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FULL PAPER

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Dikaryotic arthroconidiation of *Pleurotus* subgenus *Coremiopleurotus*

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Abstract This study was carried out to elucidate the poorly understood processes of arthroconidiation through coremium formation using *Pleurotus cystidiosus* subsp. *abalonus*. The coremia exclusively produced dikaryotic arthroconidia with the remnant of a clamp connection. Cells in the subapical zone of the hyphal bundle reduced their length by division before arthroconidiation. Approximately 400000 arthroconidia were produced by a coremium in 1 day, with constant productivity over a 2-week period. Continuous cell extension and division in the coremium stipe supplied cells for arthroconidiation at the coremium apex, which is surrounded by a liquid droplet (coremioliquid). Maintenance of moisture with coremioliquid was necessary for arthroconidiation. The coremioliquid formation was performed by active liquid transportation in the hyphae, a process that was blocked by the microtubule depolymerization agent thiabendazole.

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Introduction

Arthroconidia of basidiomycete mushrooms are usually produced on the apices of simple or branched conidiophores that formed previously on the hyphae of a vegetative mycelium (Arita 1979; Cole 1986). They can also develop directly on the vegetative hyphae without the formation of conidiophores (Hui et al. 1999). In contrast, *Pleurotus* subgenus *Coremiopleurotus*, which consists of subspecies members of *Pleurotus cystidiosus* O.K. Mill., forms arthroconidia on coremium (=synnemata). The coremium is a bundle of hyphae erected from the mycelium that produces arthroconidia from its apical surface (Moore 1977a; Guzman et al. 1991; Stalpers et al. 1991; Capelari 1999). The coremia-forming microfungus *Antromycopsis* has been reclassified as an anamorph of *Coremiopleurotus* (Moore 1977a; Guzman et al. 1991; Stalpers et al. 1991).

The coremium of *Coremiopleurotus* is 800–2500 μm tall, 150–400 μm wide, and has a drop of liquid on its apex (coremioliquid) in which a large number of arthroconidia are released (Stalpers et al. 1991). The coremia develop gregariously and haphazardly on the vegetative mycelium and, at times, on the base of the fruit-body stipe (Hilber 1982).

Cells in the coremioliquid that undergo arthroconidiation are first connected in a chain with clamps and then released into the drop as mature conidia. Mature arthroconidia are mostly ovoid to subcylindrical, pale brown to black, and glabrous, with thickened walls (Stalpers et al. 1991; Capelari 1999). The basipetal disjunction of arthroconidia has been studied focusing on the changes of the cell wall structure and the dolipore/parenthesome septa for maturation of the arthroconidia with electron microscopy (Moore 1977b). However, arthroconidiation through coremium formation is not yet fully understood. Therefore, we examined this process using two dikaryotic stock cultures of *Pleurotus cystidiosus* subsp. *abalonus* (Y.H. Han,

K.M. Chen & S. Cheng) O. Hilber ex O. Hilber, a Vietnamese strain (Blao) and a Japanese strain (S396), because these strains formed relatively large coremia.

Materials and methods

Organisms

The Japanese strain, S396, was collected in Nakasone-machi, Iyomishima city, Ehime Prefecture, Japan, by Mr. Ryokichi Kaji, and identified and isolated by H. Neda in May 1997. The Vietnamese strain Blao was collected in Bao-loc town, Lam-Dong Province, Vietnam, in 1999 by X.-T. Le. Monokaryotic strains were isolated from basidiospores of these dikaryotic strains. Sequences of the internal transcribed spacer (ITS)1 and ITS2 regions in the ribosomal RNA gene repeat of these two strains showed that these are nearly identical subspecies. However, the unusual feature of these strains was that they were dikaryons carrying two distantly related nuclei. The ITS sequences of one of the nuclei of each strain were very similar to those of *P. cystidiosus* subsp. *abalonus*. The ITS sequences of the other nuclei in these strains were also very similar to each other, showing an intermediate distance between *P. cystidiosus* subsp. *cystidiosus* and *P. cystidiosus* subsp. *abalonus* (B.-N. Truong et al., in manuscript). The properties of the two strains analyzed in this study did not show significant differences except in germination rates of the arthroconidia.

