Ann Microbiol (2015) 65:765–770
DOI 10.1007/s13213-014-0916-x

ORIGINAL ARTICLE

Morphological development of sclerotia by *Sclerotinia sclerotiorum*: a view from light and scanning electron microscopy

Claudia Ordóñez-Valencia · Ronald Ferrera-Cerrato · Rafael Emiliano Quintanar-Zúñiga · Cesar Mateo Flores-Ortiz · Guadalupe Judith Márquez Guzmán · Alejandro Alarcón · John Larsen · Oscar García-Barradas

Received: 14 February 2014 / Accepted: 6 May 2014 / Published online: 28 May 2014
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Abstract *Sclerotinia sclerotiorum* is a worldwide pathogen with a broad host spectrum pathogenic to around 400 plant species. Sclerotia formed by *S. sclerotiorum* serve as resting structures that secure fungal survival in soil for prolonged periods in the absence of a host plant or may help to overcoming periods of unsuitable growth conditions. In the present study, the morphological development of sclerotia was examined by light and scanning electron microscopy of fungal microcultures. Observations from microscopy indicated that, during the first 4 days of culture, the sclerotial primordial originate by dichotomous branching of apical hyphae and from the 5th day mycelial clusters were also observed, indicating the initiation stage of sclerotia formation. From the 6th to the 8th day, sclerotia turned from white to dark color, and water drops (exudates) were observed on their surface. The process of sclerotia formation ended at the 9th day when they were easy to detach from the culture medium and had a black coloration. All the morphological processes involved in the formation of sclerotia by *S. sclerotiorum* were observed with both light and scanning electron microscopy.

Keywords Hyphal branching · Sclerotia development · Sclerotia maturation

C. Ordóñez-Valencia · R. Ferrera-Cerrato · A. Alarcón (✉)
Área de Microbiología, Postgrado de Edafología, Colegio de Postgraduados, Campus Montecillo, Carretera México-Texcoco km 36.3, Montecillo, Estado de México 56230, Mexico
e-mail: aalarconcp@gmail.com

R. E. Quintanar-Zúñiga · C. M. Flores-Ortiz
Laboratorio de Fisiología Vegetal, Facultad de Estudios Superiores Iztacala, UNAM, Windhoek, Estado de México, Mexico

G. J. M. Guzmán
Laboratorio del Desarrollo en Plantas, Facultad de Ciencias, UNAM, México, DF, Mexico

J. Larsen
Laboratorio de Agroecología, CIEco-UNAM, Morelia, Michoacán, Mexico

O. García-Barradas
Unidad de Servicios de Apoyo en Resolución Analítica (SARA), Universidad Veracruzana, Veracruz, Mexico

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is an aggressive plant pathogen that significantly affects the yield of several crops (Hegedus and Rimmer 2005; Bolton et al. 2006). This fungus is distributed worldwide, and does not possess specificity for any particular plant species (Fernando et al. 2004). *S. sclerotiorum* may form melanized sclerotia (hyphal clusters) that are resistant to physical, chemical and biological degradation, and thus provide long term fungal survival in soil (Bolton et al. 2006; Wu and Subbarao 2006). Sclerotia play a significant role in the biological cycle and infectivity of *S. sclerotiorum* since this structure is the main propagule for fungal dispersion (Rollins and Dickman 1998).

Typically, sclerotia formation presents three stages: (1) initiation, in which hyphal tips start to aggregate; (2) development characterized by growth of hyphae and increase in size; and (3) maturation, which involves internal matrix consolidation and melanization (Rollins and Dickman 1998; Bolton et al. 2006). These three stages are processes accompanied by morphological and biochemical differentiation (Chet and Henis 1975).

Once the sclerotia have matured it is possible to observe three layers: rind, cortex and medulla (Willetts and Wong

1971), but some researchers recognize only two layers: rind and medulla (Colotelo 1974; Arseniuk and Macewicz 1994). The rind is the outer layer and is composed by hyphae closely organized to form a continuous layer with swelled tip (Colotelo 1974). The cortex, when distinguished, is the thin intermediate region composed by pseudoparenchymatous cells (Willetts and Wong 1971). Finally, the medulla covers most of the internal structure of the sclerotia, and is constituted by prosenchymatous tissue (Arseniuk and Macewicz 1994).

