Morphological Characteristics of Conidiogenesis in *Cordyceps militaris*

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(Received April 19, 2005)

Conidial development of *Cordyceps militaris* was observed from germinating ascospores and vegetative hyphae through light and scanning electron microscopy (SEM). Ascospores were discharged from fresh specimens of *C. militaris* in sterile water as well as Sabouraud Dextrose agar plus Yeast Extract (SDAY) plates. We observed ascospore germination and conidial formation periodically. Under submerged condition in sterile water, most part-spores germinated unidirectionally and conidia were developed directly from the tips of germinating hyphae of part-spores within 36 h after ascospore discharge, showing microcyclic conidiation. First-formed conidia were cylindrical or clavate followed by globose and ellipsoidal ones. Germination of ascospores and conidial development were observed on SDAY agar by SEM. Slimy heads of conidia on variously arranged phialides, from solitary to whorl, developed 5 days after ascospore discharge. Besides, two distinct types of conidia, elongated pyriform or cylindrical and globose, were observed in the same slimy heads by SEM. Conidia were shown to be uninucleate with 4,6-diamidino-2-phenylindole staining. Conidiogenous cells were more slender than vegetative hyphae, having attenuated tips. Microcyclic conidiation, undifferentiated conidiogenous hyphae (phialides), polymorphic conidia and solitary, opposite to whorled type of phialidic arrangement are reported here as the characteristic features of asexual stage of *C. militaris*, which can be distinguished from other *Cordyceps* species.

KEYWORDS: Conidiogenesis, *Cordyceps militaris*, Microcyclic conidiation

Sexual (teleomorphic) fungi are studied for their asexual (anamorphic) structures to understand their complete life cycle and to clarify their phylogenetic relationships among the related genera. Out of more than three hundred *Cordyceps* species reported, only about fifty species have been studied for their conidial structures, which have been linked to different hyphomycetous anamorphic genera (Kobayasi, 1982; Liang, 1991; Liu *et al.*, 2002; Hodge, 2003). Occasionally, more than one anamorphic name is given to a single teleomorphic species of *Cordyceps*, such as twenty-two anamorphic names for *C. sinensis* (Jiang and Yao, 2002). A potential reason for assigning multiple anamorphic names to a single teleomorphic species is mainly attributed to the possible contamination occurring due to isolation from stroma tissue or host body, rather than using ascospores for obtaining the axenic cultures (Liu *et al.*, 2002).

Conidiation of *C. militaris* was vividly described by de Bary (1887) from the germinating ascospores. He described simple undifferentiated hyphae producing dimorphic conidia in slimy heads, or rarely in short chains. However, prior to de Bary's observation, *Isaria farinosa* Fr. had been already shown as the conidial stage of *C. militaris* on the basis of chain-like conidia of both species (Tulasne and Tulasne, 1865). The view of Tulasne brothers (1865) was not accepted by de Bary (1867) at first, but was later accepted by himself (de Bary, 1887). After the conidial

structures of *C. militaris* were more clearly understood, Petch (1936) concluded that the conidial stage was rather *Cephalosporium*, and not *I. farinosa* based on the absence of regular whorls of prophialides, broader flask-shaped phialides and truly catenulate conidia. However, Brown and Smith (1957), while revising the genus *Paecilomyces*, described the conidial stage of *C. militaris* as similar to both *Cephalosporium* and *Paecilomyces*, the view similar to those of Tulasne brothers (1865) and de Bary (1887).

During the revision of *Cephalosporium*, its entomogenous species, including the asexual stage of *C. militaris*, were transferred to a newly elevated section *Prostrata* of *Verticillium* (Gams, 1971) due to the prostrate nature of conidiogenous cells. Taxonomic revision of *Verticillium*, a mega-genus comprising plant pathogens, entomogenous, nematogenous and fungicolous species, was felt overdue and found desirable to be divided into a number of distinct genera (Gams, 1988; Jun *et al.*, 1991).