Media and reagents

Potato dextrose agar (PDA) medium (Difco) was used for all experiments except for one analyzing coremioliquid obtained from the coremia of mycelium grown on MY agar, which contains 1% malt extract (Oxoid), 0.42% yeast extract (Difco), and 1.5% agar (Nacalai tesque). Thiabendazole, latrunculin A, and hydroxyurea were purchased from Sigma.

Cultivation and preparation of coremia and arthroconidia

A mycelial agar block (5 mm in diameter) of a dikaryotic or monokaryotic isolate was cut from the peripheral region of the dikaryotic or monokaryotic mycelial colony and inoculated on the PDA in a plastic petri dish (9 cm in diameter). After 7 days of incubation at 27.0° ± 0.5°C in the darkness, the cultures were grown under continuous illumination approximately at 400 lux provided by a fluorescent light tube (National FHF 32EX-N-H) at 23.0° ± 1.0°C to induce coremium formation.

Microscopic observation

Photographs of coremia were taken using a Leica MZ FLIII stereomicroscope (Leica Microsystems, Wetzler, Germany). The sectioning microscopy Delta Vision System (Applied Precision, Issaquah, WA, USA) and its associated software were used to obtain digital images of stained cells and to determine the distance between two points on the picture files. To observe nuclei, mycelia were fixed with 70% ethanol and stained with 1 µg/ml DAPI (4′,6-diamidino-2-phenylindole). In the case of conidia, they were permeabilized with iodine vapor for 8 h and stood in 70% ethanol overnight before staining with 10 µg/ml DAPI. Hyphal septa were stained with 10 µg/ml calcoflour white to measure cell length within the coremium. For scanning electron microscope (SEM) observation, samples fixed by osmic acid were 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.

Analysis of the liquid components of the coremium droplet

After centrifugation of combined droplets from coremia, 10 µl supernatant was separated by Agilent HPLC 1100 series (Agilent Technologies, Palo Alto, CA, USA) using the C18 column TSK-GEL RP-18 (ϕ4.6 mm × 150 mm; Tosoh, Tokyo, Japan) with a gradient of solvents A (99.9% H_2O and 0.01% AcOH) and B (100% ACN). The collection samples were then analyzed with a gas chromatographic column coupled to the mass spectrometer Pegasus III MS system (Leco, St. Joseph, MI, USA). The GC 6890 (Agilent Technologies) was equipped with a capillary column DB-17ms (30 m × 0.25 mm; J & W Scientific, Folsom, CA, USA; carrier He gas with speed at 1 ml/min) and the temperature was programmed from 70° to 310°C at 15°C/min. The temperatures of injector and detector were set at 200° and 250°C, respectively.

Results and discussion

Development of coremium

Coremium formation was clearly induced by light. Therefore, we were able to observe its developmental process from the early stage, which may be a process closely related to fruit-body formation. The aerial hyphae were aggregated and randomly changed growth tip orientation during the formation of spherical primordia (Fig. 1A, arrows). The loosely entangled hyphae were soon twisted tightly with many new branches to form a "prosenchyma," which then grew vertically to become a coremium stipe (Fig. 1B). This progression resulted in hyphae inside the stipe that were twisted tightly near the base and more parallel toward the apex. The growing apex was a bundle of hyphal tips (Fig. 1C). The coremium stipe elongated for approximately 7 days before conidiation occurred at the apex. Coremioliquid accumulated before the apical cells began conidiation (Fig. 1D).