Information on the structural development of sclerotia from light microscopy (Townsend and Willetts 1954; Willetts and Wong 1971) and scanning electron microscopy (SEM) studies (Colotelo 1974; Arseniuk and Macewicz 1994) is available, focusing on the analysis of the ultrastructure of *Sclerotium cepivorum* (Backhouse and Stewart 1987), *S. rolfsii* (Chet 1975; Zarani and Christias 1997), *Sclerotinia minor* (Bullock and Willetts 1996), and *Botrytis allii* (Sadeh et al. 1985). However, information on sclerotia formation of *S. sclerotiorum* is limited. Thus, the aim of the present study was to microscopically describe the morphogenesis of sclerotia of *S. sclerotiorum* by combining light microscopy and SEM.

Materials and methods

Growth reactivation of *Sclerotinia sclerotiorum*

The strain of *S. sclerotiorum* strain was previously isolated from a garlic crop at El Bajio, Guanajuato (México), identified morphologically by following specialized fungal keys and literature (Wong and Willetts 1975; Willetts and Wong 1980; Ekins et al. 2005) and maintained in the microbial culture collection. Fungal growth was achieved in potato dextrose agar medium (PDA, Merck, Darmstadt, Germany) incubated at room conditions (~22 °C) until sclerotia formation was observed (9 days). Further fungal growth was obtained for the following experimental stages.

Sclerotinia sclerotiorum microcultures for light microscopy evaluation

The formation of sclerotia was evaluated via light microscopy of fungal microcultures (Riddell 1950). Every 24 h for 4 consecutive days, fungal microcultures were prepared and

