus*) was maintained as described previously (Iwahori and Futai 1993). Plates with mycelium and nematodes were observed directly under an inverted microscope (Zeiss Axiovert 25) with a LD ACHROPLAN 20×/0.4 PH2 (D = 0.6–1.2) objective lens. A short movie of a nematode touching the toxocyst was taken with a Nikon 4500 digital camera connected to a light microscope. Safelight in the darkroom used for inoculating mycelia on agar media was a Toshiba 20 W bulb with KING Safe light glass for gaslight paper no. 3, the standard condition for handling X-ray film. For toxocyst development, an agar piece was laid on a 35-mm glass-bottomed culture dish with an uncoated no. 0 coverslip (MatTek, Ashland, MA, USA). Hyphae were fixed with 70% ethanol and stained with 1 µg/ml of 4′,6-diamidino-2-phenylindole (DAPI), and/or 10 µg/ml of calcofluor white M2R. The Delta Vision System (Applied Precision, Issaquah, WA, USA) that consists of the sectioning fluorescence inverted microscope and the associated software were used to obtain digital images of fixed cells and for time-lapse recordings of living cells. An Olympus 60×/1.40 Plan Apo or Olympus 100×/1.3 DApo objective lens was used to view fixed cells, and a Zeiss Ph2 Plan-NEOFLUAR 40×/0.75 objective lens was used for the time-lapse sequences. For scanning electron microscope observation, fresh hyphae with toxocysts were laid in a 10-cm Petri dish with a drop of 2% aqueous solution of osmium tetraoxide (Wako Pure Chemical) for 10 h at room temperature to be fixed with osmium tetraoxide vapor, coated with platinum-palladium in an ion sputterer (Hitachi E-1030; Hitachi, Tokyo, Japan), and observed with a Hitachi S-800 at 15.0 kV.

Results and discussion

Nematocidal property of *Pleurotus cystidiosus* subsp. abalonus S396

The nematocidal activity of toxocyst has never been fully described for the subgenus *Coremiopleurotus* up to the present time. *Pleurotus cystidiosus* subsp. *abalonus* S396 (subgen. *Coremiopleurotus*) forms many toxocysts along the aerial hyphae of the mycelium or on the coremium surface. The mature toxocysts consisted of a fine sterigma-like stipe (1.5–3 µm in length) protruding from aerial hyphae and an ovoid knob-like structure (up to 2.5–3.5 µm in width) surrounded by a liquid-producing droplet (up to 6 µm in diameter) (Fig. 1). When the nematodes were placed on the mycelia, less than 10% of nematodes added were trapped directly by adhesion to the structure, but most of them were paralyzed after touching it, usually with their oral tip. The nematodes moved back instantly (Fig. 2). Nevertheless, paralysis began spreading from their tails, usually completely immobilizing the worms within 50–60 s. When paralyzed nematodes were rescued into freshwater within 10 min of touching the toxocysts, most remained alive for a few days but showed no significant recovery from the paralysis. We observed infection of nematodes by specialized directional hyphae into the bodily orifices of the worm when the mycelia were nutritionally starved on WA (Figs. 3, 4). In contrast, on the rich medium, PDA, nematodes were paralyzed but not invaded by hyphae although the toxocysts of this species were also induced on the medium. In the latter case, the paralyzed worms lived for a long period of time. These observations suggest that the attractant from worm orifices that stimulates the hyphal invasion is nutrient rich and that the nematodes were killed not by the toxin but by the hyphal invasion. This observation is consistent with the



