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MYCOSCIENCE

ISSN 1340-3540 (print), 1618-2545 (online)

journal homepage: www.elsevier.com/locate/myc

Full paper

Life cycle and morphology of *Physarum pusillum* (Myxomycetes) on agar culture

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ARTICLE INFO

Article history:
Received 16 April 2011
Received in revised form
12 April 2012
Accepted 21 May 2012
Available online 1 January 2013

Keywords:
Artificial culture
Hanging drop culture
Slime molds
Spore

ABSTRACT

Myxomycetes (slime molds) are unique eukaryotic microorganisms with both characteristics of fungi and amoebae. Artificial cultures grown under controlled conditions were used to study the life cycle and morphogenesis. *Physarum pusillum* was collected from the field. Spores were inoculated and cultured with the hanging drop method. The complete life cycle was observed from spore to spore on agar without adding any solid nutrients or bacteria as food. Life cycle morphological characteristics were described for spore germination, myxamoebae, zygote, plasmodium and sporangia formation.

1. Introduction

Myxomycetes (slime molds) are a diverse group of eukaryotes characterized by spore-bearing fruiting bodies, are the diverse eukaryotes. In their life cycle they have myxamoebae, swarm cells, and plasmodia like protozoa and also produce spores like fungi. Myxomycetes are phylogenetically included in the protozoa (Cavalier-Smith 2001). Taxonomic confusion still exists, however, in the classification of myxomycetes at the genus or species level. Life cycle data are important and useful to clarify not only living strategies and ecological adaptations to environmental conditions, but also biological phenomena in each stage.

Development of the plasmodium is accompanied by a series of synchronous nuclear divisions (Everhart and Keller 2008). Nuclear division in the plasmodium of *Physarum polycephalum* Schwein. is synchronous (Guttes et al. 1961). As a result, the plasmodium becomes a multinucleate structure where individual cells are never delimited. Also, in most *Physarum* plasmodia a common spindle pole organizer is present (Rotaru et al. 1999). Agar culture is essential for better observation of synchronous nuclear divisions.

The plasmodia of myxomycetes have attracted much attention due to their apparent intelligent adaptive behavior (Niizato et al. 2010). The network formation of plasmodia is attracted and moves to food by the most efficient route (Dussutour et al. 2010). The sporangia adhere to the substratum enclose a mass of spores with reproductive capacity like fungi. Sporangia are often difficult to obtain from spore to spore culture so alternatives such as reduced air

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http://dx.doi.org/10.1016/j.myc.2012.09.001

humidity that triggered large numbers of sclerotia in *Perichaena depressa* were used to obtain sporangia (Keller and Eliasson 1992). Culture conditions of all life cycle stages of myxomycetes may also serve to obtain the sporangia in different ways.

The first spore to spore cultures of the myxomycete *Badhamia utricularis* (Bull.) Berk and *Didymium difforme* (Pers.) S. F. Gray were reported by Lister (1901). However, additional life cycles of myxomycetes were investigated and 104 species were reported from spore to spore in culture (Collins 1979; Keller and Schoknecht 1989a, b, c; Clark 1995; Ishibashi et al. 2001; Mosquera et al. 2003; Lado et al. 2007; Wrigley de Basanta et al. 2008; Liu et al. 2010). These cultures used mostly natural food sources, usually a bacterial associate, to produce sporangia. Even in the axenic cultures of 21 species, only characteristics of plasmodia or myxamoebae were reported (Hu and Clark 1986; Clark et al. 1990) except for P. *globuliferum* (Liu et al. 2010). Therefore, morphological changes in life cycles during culture still are not well understood.

Physarum pusillum (Berk. & Curt.) G. Lister belongs to Physaraceae in the Physarales and occurs on decayed wood, bark, grass, and straw on ground sites (Keller and Braun 1999). It was also found on the body of a living animal (Townsend et al. 2005). Its mating system has been reported as heterothallic based on cultures of myxamoebae (Collins et al. 1964; Clark 1995). However, its complete life cycle from spore to spore has not been described in detail. The morphology of all life cycle stages is described here on artificial agar medium.

2. Materials and methods

2.1. Samples

Samples were collected from decayed wood on ground sites at Huadian, Jilin province, P. R. China, in June 2008. They were preserved in the Mycological Herbarium of Jilin Agricultural University as specimens (HMJAU11725). These specimens were used for morphological observations for species identification and also for comparative morphology cultured on agar medium. Spores collected from these samples were used for cultures.