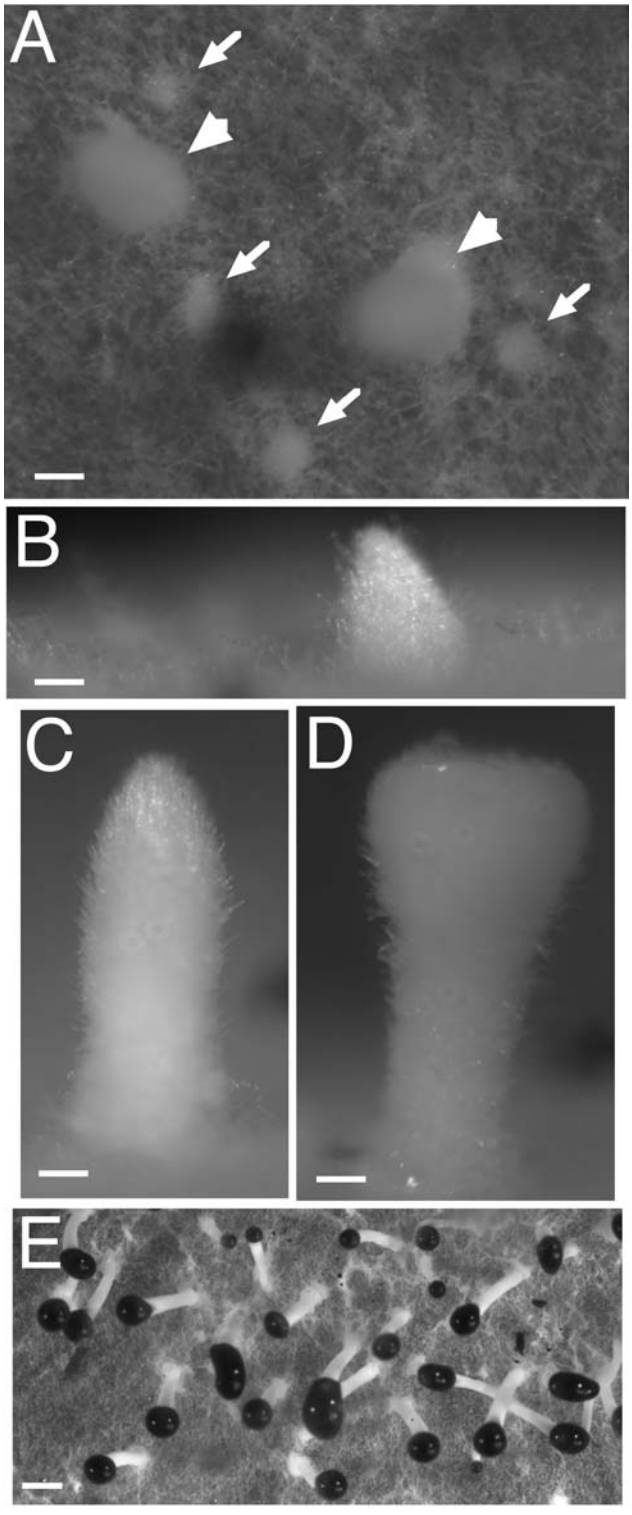

Fig. 1. Development of coremia. **A** Aerial hyphae aggregate and form spherical primodia (*arrows*). *Arrowheads* point to unfocused growing coremia. **B** Prosenchyma. **C** Growing young coremium with an apex of bundled hyphae. **D** Liquid accumulation at the apex of the coremium. Conidiation of apical cells has not yet been observed. **E** Mature conidia. Strains **A, B** Blao; **C–E** S396. *Bars* **A–D** 0.1 mm; **E** 1 mm