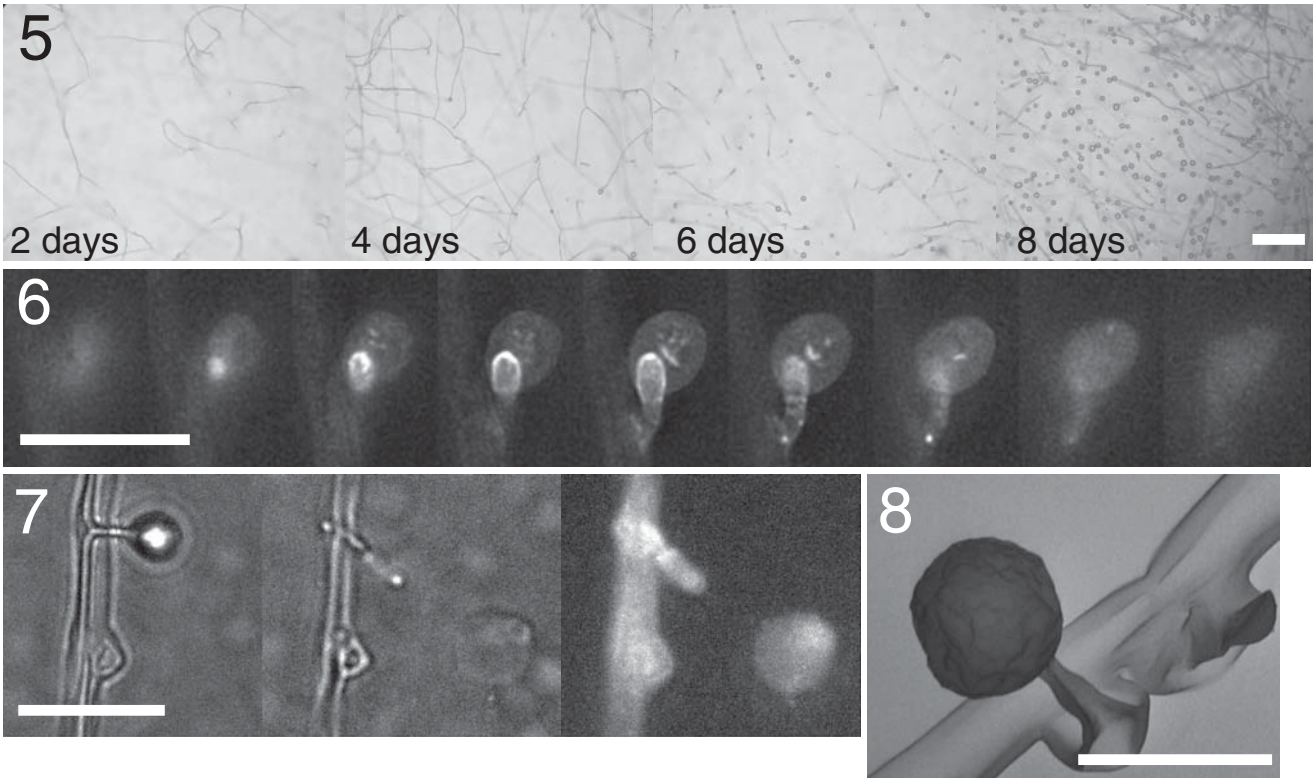
Figs. 1–4. Toxocyst of *Pleurotus cystidiosus* subsp. *abalonus* and its nematocidal activity. **1** Toxocyst formed on mycelium. **2** Time-lapse recordings of the nematode touching a toxocyst with its oral tip. The toxocyst balloon disappeared after the nematode touched it (*arrows*). **3** Early stage of hyphal invasion into the nematode on water agar (WA). **4** Terminal stage of infection. *Bars* **1** 10 μm; **2–4** 100 μm

idea that the role of toxocyst is not only to obtain nutrients from nematodes but also to defend against fungivorous nematodes or other microfauna (Barron 2003).

Environmental conditions for toxocyst formation

In contrast to the other nematode-trapping structures such as hyphal rings and networks (Drechsler 1937; Nordbring-Hertz 1973; Barron 1977; Saikawa 1985; Nakagiri and Ito 1996), the toxocysts of *P. cystidiosus* subsp. *abalonus* S396 develop well along the aerial hyphae and on the coremial surface of mycelia grown on the rich medium, PDA, in the absence of nematodes. Moreover, light is important for the toxocyst formation. On PDA plates, toxocyst formation is synchronized with the formation of coremia, which is also induced by light irradiation (Truong et al. 2006). Toxocysts were abundantly formed on the surface of the coremial stipe and on mycelia near the coremia (within about a 2-mm radius from the coremium base). On the other hand, neither toxocysts nor coremia were formed in the dark on PDA-grown mycelia even after adding nematodes, although stimulation of toxocyst induction by nutritional starvation was evident. Toxocysts were formed in the dark on WA along the hyphae that originated in the mycelium developed from the inoculated piece of PDA with the fungus culture. In this condition, coremia developed on the PDA piece, even when the master mycelium had been manipulated and maintained under darkroom safelights. Thus, the toxocysts were not formed on WA without the tiny piece of PDA, suggesting that a certain amount of nutrients is still necessary for toxocyst formation during starvation. Conversely, even in the presence of nematodes, neither toxocysts nor coremia were induced on nitrogen-rich YPDA medium on which the aerial hyphae filled up the entire space in the Petri dish.