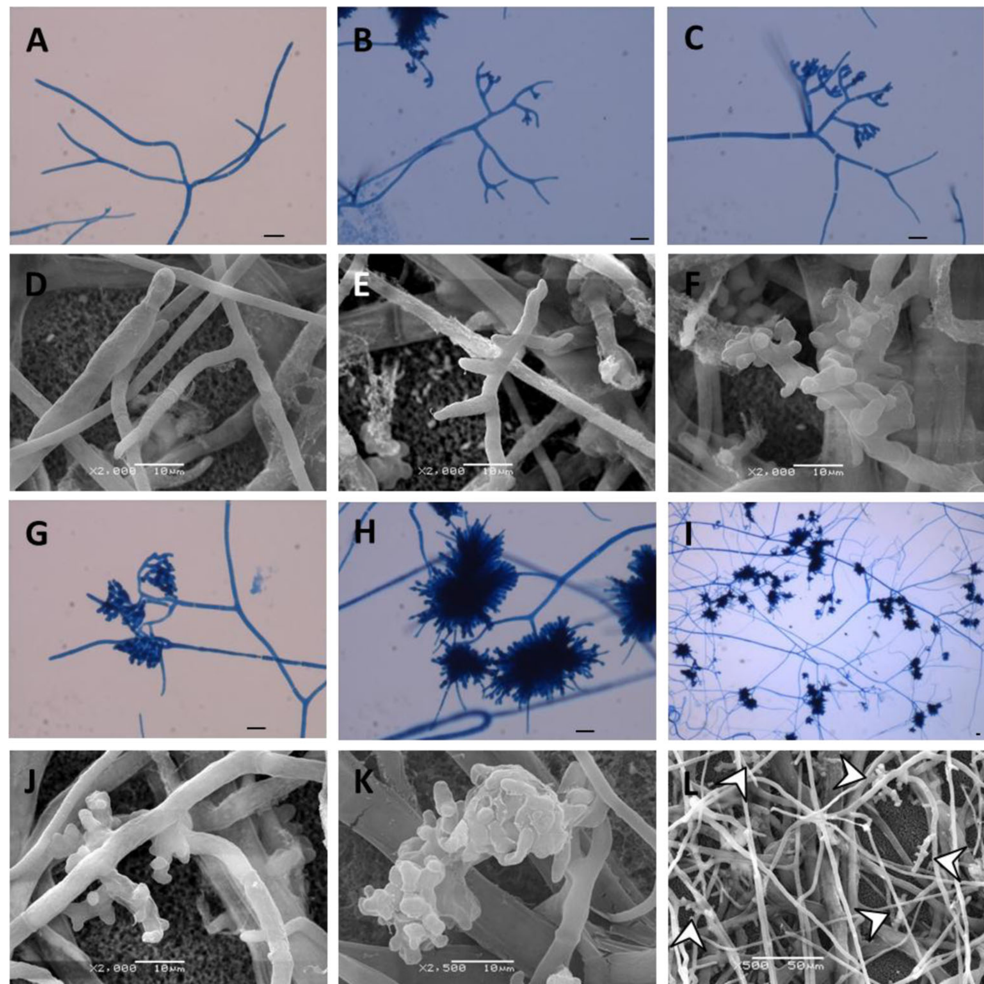

Fig. 1 Microphotographs of light microscopy (**a**–**c**, **g**–**i**) and scanning electron microcopy (SEM) (**d**–**f**, **j**–**l**) showing the early development of sclerotia by *Sclerotinia sclerotiorum*, prior to the initiation stage. **a**, **d** Growth of hyphae tips (24 h). **b**–**c**, **e**–**f** Dichotomous branching of hyphae (48 h). **g**, **h** Initial formation of hyphal clusters (sclerotia primordia) and pigmentation (72 h). **i**–**l** Formation of several sclerotial primordia. *Bars* (**a**–**c**, **g**–**i**) 50 μm

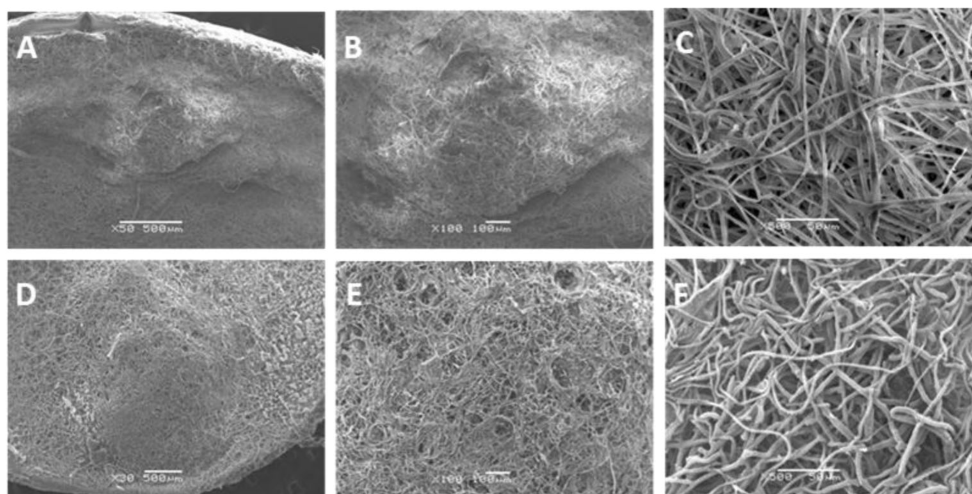

Fig. 2a–f Differentiation of sclerotia by *S. sclerotiorum*. **a**–**c** Initiation stage, after 5 days of incubation. **d**–**f** Slight knob with intrincated mycelium, after 6 days of incubation

observed with an optical microscope (Carl Zeiss model III, Zeiss, Jena, Germany).

Sclerotinia sclerotiorum microcultures were prepared for SEM evaluation. Petri dishes (n=6) with PDA were utilized, and four sterile nylon membranes (1 cm of diameter) were placed on the agar surface near to the edge of each Petri dish. Then, an agar disk with fresh growing mycelium of *S. sclerotiorum* was placed in the center of each Petri dish. The fungal cultures were incubated at room temperature (~22 °C). Membranes were collected from fungal cultures starting at the 4th day to the 9th day when mature sclerotia were observed. Each membrane was fixed in FAA (formaldehyde, acetic acid, ethyl alcohol). Furthermore, membranes were exposed to a dehydration process consisting in immersion in consecutive solutions of ethanol (30 %, 50 %, 70 %, 80 %, 90 %, and 100 %) for 1 h each, but with two changes for the 100 % ethanol solution. Once dehydrated, membranes were critical-point dried with CO_2 (Tousimis, Samdri-780A, Rockville, MD). Membranes were then coated with gold by means of an ionization chamber (Denton Vacuum Desk IV, Moorestown, NJ), and observed by SEM (JEOL JSM-6380LV, Peabody, MA).

Results and discussion

The maximum fungal growth in Petri dishes was achieved after 4 days, and aerial hyphae and aggregation were observed at the edge of Petri dishes, where white dense hyphal aggregations provided indications of the initiation of sclerotia formation. After day 5, the sclerotial primordial turned grayish