2.2. Morphological observations

Morphological observations were made by dissecting and light microscopy. Spores obtained from dried specimens on decayed wood and cultured from artificial agar medium were mounted in water on glass slides and were observed with a Jnoec dissecting microscope (JSZ4) and a Jnoec light microscope (XS-213). Photographs were taken with a Canon A2000 camera. 2–5 fruiting bodies, 20 capillitia and 20 spores were observed, obtaining average values (Wang and Li 2006).

2.3. Hanging drop culture

Hanging drop described by Keller and Schoknecht (1989a) were prepared for observation of spore germination with some modifications. Spores obtained from a sporangium were mixed in a droplet of nutrient solution on the undersurface of a 22-mm square cover glass. The cover glass was then inverted over a depression slide. Vaseline was used to ring the edges of the cover glass. Spores were freely suspended in the nutrient solution water droplet and cultured at 26 °C. Nutrient solution was prepared as follows: 40 g dried pine needles were boiled in 1000 ml water for 2 h, and this nutrient solution was filtered through multi-layer gauze and autoclaved for 20 min at 121 °C, and cooled at room temperature.

2.4. Oat–agar culture

The agar culture was prepared as described by Keller and Schoknecht (1989a) with some modifications. A hole was made in the middle of the agar surface with a punch (1 cm in diameter) then filled with 1–2 ml nutrient solution. Then, the spores from crushed sporangia were sown in nutrient solution. Petri dishes were cultured in an incubator in the dark at 26 °C. Plates were observed daily in the morning with a dissecting microscope and photographs taken.

Oat–agar medium was prepared as follows: 30 g SeaMild oats were boiled in 1000 ml water, adding 20 g agar to the filtrate. After the agar was dissolved fully water was added to bring the volume to 1000 ml. This mixture was poured into a flask and autoclaved for 20 min at 121 °C. After autoclaving the 10 ml of oat–agar mixture was poured into a 90 mm glass Petri dish.

3. Results

3.1. Identification and description of specimens

Sporangia gregarious, the spore case globose or oblate to subglobose, slightly umbilicate below and 0.3–0.5 mm in diameter. Stalk erect, tapering slightly, 0.5–1.4 mm in total height, wrinkled, dark brown in reflected light and red brown in transmitted light, with a brown, thickened and often persistent base. Peridium thin, white, membranous, densely encrusted with calcareous granules and with petaloid dehiscence. The base often like *Craterium*. Columella lacking. Capillitium consists of polygon white or yellowish calcareous nodes connected by hyaline threads. Spores dark brown in mass, light violaceous brown by transmitted light, asperulate to verruculose, 9–12 μm in diameter. Cultured sporangia compared to the descriptions in Flora Fungorum Sinicorum (Li et al. 2007) and the Myxomycete Biota of Japan (Yamamoto 1998), had characteristics similar to *P. pusillum*, but with some differences. The diameter of the sporangia on agar (0.3–0.5 mm) was smaller than its field-collected counterpart (0.4–0.6 mm). The total height of sporangia on agar (0.8–1.9 mm) was shorter than the field-collected sporangia (1–2 mm). Based on the morphological characteristics and microstructure of sporangia, the specimen from Huadian was identified as *P. pusillum* (Berk. & Curt.) G. Lister (Yamamoto 1998; Li et al. 2007).

3.2. Life cycle and morphology in culture

Spores germinated in 2 d and produced one unwalled myxamoeba by the split method typical of the Physarales (Fig. 1a). The myxamoeba changed into a swarm cell with a single