Toxocysts developed only from the aerial part of hyphae, as was clearly seen by the hyphae that alternatively sank into and reappeared on the surface of the WA plate. Toxocysts were absent in the frontal zone of growing mycelia on all media used. On WA, the toxocyst-positive zone expanded behind the mycelial growth edge at least after 4 days (Fig. 5). These data suggested that only aerial hyphae in which cell division had ceased had the ability to form toxocysts. However, cells that formed a toxocyst did not lose their potential to divide. When inoculated onto a new medium, they restarted cell division although they showed a longer lag phase than the neighboring toxocyst-negative cells (data not shown). The toxocysts were also induced by blocking the mycelial growth by cutting the mycelia with a blade. Taking advantage of this property, the toxocysts were allowed to develop on aerial hyphae that extended from the



Figs. 5–8. Toxocyst formation on aged but not young hyphae. **5** Mycelium growing on WA was photographed from the growth edge to the center. Approximate days after the time that the photographed area had been the growth edge of mycelium are indicated. **6** Serial sectional view of the toxocyst stained with calcofluor white. Serial sectional images deconvolved using the Delta vision system are aligned. **7** Removal of the toxocyst balloon. *Left:* the toxocyst before balloon removal. *Center:* the toxocysts after balloon removal. The balloon envelope remains near the toxocyst. *Right:* view of the same sample with the intrinsic red fluorescence. **8** Scanning electron microscopic view of an osmium-stained toxocyst. *Bars* **5** 100 μm; **6–8** 10 μm

cut edge of the agar close to the coverslip to facilitate time-lapse videomicroscopy. The time-lapse recordings also showed that toxocysts developed only when hyphae had ceased growth (for example, compare Figs. 9, 10).

