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Involvement of autophagy and apoptosis and lipid accumulation in sclerotial morphogenesis of *Morchella importuna*

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ABSTRACT

Sclerotial formation is a key phase of the morel life cycle and lipids have been recorded as the main cytoplasmic reserves in sclerotia of *Morchella* fungi without any experimental verification. In this study, the ultrastructural features of the undifferentiated mycelia stage (MS) and three main sclerotial differentiation states (sclerotial initial [SI], sclerotial development [SD] and sclerotial maturation [SM]) were compared by transmission electron microscopy. The nature of the energy-rich substance in hypha and sclerotium of *Morchella importuna* was qualitatively investigated by confocal laser scanning microscopy and quantitatively studied by extraction of lipids. Sclerotia were observed to form from the repeated branching and enlargement of either terminal hyphae or subordinate hyphal branches, indicating a complex type of sclerotial development. Autophagy and apoptosis were involved in the sclerotial metamorphosis of the cultivated strain of *M. importuna*. During the SI phase, the characteristic features of autophagy (vacuolation, coalescence of small vacuoles, existence of autophagosomes and engulfment of autophagosomes by vacuoles) were observed. At the SD phase, apoptotic characteristics (condensation of the cytoplasm and nucleus, shrinkage of plasma membrane, extensive plasma membrane blebbing and existence of phagosomes) could be seen in some developing sclerotial cells. In the final stage of sclerotial morphogensis, the sclerotial cells showed a necrotic mode of cell death. In addition, confocal laser imaging studies of live cells indicated that the energy-rich substance in morel hyphae and sclerotia was lipid. The lipid content in sclerotia was significantly more than that in hyphal cells. To the best of our knowledge, this is the first detailed ultrastructural description highlighting the involvement of autophagy and apoptosis in sclerotial metamorphosis of *Morchella* species and lipid accumulation during morel sclerotial development was also first experimentally verified. This work will promote a better understanding of the mechanism of morel sclerotial metamorphosis.

1. Introduction

True morels (*Morchella* spp., Pezizales, Ascomycota) are edible and medicinal mushrooms appreciated worldwide for their savory flavor and multiple bioactivities, including anti-oxidative, anti-inflammatory, anti-microbial, immunostimulatory and anti-tumor properties (Heleno et al., 2013; W. Liu et al., 2017; Q. Liu et al., 2018; Rossbach et al., 2017; Tietel and Masaphy, 2017; Vieira et al., 2016). On the basis of Ower's pioneering study (Ower, 1982), artificial production of *Morchella* mushrooms in fields has been realized since 2012 in China (Du et al., 2015; W. Liu et al., 2017; Q. Liu et al., 2018). Some strains of *Morchella importuna*, e.g. #1, #2 and #4, are widely cultivated and account for about 95% of total production (He et al., 2017; W. Liu et al., 2017). Despite obvious commercial applications and successful artificial cultivation of morels, many aspects of their genetics and biology remain poorly understood, e.g. mechanisms of fructification, metamorphosis of sclerotium, spawn ageing and exogenous nutrition, role of microconidium in life cycle etc.; these knowledge gaps restrict the healthy and long-term development of morel farming. Enhancement of fundamental research on morels will be beneficial for promoting technological progress of morel artificial cultivation (Du et al., 2015; W. Liu et al., 2017; Q. Liu et al., 2018).

Sclerotial formation is a key phase of the life cycle of *Morchella* spp. (Alvarado-Castillo et al., 2014; Volk and Leonard, 1990). In *Morchella* species, sclerotia are presumed to be nutrient storage organs that can tolerate adverse conditions such as low temperature and desiccation,

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properties which are believed to enhance overwinter survival (Alvarado-Castillo et al., 2014; W. Liu et al., 2017; Q. Liu et al., 2018; Volk and Leonard, 1990). Lipids have been recorded as the main cytoplasmic reserves in sclerotia of *Morchella* fungi (Scott and Mohammed, 2004; Volk and Leonard, 1990), however, we could not locate any experimental verification of this. Unlike sclerotia in most other fungi, morel sclerotia lack differentiation of the rind and medulla, and thus are actually pseudosclerotia which form from the repeated branching and enlargement of either terminal hyphae (Volk and Leonard, 1990), or subordinate hyphal branches (Amir et al., 1993). Nonetheless, the formation of pseudosclerotia is a metamorphic process for *Morchella* fungi (Amir et al., 1993; W. Liu et al., 2017; Volk and Leonard, 1990). The involvement of autophagy and apoptosis in the metamorphosis of a subset of animals, including insects, amphibians, some fish and many marine invertebrates, has been thoroughly investigated (Bartolomeo et al., 2010; Messner et al., 2016; Romanelli et al., 2016; Suzanne and Steller, 2013). However, the involvement of autophagy and apoptosis in the biogenesis of fungal sclerotium has been poorly studied (Ren et al., 2017; Sumita et al., 2016).

Morphogenesis and some aspects of the biology have been studied in other fungal sclerotia, e.g. *Botrytis allii* (Sadeh et al., 1985), *B. cinerea* (Nair and Martin, 1987; Willetts and Bullock, 1982), *Claviceps purpurea* (Lösecke et al., 1980), *Grifola umbellata* (Xing and Guo, 2005), *Sclerotinia minor* (Bullock et al., 1980), *S. sclerotiorum* (Ordóñez-Valencia et al., 2015) and *Sclerotium rolfsii* (Chet et al., 1969). However, a detailed description of ultrastructure during the various stages of sclerotial formation in morels is lacking. In this study, we investigated these ultrastructural changes and revealed the involvement of autophagy and apoptosis in sclerotial metamorphosis of the artificially cultivated species of *M. importuna*. Moreover, the energy-rich substance in morel sclerotia was determined experimentally. Our work will provide useful enlightenment for the improvement of morel production technology.