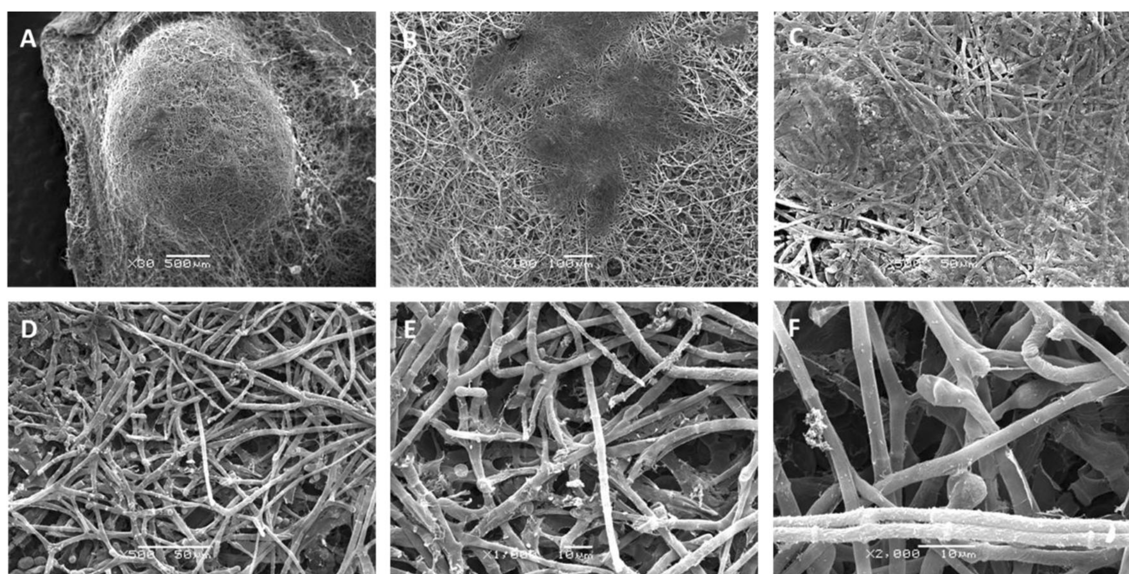

Fig. 3a–f Sclerotium in the developmental stage, after 7 days of incubation. **a** Bigger knob of developing sclerotium. **b** Intricate mycelium that will form the origin of the sclerotium rind. **c**–**f** Magnification of the outer layer of the sclerotium

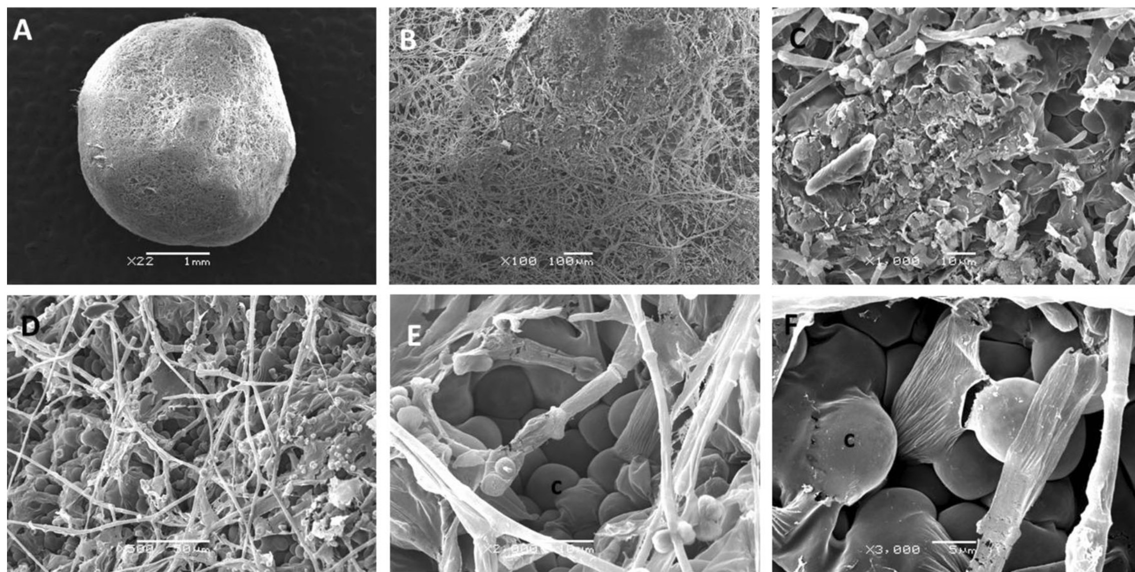

Fig. 4a–f Sclerotium at maturation stage, after 8 days of incubation. **a** Rounded structure. **b** Mycelium covering the sclerotium surface. **c** Membranous layer developing on the sclerotium surface. **d** Hyphae protruding into the outer surface. **e**–**f** Swelling cells (*c*) of hyphal tips

and had water drops on their surface. At day 9, sclerotia turned dark with a hard consistency, and no water secretions were observed (data not presented). The above mentioned preliminary observations determined the best days to perform microscopic analysis for monitoring sclerotial formation by *S. sclerotiorum*. In accordance with Rollins and Dickman (1998) and Saharan and Mehta (2008), sclerotia typically form when the mycelia completely covers the agar surface, due to limited nutrient availability or as a response by the mycelia to unidentified metabolites, or when the fungal colony reaches a particular physiological stage. Additionally, the pH of the growth medium can significantly influence sclerotia formation (Bolton et al. 2006; Ordóñez-Valencia et al. 2009).