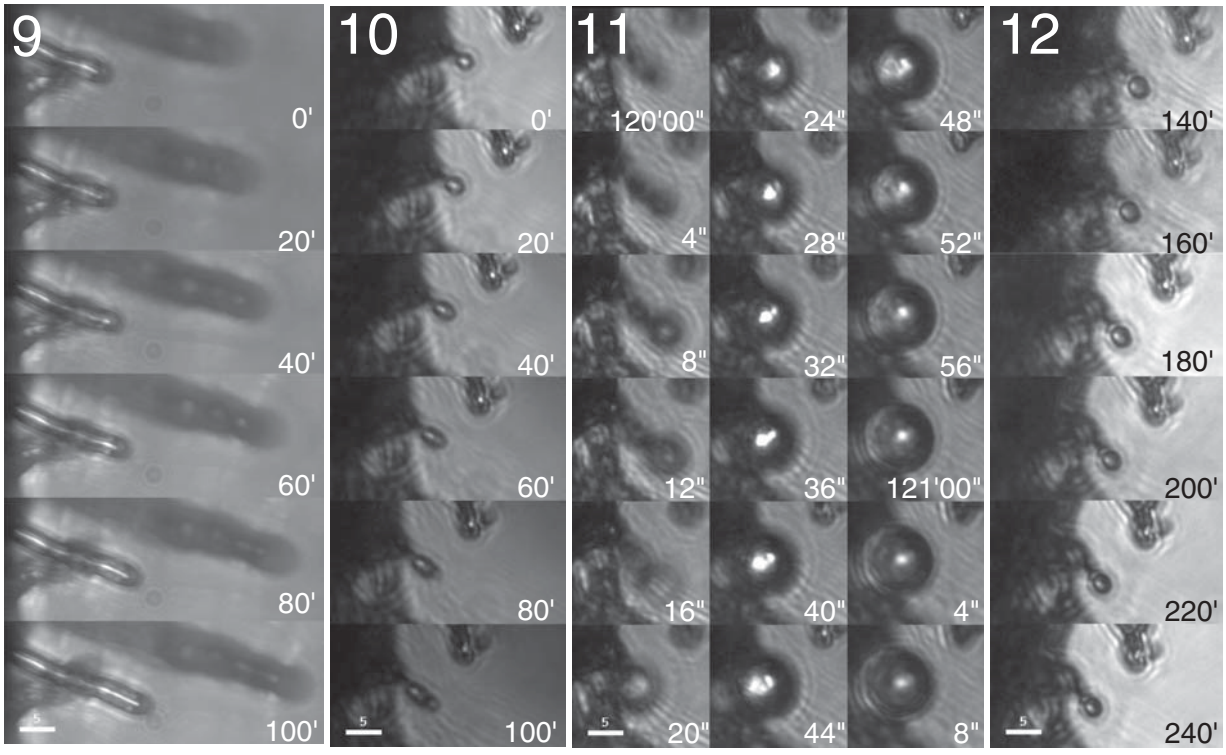
Toxocyst structure

By placing the hyphae in water in the microscopic preparation, the droplets of toxocysts burst from hypoosmolarity. The ovoids became naked and were not surrounded by slime material. In addition, the droplets were found to shrink during periods of dehydration, and upon rehydration, they enlarged again. Therefore, the surface of the droplet appears to be an elastic envelope containing an aqueous solution. Thus, hereafter we designate the liquid droplet covered with the envelope as a toxocyst balloon.

The balloon envelope was removed by dimethylsulfoxide (data not shown) but could be found intact after fixation with ethanol. To clarify the topology of the balloon and the toxocyst, their cell surfaces were stained with calcofluor white after ethanol fixation. Deconvolved images encompassing whole focal planes of the structure showed that the balloon envelope enclosed the ovoid entirely (Fig. 6). The liquid containing the toxin should be secreted into the space surrounded by the balloon envelope. To understand how toxocyst balloons disappear after the touch of a nematode (see Fig. 2), we broke the balloon with a human hair by freehand manipulation under the inverted microscope. The balloon was stripped from the other part of the toxocyst and left as a flat membrane (Fig. 7). Therefore, we presume that the balloon had burst and detached from the mycelium upon being touched by the nematode. The balloon appeared adhesive, because some nematodes adhered to the toxocyst. If so, the balloon detaches from the mycelium but adheres to the nematode cuticle. We speculate that the elastic balloon envelope is made of a certain latex-like substance secreted from the toxocyst based on the following facts. Latex is also present in fungi. The mushroom species *Lactarius volemus* produces polyisoprene rubber with a molecular weight of more than 10^4 daltons in its fruit body (Ohya et al. 1998). Polyisoprene rubber is known to be susceptive to OsO_4 staining (Subramaniam et al. 2004). Dark staining of the toxocyst envelope with OsO_4 (Fig. 8) supports this idea.

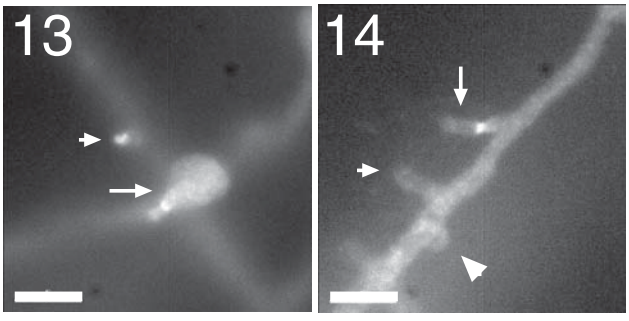
Process of toxocyst formation

The ovoid in the balloon varied in size, and some toxocysts did not contain the ovoid inside the balloon. Liquid was suddenly secreted as a droplet at the tip of the sterigma