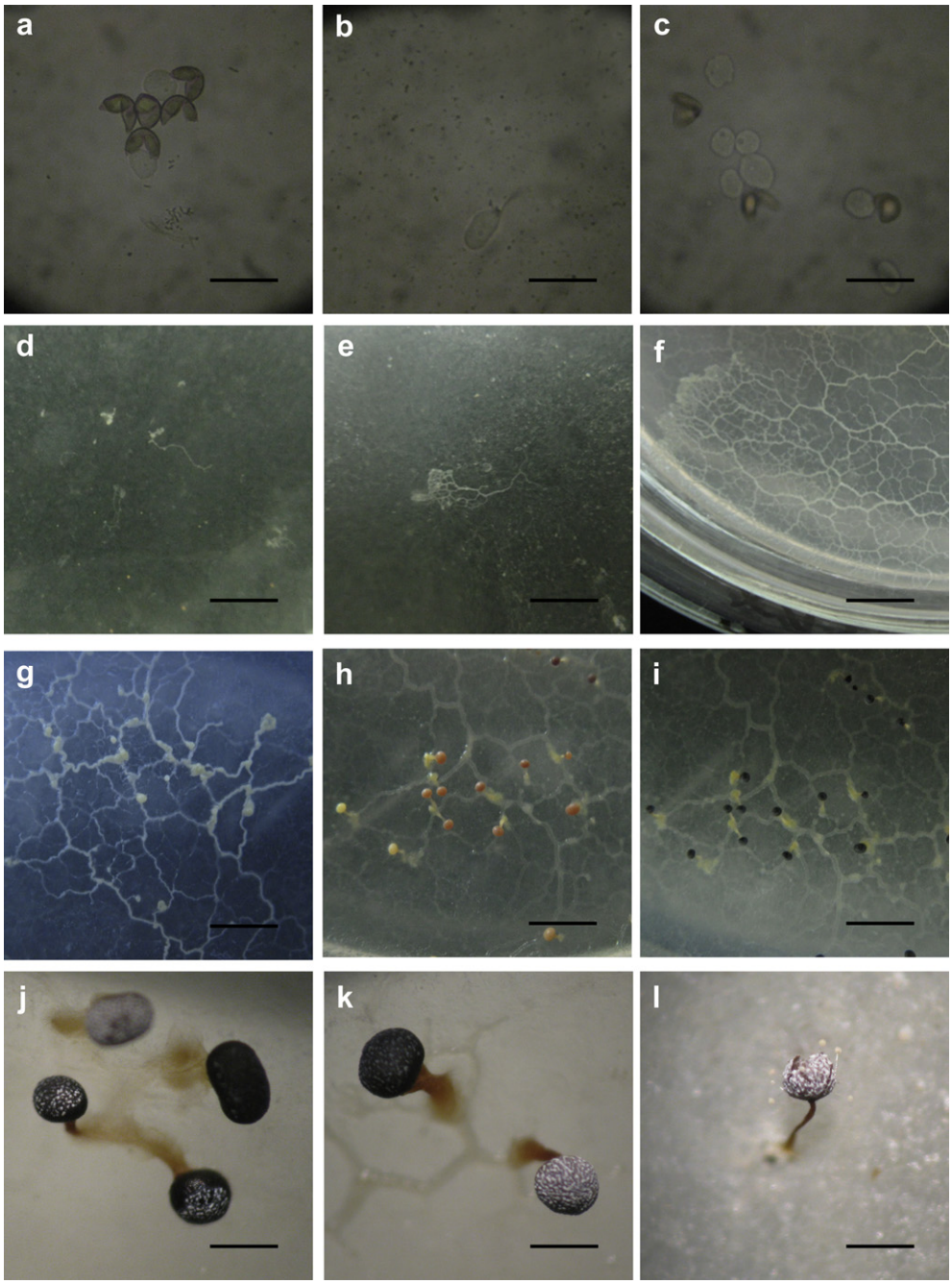

Fig. 1 – Process of *Physarum pusillum* development. a, spores germinating; b, swarm cell; c, myxamoebae; d, e, young phaneroplasmodia; f, plasmodia; g, plasmodia and protuberances; h, i, young stalked sporangia; j–l, mature stalked sporangia. Bars: a, 20 μm; b, 8 μm; c, 20 μm; d, 5 mm; e, 1 cm; f, 2 cm; g, 10 mm; h and i, 2.5 mm; j–l, 0.5 mm.

flagellum in 2 h. Only one flagellum was observed even though myxomycete swarm cells typically are unequally biflagellate. The short flagellum was possibly recurved and appressed to the surface of the swarm cell making it difficult to observe.

The swarm cells were colorless (Fig. 1b), 8.2–11.5 μm (average 10.2 μm) long and 2.6–4.1 μm (average 3.4 μm) wide. The swarm cell resorbed its flagellum and converted to a myxamoeba. When a mass of myxamoebae or swarm cells were present two swarm cells or two myxamoebae fused to form a zygote. The shape of the zygote was subglobose and the color was similar to the myxamoeba. Zygotes through a sequential nuclear division formed a multinucleate phaneroplasmodium.

Twelve days after spore inoculation, several young phaneroplasmodia (Fig. 1d and e) were found on the oat agar. Young phaneroplasmodia were thin, white and with a network of veins. As the phaneroplasmodia grew bigger, migrated together, and fused, the increase in size formed a mature phaneroplasmodium. After another 9 d, the mature phaneroplasmodia covered the oat–agar medium.

Mature plasmodia were white with a large fan-shaped network (Fig. 1f) and an anterior, thick feeding edge. When the phaneroplasmodium migrated across the surface of the oat agar there was excreted matter left behind along the margin of the veins that created tracks that easily were observed.