Despite vast amount of work on the conidial stage of *C. militaris*, there was a lack of detailed microscopic studies on ascospore germination and microcyclic conidiation. In addition to light microscopy, electron microscopic tools such as TEM and SEM are often very useful in revealing minute details of fungal structures. The objectives of this study are to describe periodic conidiation processes of *C. militaris* from ascospores using light and scanning electron microscopy and to observe nuclear conditions of conidia.

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Table 1. List of *Cordyceps militaris* specimens used in this study

Specimen no.	Date of collection	Collection site
EFCC11255	May 2004	Hanla Mt., Jeju-Do
EFCC11338	June 2004	Hanla Mt., Jeju-Do
EFCC12179	July 2004	Hanla Mt., Jeju-Do
EFCC12445	August 2004	Hongcheon-Gun, Kangwon-Do
EFCC12448	August 2004	Hongcheon-Gun, Kangwon-Do
EFCC12449	August 2004	Hongcheon-Gun, Kangwon-Do
EFCC12451	August 2004	Hongcheon-Gun, Kangwon-Do

Materials and Methods

***Cordyceps militaris* specimens.** Ascospores discharged from fresh stroma of *C. militaris* specimen EFCC 11255 were observed for germination and conidiation periodically through the light microscope and SEM. In addition to EFCC 11255, a few more *C. militaris* specimens were also observed for ascospore germination and conidial formation through the light microscope, following the concept of Samuels (1979). Collection sites and dates for different *C. militaris* specimens are given at Table 1 and have been preserved in air-dry condition in Entomopathogenic Fungal Culture Collection (EFCC), Kangwon National University, Korea. All the specimens were found growing on Lepidopteran pupae in nature.

Light microscopy. Ascospore filaments of *C. militaris* specimens (Table 1) were discharged on dry glass slides and observed for their morphological characters. Ascospores were also observed for germination, hyphal growth and conidial formation starting from 12 h after discharge in submerged condition of sterile water without any nutrition for 5–6 days. All the preparations were stained by 2% cotton blue in Lactophenol and observed with a Zeiss AxioLab Microscope. Microscopic measurements were made under oil immersion at $\times 1000$. Photographs were taken with Elite Chrome DX Process E-6 35 mm film. The contrast and brightness of photographs were adjusted using Adobe Photoshop 7.0.

Scanning Electron Microscopy (SEM). Single layers of dialysis tubing cellulose membranes (Sigma-Aldrich MW03123) were autoclaved with 0.5% locust bean gum (Sigma) and spread over Sabouraud Dextrose agar plus Yeast Extract (SDAY; dextrose 40 g, peptone 10 g, yeast extract 10 g, agar 15 g per 1000 ml; pH 5.6) plates. Ascospores from *C. militaris* EFCC 11255 were discharged over the membranes and left at room temperature under light. The membranes containing growing mycelia were cut with sterile scalpels into small pieces 1, 3 and 5 days after ascospore discharge and fixed in 4% (v/v) glutaraldehyde with 0.5% Triton-X 100 in 0.1 M cacodylate buffer (pH 7.2) for 2 h at room temperature,

then dehydrated in a graded ethanol. The dehydrated materials were dried in Hitachi HCP-2 critical point dryer using carbon dioxide. They were mounted on aluminum stubs and coated with gold-palladium alloy (Ion Sputter E 1010) and examined with Hitachi S-3500N scanning electron microscope operated at 15 kV. The images were captured and digitized by a computer coupled to the microscope. The contrast and brightness of photographs were adjusted using Adobe Photoshop 7.0.