Figs. 9–12. Time-lapse recordings of liquid secretion from the toxocyst. Toxocysts were induced along the cut edge of a piece of potato dextrose agar (PDA) laid on a coverslip. Time-lapse recordings were taken at 20-min intervals. At each time point, 20 photographs were taken, 1 for each change of focus at 1-μm intervals. **9** Hyphal growth continued in the early state. No toxocyst was observed on the growing hyphae. **10** Toxocysts began to develop after cessation of hyphal growth (**9** and **10** show different areas but with the same time scale). **11** During focal scanning of the toxocyst shown in **10**, a liquid droplet suddenly formed at the toxocyst. **12** The liquid droplet had disappeared by the next time point. *Bars* 5 μm

and then suddenly disappeared (Figs. 11, 12). This action occurred repeatedly before and after the ovoid formation. Therefore, liquid secretion might be a process involved in the balloon formation, which proceeds simultaneously with enlargement of the ovoid. Intrinsic fluorescence was observed at the tip of the sterigma or at the neck of the ovoid (Figs. 13, 14). The fluorescence corresponded to the sealed-edge portion of the balloon envelope. Although we do not know exactly what kind of chemical is responsible for the fluorescence, its presence suggests that the area is in accord with the portion active for the secretion of the fluorescence substance, the balloon envelope precursor, and the toxin. The toxocyst fluorescence was seen with all the filter sets for DAPI, fluorescein isothiocyanate (FITC), Cy3, and Cy5 (data not shown). The fluorescence marked the position of the toxocysts most brilliantly when observed with the Cy3 filter set. The fluorescence was used to identify the position of early-stage toxocyst formation (Fig. 13). This technical advantage made it possible to examine the development of the toxocyst (see next paragraph). The subgenus *Coremiopleurotus* is known to secrete liquid from the coremial apex as coremioliquid. The liquid secreted from the toxocyst is distinct from the coremioliquid, because the nematodes placed in coremioliquid were not paralyzed (data not shown). We have shown that the transport of the coremioliquid in the hyphae can be blocked by the microtubule depolymerization agent thiabendazole (Truong et al. 2006). The liquid droplet at the tip of the toxocyst sterigma might be transported by a similar mechanism, but we did not verify this possibility because of technical difficulty.



Figs. 13, 14. Intrinsic red fluorescence of toxocysts. Red fluorescence observed with 541–569 nm excitation and 581- to 653-nm emission filters specifically marks toxocysts. **13** Mature toxocyst (*long arrow*). Strong fluorescence exists at the neck. The shape of the ovoid is faintly distinguishable. Early stage of toxocyst formation (*short arrow*). Strong fluorescence is seen at the tip of the sterigma-like stipe. **14** Mature toxocyst from which the balloon has been stripped (*long arrow*). Strong fluorescence is present at the neck of the ovoid. The fluorescence clearly distinguishes the toxocyst from the short branch (*short arrow*) and the clamp connection (*large arrowhead*). *Bars* 5 μm

Control of toxocyst number by the nucleus

Each dikaryotic cell predominantly formed one or two toxocyst(s) whereas each monokaryotic cell predominantly formed one (Table 1). The position of toxocyst emergence was biased toward the apex of the hyphal tip cell, especially for hyphae under coremia, but was random in medial cells (Fig. 15). We noticed that nuclei tend to locate near the toxocyst. To evaluate this possibility, dikaryotic cells forming only one toxocyst in the mycelial area near the cut edge of the agar were used to obtain the statistical data. Nuclear pairing was sometimes lost in the cell forming the