2. Materials and methods

2.1. Fungal strain, growth conditions, and development stages

The tested strain of *M. importuna* #1 is a production one selected from wild morels occurred in Sichuan province, China, and has been applied in soil cultivation since 2010 (W. Liu et al., 2017). It is available from Peixin He and Wei Liu in Zhengzhou University of Light Industry, China. The strain was grown on Complete Yeast extracts Medium (CYM) in sterile Petri plates (glucose 20 g l^{-1}, yeast extracts 2 g l^{-1}, peptone 2 g l^{-1}, K_2HPO_4 1 g l^{-1}, $MgSO_4$ 0.5 g l^{-1}, KH_2PO_4 0.46 g l^{-1} and agar 20 g l^{-1}) at 23 °C; mycelia initially covered the Petri dish with sclerotium undifferentiated mycelial stage (MS) in 3d. Four days later, sclerotia appeared on the surface of the medium. Their formation involved three main sclerotial differentiation stages: sclerotial initiation (SI), sclerotial development (SD) and sclerotial maturation (SM) after cultured at 23 °C for 7, 10 and 14 d, respectively (Fig. 1). The cultivation was repeated 3 times with different stock culture, and 3 plates were inoculated for each batch culture.

2.2. Light microscopy

Colonies on the plates were observed and recorded directly or via stereo microscope (Lecia M205FA, Germany). Mycelia and sclerotia were excised from the media and placed on glass slides for microscopic observation. Three batch of plate cultures from different stock culture were observed, and at least three slides for each cultures were examined under bright field illumination on an optical microscope with a DIC module (Carl Zeiss Axio Observer Z1, Germany) (He et al., 2015).

2.3. Transmission electron microscopy

The mycelia or sclerotia were chopped into 1-mm blocks. The sample preparation and viewing with a transmission electron microscope were conducted according to the procedure of He et al. (2015). Briefly, the blocks were cleaned with distilled water, fixed in 2.5% glutaraldehyde, rinsed with distilled water and post-fixed in 1% osmium tetroxide. After rinsing with 0.1 M phosphate buffered saline (PBS, pH 7.0) three times and dehydration in a graded ethanol series, the fixed samples were embedded overnight. The samples were transferred into embedding mold and polymerized for 48 h at 65 °C. Ultrathin sections were prepared using a Leica UC7 ultramicrotome with a diamond knife. After staining with uranyl acetate and lead citrate, the ultra-thin sections were viewed with a transmission electron microscope (Hitachi HT-7700, Japan).

2.4. Confocal laser scanning microscopy

Cultures containing mycelia, sclerotia and a few agars were sliced into 8-μm sections using a cryostat microtome (CryoStar NX50, Thermo, USA), transferred onto glass slides, and then stained with 0.5 μg mL^{-1} Nile red solutions (Sigma-Aldrich) for 10 min away from light (Chen et al., 2009). After rinsing with PBS and covering with a cover slip, the samples were examined with a confocal laser scanning microscope (Carl Zeiss LSM 710, Jena, Germany) using the 40 × objective (numerical aperture of 0.75). The samples were visualized with an Argon laser excitation wavelength of 514 nm and with a 540–600 nm filter. To enhance the bright field contrast, differential interference contrast (DIC) was used for observation and recorded under 40× objectives (He et al., 2017). Three batch of plate cultures from different stock culture were observed and recorded. For each culture, the samples were repetitively prepared and observed for five times and six different fields in each observation were recorded. The images were merged and exported by zen 2010 software package.

2.5. Extraction and determination of lipids

Mycelial disks excised from the cultures were inoculated on autoclaved cellophane membranes placed on the surface of CYM plates. Cultures with colonies at the MS, SI and SM stages were scraped from the membranes after incubation at 23 °C in darkness for 3, 7 and 14 d, respectively. The experiments were repeated 3 times for three batch of plate cultures from different mother culture. For each batch culture, six plates were inoculated, and the cultures were combined after incubation. After drying at 50 °C, the cultures were ground to a particle size of 125 μm and stored in an airtight container at 4 °C for further use. About 200 mg of powdered sample and 2 mL of 4 M hydrochloric acid were placed into a 10-mL centrifuge tube. After incubating at 37 °C for 30 min in a rotary shaker at 160 r.p.m., the suspension was incubated in a water bath for 3 min at 100 °C, and then cooled in ice water. A 4-ml mixture of chloroform:methanol (1:1, v/v) was added and incubated for 10 min at ambient temperature (ca. 20 °C). After centrifugation for 10 min at 5000 × *g*, the layer of chloroform was rinsed with equal volume of sodium chloride solution (0.1%, w/v), and then dried to a constant weight with nitrogen at 50 °C using a Thermovap Sample Concentrator. The yield of lipids from dried cultures with different stages of sclerotial development of *M. importuna* was calculated by the following formula: Lipid yield (%) = [(lipid weight of extract)/dry weight of cultures] × 100%. The extraction and determination of lipids for each batch of plate cultures were repeated 3 times, the means of three batches of cultures were evaluated and the value of the final lipid yield was expressed as mean ± standard deviation (SD). The means of the final lipid yields of different stages of sclerotial development were compared using the Student's *t*-test and $p < 0.05$ was taken as significant difference. Statistical analyses were carried out using SPSS 12.0 for Windows (SPSS Inc, Chicago, USA).