Arthroconidiation of bundled hyphae in droplets at apex of coremia

The arthroconidia were produced by a sequential secession of apical cells of the bundled hyphae of the coremium. Conidiation proceeded in a train of cells near the hyphal apex surrounded by coremioliquid. Up to maturation, they expanded in both length and width to become mostly ovoid to subcylindrical in shape (Fig. 2A,B) and accumulated a black pigment. The long cells constituting hyphae divided to reduce cell length just before the initiation of conidiation (Fig. 2B,C). Every cell division for arthroconidiation accompanied formation of a clamp connection (Fig. 2C). Staining with DAPI showed that the arthroconidia contained exclusively two nuclei [Fig. 2A(right),C,D]. The nuclei within the mature conidia were condensed and usually paired in the middle of the cell (Fig. 2D), with an average distance between the nuclei centers of 2.26 µm. The mature conidia were bent ovoid because of vestigial clamp connections (Fig. 2E). Hyphae from germinating arthroconidia always formed clamp connections (Fig. 2F). Therefore, the coremia of the *P. cystidiosus* subsp. *abalonus* dikaryon strains produce exclusively dikaryotic arthroconidia, which is in contrast to the dikaryotic mycelia of many other basidiomycetes that also produce monokaryotic conidia. The arthroconidia produced by the monokaryotic coremia contained a single nucleus and was accompanied with no clamp connections (Fig. 2G).

Continuous supply of cells for arthroconidiation

The apical cells for arthroconidiation in the coremioliquid were continuously delivered by hyphal extension. The number of conidia produced by a coremium in a day was counted with a hemocytometer (Fig. 3). The diameter of the coremia changed little during the experiment. The average number of hyphae bundled at the apex of a coremium was estimated as 20 000 hyphae/coremium from the diameter of the coremia (0.81 ± 0.15 mm) and hyphal density (about 40 000 hyphae/mm^2 in cross section). Based on these estimated numbers and data in Fig. 3, about 20 cells were released as arthroconidia from a hypha each day, which corresponds to a hyphal length of about 200 µm (based on Fig. 2B). However, instead of shrinking during the experiment (200 µm × 20 days = 4 mm), the coremia maintained a constant height or, rather, experienced a little elongation after conidiation started (5.0 ± 0.9 mm in height on the last day of this experiment). A statistical comparison of the stipe cell length suggested that the cells in the middle of hyphae underwent extension and division (Fig. 4).

This pattern of cell growth is in clear contrast to that of mycelia, in which cell elongation and division occur only at the apical cells of either the mother hyphae or the branches. The stipe elongation of the fruit body of *Coprinus cinerius* (Schaeff.) Gray (Kamada 1994) is performed by hyphal cell extension, which produces a number of multinucleated cells (Stephenson and Gooday 1984; Kamada 1994). In contrast, DAPI staining of the coremia stipe showed that the hyphal