When mass phaneroplasmodia covered the whole oat agar medium cultures were put in a lower temperature incubator (20 °C) with diffused light that stimulated sporangia formation. About 21 d later, the mass phaneroplasmodia began to gather and formed several protuberances within the veins (Fig. 1g). Blebs increased in size and gradually formed the regular globose, young, stalked sporangia (Fig. 1h) in 2–4 h. Young stalked sporangia were bluish white, like the mature plasmodia. The diameter of the young sporangia was 0.9–1.3 mm, and the young stalks were no more than 1 mm in height. The type of sporangia development was myxogastroid (subhypothallic stalk development). This type was found in all orders of myxomycetes except the Stemonitales.

During the development of stalked sporangia the color of sporangia changed from orange, brownish red, brownish-black to black in mature sporangia (Fig. 1h and i) in 4 h, and the stalks changed in colors from entirely white to dull red at the base. With the loss of inner moisture the sporangia gradually dried undergoing color changes producing a gray peridium with calcareous deposits. The stalks eventually turned brownish (Fig. 1j and k). The life cycle of *P. pusillum* was completed in 44 d. Twenty days after sporangia matured dehiscence began (Fig. 1l) releasing black spores with reproductive capacity.

4. Discussion

Spores of myxomycetes germinate in water but in habitats the nutrient solution of the substrate like decayed bark, pine needles, or leaves apparently promote their spores germination. Shi and Li (2003) reported that the decayed pine bark or needles were better used to promote spore germination of most Physarales. In this study the spores of *P. pusillum* germinated in pine needles nutrient solution. The optimal germinating temperature of spores was usually at 22–30 °C. Spores of the Physaraceae did not germinate above 28 °C (Smart 1937) and spores of *P. pusillum* germinated in this study at 26 °C. The spores of virtually all Physaraceae germinated easily, therefore no addition of bilirubin (Elliott 1948) or cellulase (Koevenig 1964) was included.

Previous studies showed that (Collins and Clark 1966; Collins et al. 1978; Clark 1980; Clark et al. 1991; Liu et al. 2010), the young plasmodia migrated and fused to form a mature plasmodium. As the phaneroplasmodium grew bigger it usually migrated toward the drier inner wall of the agar plate. Therefore parts of the sporangia grew on the sides and lid of the plate. Hok (1954) reported the plasmodium would form sporangia when the temperature (22 °C) was controlled while at a lower temperature (under 20 °C) the plasmodium died.

Light is required to initiate reproduction in pigmented phaneroplasmodia, but that nonpigmented form sporangia equally well in light or in darkness (Gray 1938; Keller and Schoknecht 1989a,b,c). In our study, the young growing phaneroplasmodia of *P. pusillum* were comma like, growing quickly and fusing into a fan-shaped network plasmodium. Oat agar culture of *P. pusillum* produced plasmodia that grew quicker and fused better. When larger phaneroplasmodia of *P. pusillum* formed on agar the culture plate was moved to ambient light conditions. Five days later the phaneroplasmodium formed sporangia and the results were something similar as previously described. When the moisture was lowered, the peridium began to form calcareous deposits slowly. Removal of the plate lid induced drying of the sporangia more quickly. This demonstrated that the formation of calcareous deposits was related in part to both moisture and temperature.

The presence of calcareous deposits is usually an obvious feature of the Physarales (Keller and Braun 1999). The species resembles P. *nutans*, which may be found in the same habitat and sometimes appears mixed with it. However it can be distinguished by the translucent reddish stalk without calcareous deposits (Nannenga-Bremekamp 1991). The colors of sporangia of *P. pusillum* obtained from agar culture were different from those obtained from natural substrates. This may be related to the formation time of calcareous deposits. When the formation time of the calcareous deposit was long, the sporangium was white, rugose, and fragile. In contrast, when the formation time of calcareous deposit was short, the color of the sporangium was black, smooth, and unbroken. Other characteristics of this species from agar culture were the same as for mature stalked sporangia obtained from the field and in both a brown, thickened and often persistent base were present (Martin and Alexopoulos 1969). The capillitium consisted of irregular or stellate calcareous nodes connected by hyaline threads (Keller and Braun 1999).

The culture conditions of axenic culture for myxomycetes are changeless, and the characteristics of this species from agar culture are the same as for mature stalked sporangia obtained from the field. Through the observation of life cycle stages, finding the classification characteristics of myxomycetes to clarify the classification.

Acknowledgments

We gratefully acknowledge many helpful discussions with Prof. Harold W. Keller. This study was supported by the National Natural Science Foundation of China (Project Nos. 70670015 & 71070012).

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