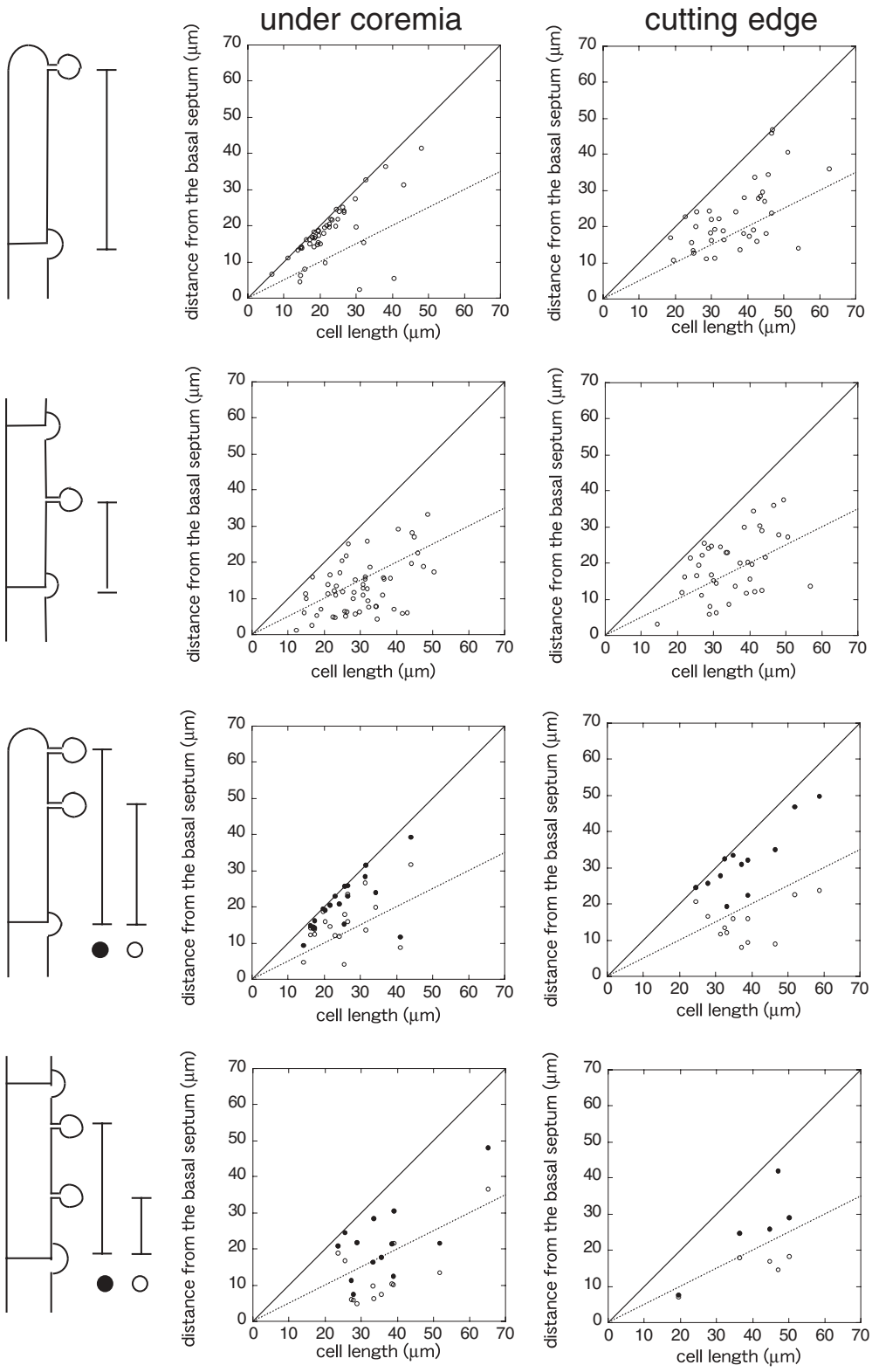


Fig. 15. Cellular position of toxocyst emergence from dikaryotic hyphae. The positions of toxocyst emergence were measured from the basal septum and plotted against cell length. Data were obtained from the toxocyst-rich area in the mycelia grown on PDA, either under the coremia or near the cut edge of the agar. *Lines* indicating the apical cell end and the cell center are drawn in the graphs. When cells formed two toxocysts, distances for the apical and basal toxocysts are shown as *closed circles* and *open circles*, respectively