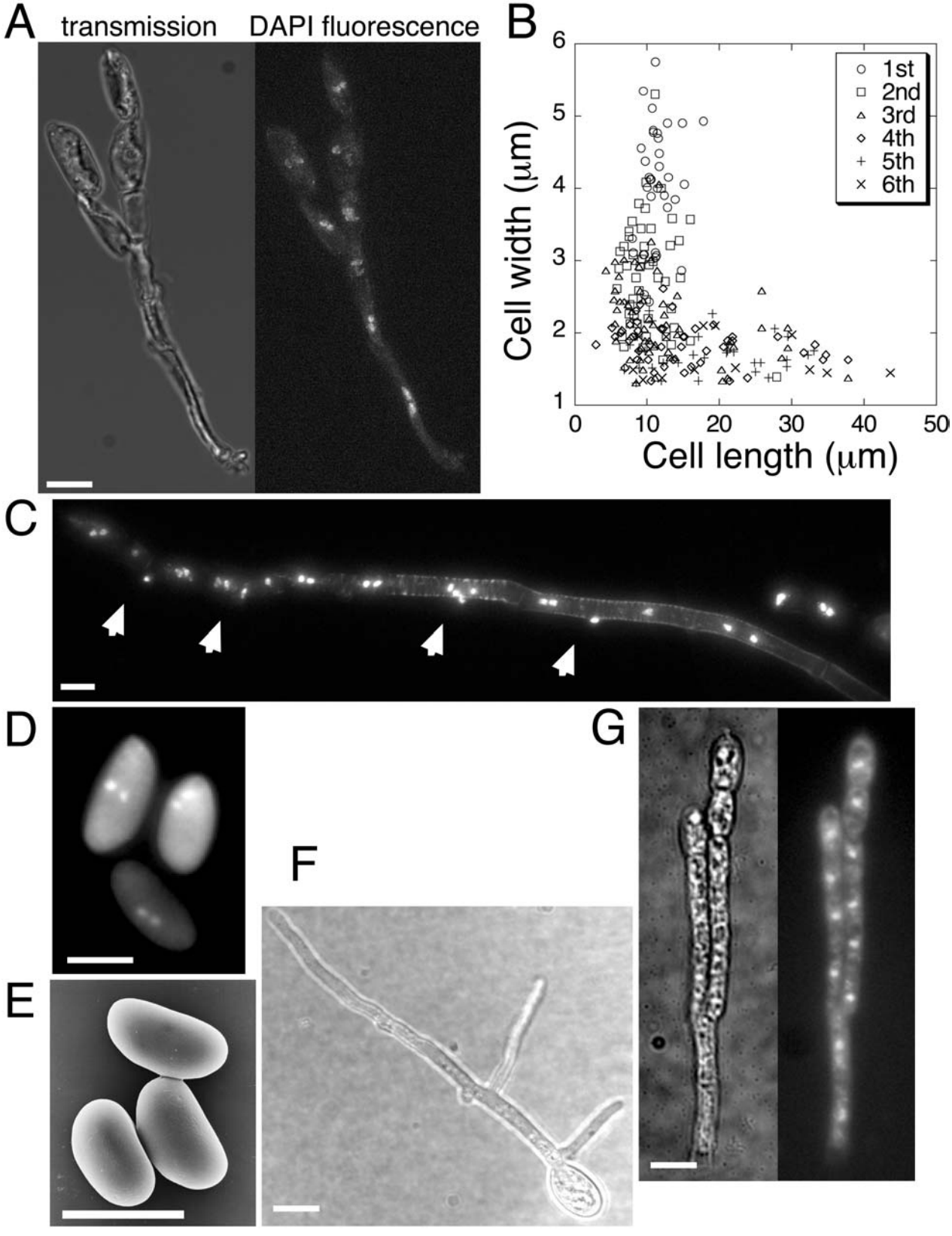

Fig. 2. Division with clamp connections and sequential arthroconidiation of subapical cells. **A** Conidiophore of dikaryotic coremium stained with DAPI (4′-6′-diamidino-2-phenylindole). *Left*, transmission light view; *right*, fluorescent view. **B** Size distribution of subapical cells. Length (*x*-axis) and width (*y*-axis) of conidiophore cells are plotted. Cells are categorized based on the order from apical to basal regions. **C** Conjugate divisions in subapical cells. Four subapical cells are dividing with clamp connections still containing nuclei (*arrowheads*). Note that the two divisions on the *left* are producing cells similar in length to conidia, whereas the two divisions on the *right* are producing cells nearly twice that length. **D** DAPI staining of mature conidia. **E** Scanning electron microscopy of mature conidia. **F** Germination of conidia. **G** Conidiophore of monokaryotic coremium stained with DAPI. *Left*, transmission light view; *right*, fluorescent view. Strains **A**, **B**, **D–G** S396; **C** Blao. *Bars* 10 μm