Nuclear staining. Hyphae of *C. militaris* EFCC 11255 were grown by slide culture technique (modified from Booth, 1971). For this, mycelial discs cut from growing colonies of *C. militaris* on SDAY agar were inoculated on sterile glass slide upside down, with mycelial layer in contact with the slide surface and placed at room temperature for 5 to 6 days when mycelia were observed growing out from the mycelial disc. For nuclear staining, mycelial discs were removed, and mycelia attached on the slide glass were stained with 4,6-diamidino-2-phenylindole (DAPI) at the concentration of 2 mg/ml and observed with a fluorescence light microscope (Reichert-Jung Polyvar Type A).

Results and Discussion

Observation by light microscopy. Freshly discharged ascospores of *C. militaris* either remained intact or broken into short fragments containing various numbers of part-spores. The number of part-spores in single ascospore filaments was one hundred twenty-eight, but few consisted of less than that. Ascospore filaments were discharged individually, but few were observed overlapping each other at the ends of filaments (Fig. 1). Part-spores were cylindrical or oblong in shape, but tapering at the

Fig. 1. Freshly discharged ascospores of *Cordyceps militaris* specimen EFCC 11255, showing overlapping ascospores at ends (pointed by arrows). (Scales: 50 μ m in A; 10 μ m in B).

Fig. 2. Ascospore germination and conidial formation of different specimens of *Cordyceps militaris* 36 to 48 h after ascospore discharge in submerged condition of sterile water (pointed by arrows). A, Conidia formed directly after germination of part-spore after 36 h (specimen EFCC 11338). B, Conidia formed on single germinating hyphae of part-spore after 48 h (specimen EFCC 12179). C, Conidia formed on branched germinating hyphae of part-spore after 48 h (specimen EFCC 12445). D, Conidia formed in globose head after 48 h (specimen EFCC 12448). E, A single part-spore germinating at two opposite points after 36 h (specimen EFCC 12449). F, Conidia formed in short chains after 48 h (specimen EFCC 12451). (Scales: 10 μm).

ends of the filaments; mostly 3 μm long, with a few ranging from 2–4.5 μm , with the width of 1 μm or slightly wider. Freshly discharged ascospores were all darkly stained by cotton blue, but after 12 h in submerged condition in water they varied in staining properties; pale stained part-spores were also frequently observed. Faintly stained part-spores were observed when stained after long hours of submerged conditions in water. Part-spores started germination after 12 h of submerged condition, mostly in one direction (Fig. 2A and B), but a few in two (Fig. 2E) or more than two directions (figure not shown), thus appearing polygonal in shape.

Some of the germinating part-spores produced conidia microcyclically at the end of germ tubes, 2–8 μm long, as early as 36 to 48 h after ascospore discharge in sterile water (Fig. 2A, B and E). When observed after 48 h in submerged condition in water, more and more part-spores

germinated and produced long hyphae (Fig. 2B, D and F), sometimes branched (Fig. 2C), which produced conidia in slimy heads (Fig. 2D), but rarely in short chains (Fig. 2F). Usually, first formed conidia were somewhat cylindrical or clavate, while the succeeding conidia were nearly globose or ellipsoidal (Fig. 2B, C and E). Conidia usually resembled part-spores in shape and size. A few part-spores germinated normally, but developed densely stained, blunt sterile ends without conidia, sometimes dichotomously branched. Globose conidia were 1.5–2 μm in diameter, whereas the oval or broadly ellipsoidal conidia varied from 2–4 $\times$ 1.5–2 μm in size, first formed cylindrical conidia as large as 7 $\times$ 3 μm .

The finding that the number of part-spores in single ascospore filaments of *C. militaris* was one hundred twenty-eight, a multiple of eight, is similar to the previous report (Hywel-Jones, 2002). Filamentous ascospores,

during discharge, sometimes overlap at their ends, appearing abnormally long containing erroneously higher number of part-spores; for example, as many as one hundred sixty per ascospore filament was reported by de Bary (1887). Such events have been shown in other clavicipita-

ceous fungi, such as *Epichloe typhina* (Welch and Bultman, 1993). Germinating part-spores were mostly deeply stained, but few were faintly stained, may be due to vacuoles (Pettit, 1895). Sparse hyphal growth and conidial formation directly from the germinating part-spores in water

Fig. 3. Scanning electron micrographs of part-spore germination of *Cordyceps militaris* EFCC 11255 one day after discharge on SDAY agar. A. Part-spores germinating in one direction. B Part-spores germinating in same or opposite directions; germinating part-spores swell and are bigger in size than non-germinating part-spores, which are joined together, usually in pairs (Scales: 5 μm in A; 10 μm in B).