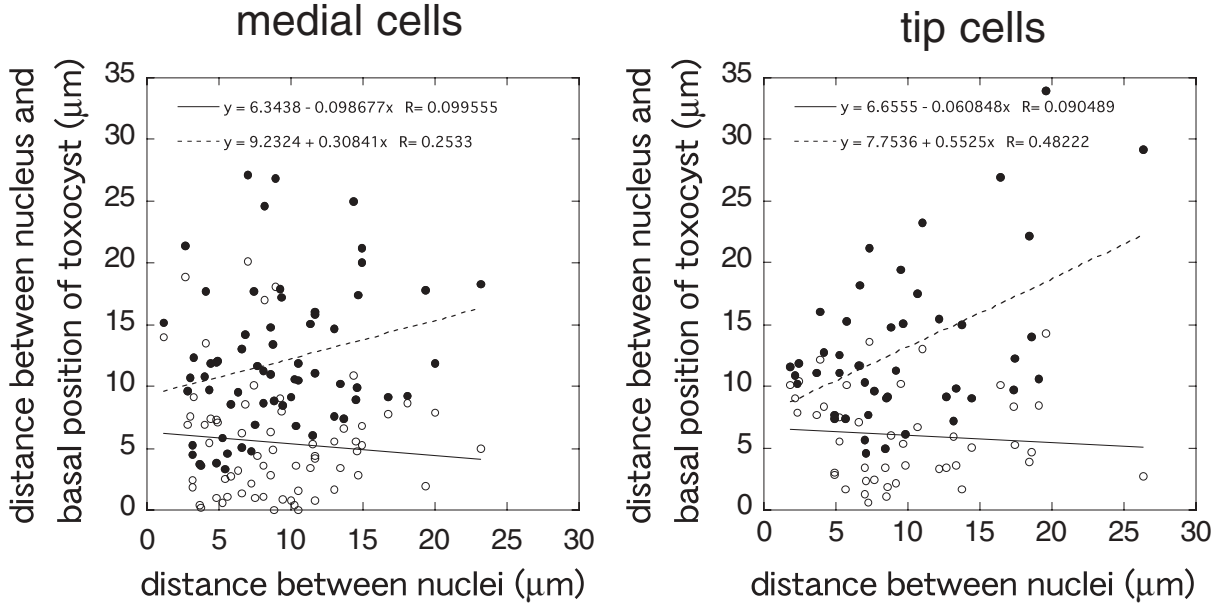


Fig. 16. Proximity of one of the nuclei of a dikaryon to a toxocyst. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Distances between the center of the nucleus and the position of toxocyst emergence were plotted against the center distances of the two nuclei with linear regression lines. Cells forming one toxocyst in the mycelial area near the cut edge of the agar were used to obtain the data. Data from nuclei proximal (*open circles with solid regression line*) and distal (*closed circles with broken regression line*) to the toxocysts are shown. The lengths of the medial and tip cells were 32.6 ± 10.0 μm and 34.7 ± 9.7 μm (average ± standard error), respectively

Table 1. Number of toxocysts formed from each cell[a]

Nuclear phase	One toxocyst	Two toxocysts	Three toxocysts	Four toxocysts	Five toxocysts	Toxocyst formation
Dikaryon	130/229 (56.8%)	85/229 (37.1%)	11/229(4.8%)	1/229 (0.4%)	2/229 (0.9%)	229/447 (51.2%)
Monokaryon	58/62 (94%)	4/62 (6%)	0/62	0/62	0/62	62/195 (31.8%)

[a]Toxocysts were formed on the mycelium grown on water agar (WA). Data above include early stage of the toxocyst development. For detection of the toxocysts, red fluorescence was used as the marker for the toxocysts in early stage of development

toxocyst. However, one of the nuclei maintained close proximity to the mature toxocyst (Fig. 16). The distance between the toxocyst and the proximal nucleus was statistically close in cells with toxocysts in an early developmental phase compared to those with mature toxocysts (4.8 ± 4.0 μm vs. 6.7 ± 4.7 μm, $P < 0.0001$) (Fig. 17). These data may suggest nuclear involvement in the determination of toxocyst position, or attraction of the nucleus by the toxocyst.