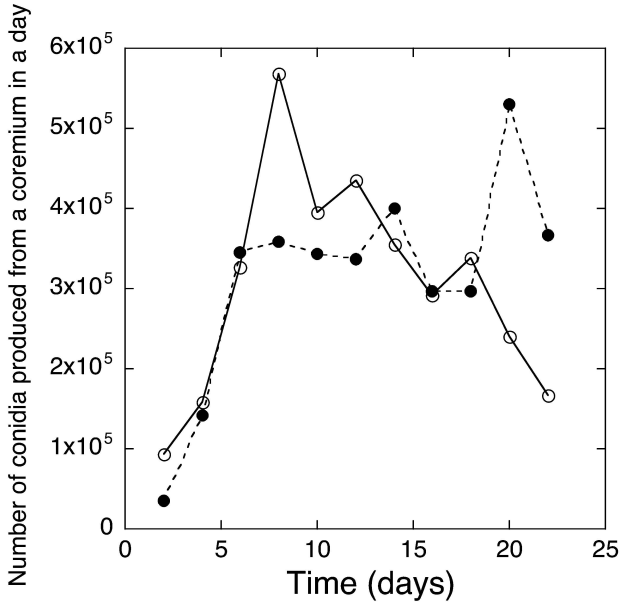

Fig. 3. Continuous production of arthroconidia by coremia. The liquid was removed from the apex of young coremia on the first day. Every 2 days, the liquid containing conidia was harvested from 15 (experiment A, *open circles*) or 18 (experiment B, *closed circles*) coremia, combined, and the total volume measured (5–26 μl). Conidia were counted with a hemocytometer. Calculated numbers of conidia produced by a coremium in a day were then plotted. Strain S396

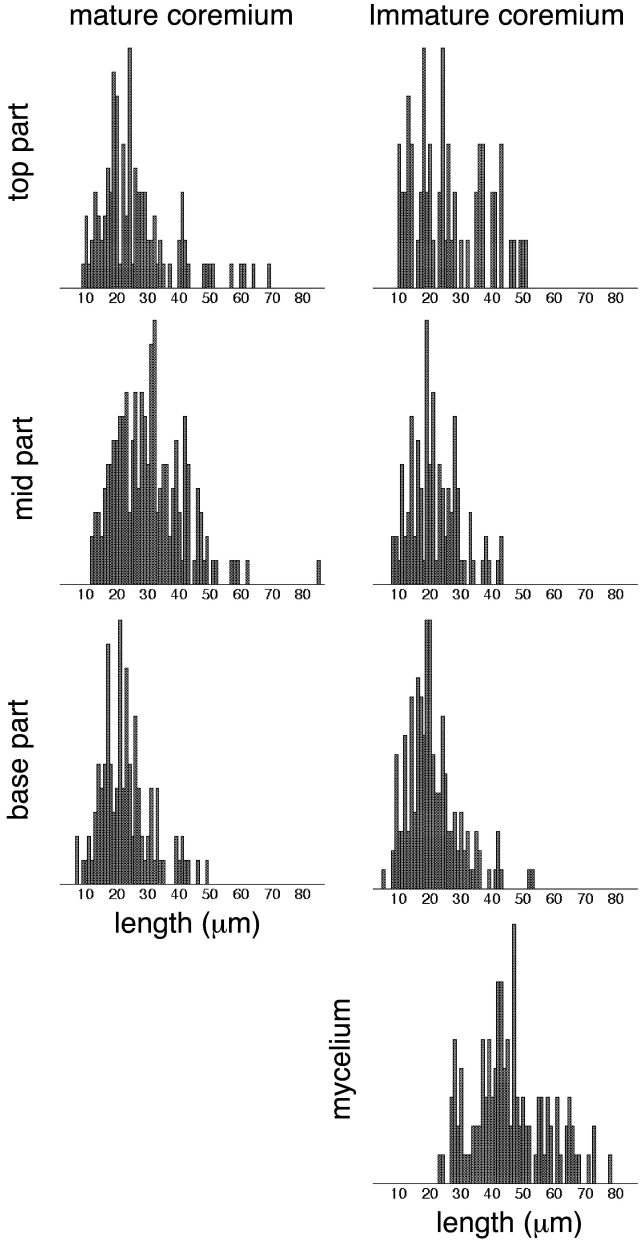

Fig. 4. Histogram of hyphal cell length within a coremium. A mature coremium (3 mm in height) actively producing conidia (*left*) or an immature coremium (2 mm in height) before producing conidia (*right*) was cut into three parts. Hyphae were stained with calcoflour white to distinguish septa. Strain S396