Fig. 4. Scanning electron micrographs of undifferentiated hyphae of *Cordyceps militaris* EFCC 11255 producing conidia on tips three days after discharge on SDAY agar. First formed conidia are usually somewhat pyriform (A and B), or cylindrical (C and D). (Scales: 5 μm in A and C; 2 μm in B; 3 μm in D).

shows its microcyclic conidiation character.

Observation by SEM. Part-spores of *C. militaris* started to swell and germinate unidirectionally 1 day after discharge on SDAY agar, when observed through SEM (Fig. 3). Oval part-spores, relatively smaller than cylindrical ones, were usually observed as jointed, mostly in pairs and did not germinate, whereas cylindrical part-spores, which were usually detached from adjacent part-spores, swelled and mostly germinated (Fig. 3B).

Elongated pyriform, cylindrical and globose conidia were produced on tips of simple and undifferentiated hyphae 3 days after ascospore discharge. First-formed conidia were somewhat pyriform or cylindrical (Fig. 4). Conidiogenous hyphae, i.e., phialides, were very similar to vegetative hyphae, but more slender (Fig. 4). More phialides, either in solitary, opposite direction or in verticillate forms, were observed 5 days after ascospore discharge (Fig. 5). Slimy conidial heads were frequently observed, instead of chains. Even thin slimy film could be observed between an immature globose conidium and a mature broadly oval conidium (Fig. 6).

In this study, oval part-spores, frequently connected together mostly in pairs, did not germinate, when observed through SEM, as described earlier by Petch (1936). Part-spores of *C. militaris* are known to germinate usually in a short period of time (Tulasne and Tulasne, 1865; Petch, 1936; Shanor, 1936), changing into fusiform (Tulasne and Tulasne, 1865), except a few which changed to irreg-

ular, elongated or pentagonal forms (Petch, 1936). Various staining behavior and germination ability of part-spores of *C. militaris* might be due to the unequal nuclear divisions during somatic division of secondary part-spores in ascospores, known as karyochorisis (Moore, 1964).

Conidia of *C. militaris* have been variously described as globose (Tulasne and Tulasne, 1865), cylindrical and spherical (de Bary, 1887), nearly spherical (Pettit, 1895), pyriform, oval or globose (Petch, 1936), subglobose, elliptical or cylindrical (Brown and Smith, 1957), large or small (Wang, 1989), etc. First-formed conidia of *C. militaris*, clavate in shape and $5\sim9 \times 2\sim2.5 \mu\text{m}$ in size, are the largest ones, reported till date (Gams, 1971). In our study also, first-formed conidia were ovoid, pyriform or cylindrical, with the succeeding conidia being globose, nearly subglobose or ellipsoidal. In this study, we found that phialides of *C. militaris* vary considerably in length, complying with the descriptions of Petch (1936), Brown and Smith (1957) and Wang (1989). The production of blunt ends, by germinating tubes and hyphae is another characteristic of *C. militaris* (Pettit, 1895; Gams, 1971), which was also observed in this study.