Consideration of evolutional relevance of the toxocyst and blastoconidium

Most of the toxocysts in this study contained no DAPI-positive material (Table 2). Therefore, the toxocyst is neither a blastoconidium nor a cell as implicated in previous reports (Han et al. 1974; Moore 1977; Nair and Kaul 1980; Hilber 1982; Stalpers et al. 1991; Reid et al. 1998; Capelari 1999; Clémençon 2004). However, DNA was observed in the ovoid in rare cases (Fig. 18). Only one nucleus was seen in the dikaryotic mother hyphal cell (Fig. 18, large arrow), which suggests that one of the nuclei can migrate into the toxocyst without accompanying nuclear division. The toxocysts of a noncoremial fungus, *Pleurotus ostreatus*, also usually contained no nuclear DNA, but the frequency of nucleus-containing toxocysts was higher than in *P. cystidiosus* subsp. *abalonus* (see Table 2). Therefore, we think that the toxocyst may be a vestigial blastoconidium and that devolution may have progressed further in *P. cystidiosus* subsp. *abalonus*, which produces tremendous numbers of arthroconidia from coremia. Kamzolkina et al. (2006) has also reported the absence of a nucleus in the toxocysts of *P. ostreatus* and *P. pulmonarius*.

Table 2. Number of toxocysts containing a nucleus[a]

Fungal taxa	Containing nucleus	No nucleus
P. cystidiosus subsp. *abalonus*	1 (0.7%)	142 (99.3%)
P. ostreatus	6 (14%)	38 (86%)

[a] Only the ovoids of the mature toxocyst were counted

The toxin might only accumulate in the balloon after maturation, because some nematodes were not immobilized even when attacked by multiple toxocysts. We recorded a worm immobilized after touching 17 toxocysts. The toxicity may attack not only nematodes but also the fungus itself

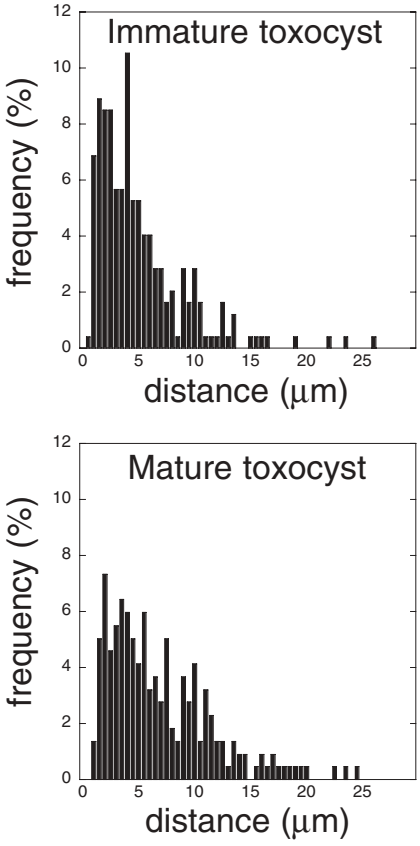


Fig. 17. Closer nuclear positioning to the toxocyst in the early state of toxocyst development. Toxocysts were identified by their intrinsic red fluorescence and categorized into two groups, immature (without the ovoid, n = 247) and mature (with the ovoid, n = 218). Nuclei were stained with DAPI. For each dikaryotic cell forming one toxocyst, the distance between the position of toxocyst emergence and the center of the proximal nucleus was measured. The histograms show a comparison of the distance between the early and late stages of toxocyst development

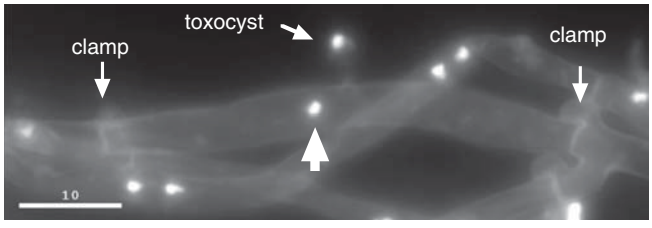


Fig. 18. A rare example of presence of nucleus within the blastoconidium-like ovoid part of the toxocyst. Nuclei were stained with DAPI. Only one nucleus is seen in the dikaryotic mother hyphal cell (*large arrow*) that has the toxocyst containing nucleus. *Bar* 10μm

(Kwok et al. 1992; Barron 2003). Therefore, not only storage but also production of the toxin might be better limited within the toxocysts. In yeast, *ASH1* mRNA that encodes a daughter-specific protein is transported from the mother cell into the bud (for review, see Cosma 2004). This is an attractive mechanism that might explain the details of toxin production and secretion from toxocysts that contain no nucleus.

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