cells were exclusively dikaryotic (data not shown). Time-lapse recording of coremia showed that hyphal cells both inside and on the surface of the stipe migrated into the coremioliquid droplet (data not shown). Exclusive recovery of conidiated cells from the coremioliquid suggested that almost all the stipe cells underwent arthroconidiation after migrating into the coremioliquid droplet.

Supply of liquid to apex of coremia

The coremioliquid was continuously supplied during conidiation. It overflowed when the size of the droplet exceeded a certain criterion. The liquid does not contain concentrated hygroscopic materials to absorb humidity from the air, because the liquid dried as rapidly as pure water. The components of the liquid were analyzed to pursue the source of coremioliquid. The pattern of the chromatograph changed according to the medium in which the mycelia had grown. Gas chromatographic peaks corresponding to D-mannose, D-galactose, myo-inositol, 3-amino-1,2,4-triazole, oxalic acid, L-malic acid, and uridine triphosphate (UTP) were detected, as well as other unidentified substances in the liquid from S396 coremia grown on PDA. However, the peaks on the chromatograph of liquid from coremia grown on MY agar medium did not clearly match with those of our library (data not shown).

We next investigated whether the liquid is transported actively by hyphae or simply by capillary action. To block the cellular energy source, a piece of PDA agar (2 × 2 × 0.5 cm = 2 ml) on which the mycelium had grown to form coremia was soaked with 0.2 ml 10% sodium azide solution. When the coremioliquid was removed with filter paper, it did not reappear, although the liquid soon reappeared on the apices of the control coremia. To estimate which cellular machinery is involved in the liquid transportation, 0.2 ml of the following drug solution was allowed to soak into the 2-ml agar piece: 0.5 mg/ml thiabendazole (microtubule inhibitor), 0.3 mM latrunculin A (actin polymerization inhibitor), or 0.5 M hydroxyurea (DNA synthesis inhibitor). Thiabendazole significantly reduced the reaccumulation of