Duration of ascospore germination and growth on nutrient medium such as SDAY agar had effect on arrangement of conidia and conidiogenous cells on subtending hyphae. At early age, conidia formed in solitary or simple hyphae, after which phialides developed on hyphae in single or in opposite directions or formed in verticils with conidia adhering in heads, similar to early descriptions

Fig. 5. Scanning electron micrographs of profuse vegetative and conidial hyphae of *Cordyceps militaris* EFCC 11255 five days after ascospore discharge on SDAY. Conidial hyphae are produced in single (A), alternately (B), in whorls originating at various levels of the subtending hyphae (C), in whorls or in opposite sides (D). (Scales: $10 \mu\text{m}$ in A, C and D; $5 \mu\text{m}$ in B).

Fig. 6. Scanning electron micrographs showing conidia of *Cordyceps militaris* EFCC 11255 at various stages on five days after ascospore discharge on SDAY. A. First formed conidium, being displaced downwards, by the succeeding conidium, both of them linked by a thin mucilaginous layer. B. Conidia adhered in slimy heads. (Scales: 2 μm in A; 5 μm in B).

(Pettit, 1895; Petch, 1936). The dominant modes of conidiogenesis in the Clavicipitaceae have been shown as enteroblastic (phialidic) and holoblastic, with sympodial proliferation (Seifert and Gams, 2001).

Nuclear behaviors. Conidia were observed as uninucleate under the UV after staining with DAPI, despite their dimorphic shape and size (Fig. 7). Conidia formed in the

liquid culture of *C. militaris* have been shown as uninucleate (Carilli and Pacioni, 1977), which is in agreement with our observation of conidia formed on agar plate. Other studies on nuclear condition of conidia of *Cordyceps* species are lacking, but studies on other anamorphic fungi, such as *Verticillium* species, have reported uninucleate conditions of conidia (Isaac, 1967).

Different methods have been proposed to link any sex-

Fig. 7. DAPI staining of hyphae and conidia of *Cordyceps militaris* EFCC 11255 under fluorescent (A and C) and UV (B and D).

ual fungus with its asexual partner, such as producing two forms reciprocally from each other in culture, or observing the microcyclic conidiations or anamorph states, etc. (Samuels, 1979; Liu *et al.*, 2002; Hodge, 2003; Stensrud *et al.*, 2005). Recently, however, molecular techniques have been used to elucidate anamorph-teleomorph relationships, including those of *Cordyceps* species (Liu *et al.*, 2002). A new generic name *Lecanicillium* has been recently proposed to include the asexual stage of *C. militaris* and other entomogenous species of *Verticillium* section *Prostrata* based on molecular techniques (Zare *et al.*, 2000; Gams and Zare, 2001; Sung *et al.*, 2001; Zare and Gams, 2001). Giving a distinct taxonomic name for the anamorph of *C. militaris* seems to be unnecessary for practical purposes as this fungus is normally dominant in nature as a teleomorph (Zare and Gams, 2001).

Observations made in this study disclosed the remarkable characters of conidial structures of *C. militaris* such as microcyclic conidiation from ascospores, simple and undifferentiated phialides arranged variously in subtending hyphae and polymorphic conidia (de Bary, 1887; Pettit, 1895; Petch, 1936; Shanor, 1936). Also, other interesting observations such as presence of polymorphic conidia clustered in slimy heads, instead of conidia in chains, and blunt hyphal ends, etc., were made, as described by previous authors. The present study has prompted more interest in the study of conidiation cycle of other undescribed *Cordyceps* species in future.

Germination of the ascospores and their subsequent development should be followed to avoid error caused by mixed infection, contamination, and other occurrence of some clavicipitaceous forms as parasites on their close relatives (Hodge, 2003). Ascosporal microcyclic conidiation has also been found useful for the identification of *Cordyceps* species (Liu *et al.*, 1997).

Acknowledgement

The authors are thankful to Misun Park in Korea Basic Science Institute, Chuncheon Branch for help for scanning electron microscopy. This work was supported by grants from the Strategic National R&D Program of the Genetic Resources and Information Network Center funded by Korean Ministry of Science and Technology and Regional Strategic Industry Program funded by Korean Ministry of Commerce, Industry and Energy.

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