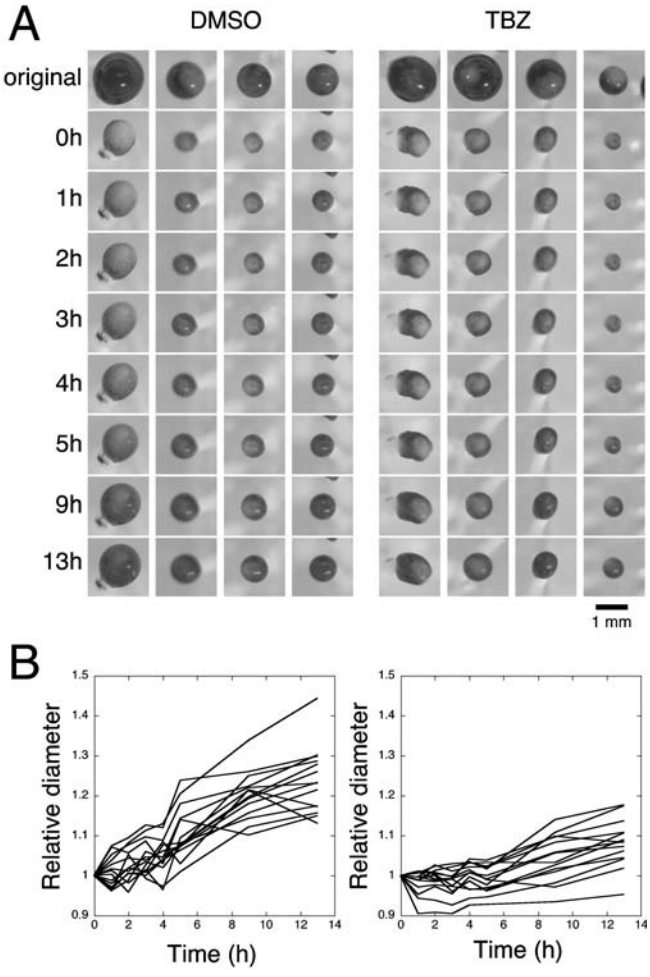

Fig. 5. Reduction of rate of liquid transportation to the apex of coremia by microtubule depolymerization. Thiabendazole (final concentration 50 µg/ml in 0.25% dimethylsulfoxide, DMSO) or the control DMSO (final concentration 0.25%) was soaked into potato dextrose agar (PDA) on which coremia were erected from the covered mycelium when the liquid at the apex of coremia was removed (time "zero"). **A** Photographs of the apex of each coremium. **B** Relative diameter referred to the size at time "0" are plotted against incubation time after subjection to thiabendazole or DMSO. Strain S396.

liquid at the apex of the coremia (Fig. 5). No acute effect of latrunculin A or hydroxyurea on reaccumulation was observed, although secondary effects of cellular toxicity resulted in dried coremia apices after 2 days. We suggest that liquid derived from the medium might be incorporated into certain organelles, which are transported within the hypha by cytoplasmic flow (Hickey et al. 2002) and then secreted from the apex of the coremium.

Role of the coremioliquid in conidiation

Conidiation takes place within the coremioloquid. Just before the initiation of conidiation, we removed the liquid with filter paper from the apex of the coremia and kept the apex dry by occasional blotting. The color of the coremium apex remained white, and no free cells were released. Instead, the apices of the coremia continued a small amount of growth as a bundle of hyphae. Next we examined whether moisture is enough for conidiation or if some other substance(s) is (are) involved in the process. After removal of the apical liquid with filter paper, the apex of an immature coremium was immersed into a large drop of water (40 µl) on the ceiling of the petri dish. The drop on the ceiling was recovered every day and the apex was immersed again with fresh water. After 3 days, the color of the coremium apex remained white. However, some arthroconidia were released into the water. The color of the conidia was also pale under the microscope (Fig. 6). Usually, conidia accumulate pigment before release into the drop. Therefore, moisture is necessary for conidiation, and some substance(s) in the droplet may be involved in the maturation of conidia, including accumulation of pigment.

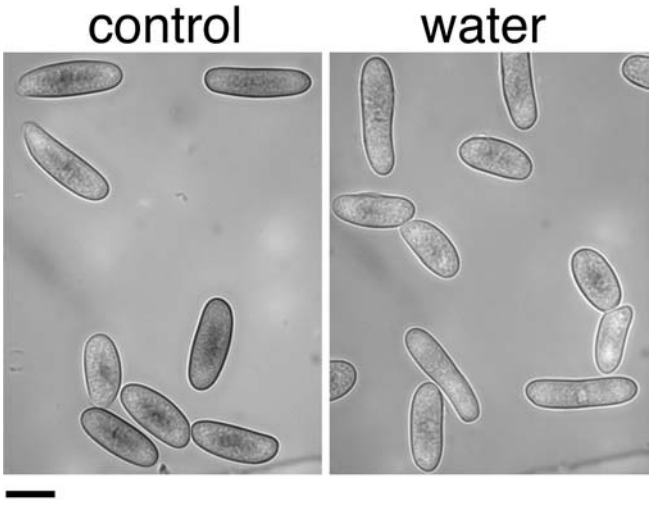

Fig. 6. Production of pale conidia in water instead of coremioliquid. Photographs of conidia under the light microscope were taken at 550 nm. Strain S396. *Bar* 10 µm

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