Observations of the initiation stage of sclerotia formation are limited since it is difficult to predict the specific sites of mycelium in which sclerotia originate (Willetts and Bullock 1992). Based on light microscopy and SEM observations (Fig. 1) in the present study, it was determined that the mycelia form small sclerotial primordia at the initiation stage in which hyphal tips showed repeated dichotomous branching (Fig. 1a–f), and, as growth continues, hyphal clusters are also formed from which sclerotia develop (Fig. 1g–l). Similarly, Willetts and Wong (1971)

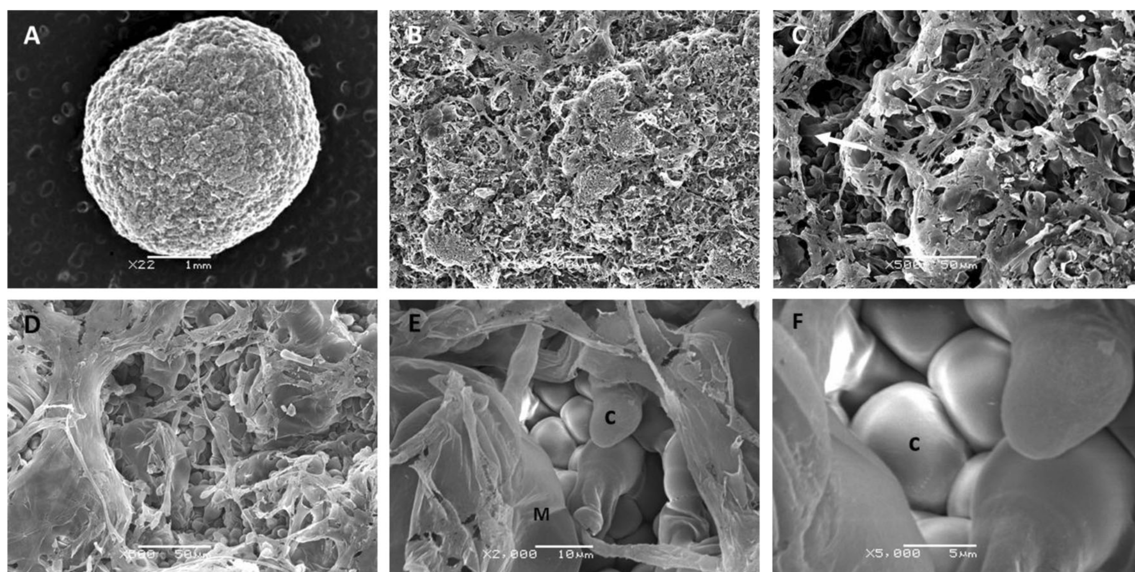

Fig. 5a–f Mature sclerotium, after 9 days of incubation. **a** Mature sclerotia. **b** Rind with rough texture. **c** Surface cavities (*arrow*). **d** Inner cortex. **e**, **f** Swelling cells (*c*) with smooth texture often covered by a membranous material (*M*)

described sclerotial primordia and further branching in some species of *Sclerotinia* (*S. sclerotiorum*, *S. trifoliorum*, *S. libertiana* and *S. minor*). At 24 h, the branching of primary hyphae was characterized by acute angles (Fig. 1a,d), and sclerotial pimordia started when hyphal growth stopped, and tips showed continuous branching (Fig. 1b–c, e–f). This branching initiated numerous pigmented hyphal masses (Fig. 1g–i, j–l). It seems that, as discussed by Erental et al. (2008), branching and fused hyphae are the main process for sclerotia formation. Figure 1h, i and k show several sclerotial primordia developing in close proximity, by which they may fuse to form one sclerotium, whose size depends on the number of fused primordia as described by Willetts and Wong (1971). Sclerotia may be involved during the infection process of the pathogen in planta, as reported by Garg et al. (2010), who observed sclerotia after 2 days of infection by *S. sclerotiorum* and *S. minor* in *Brassica napus* and *Phaseolus vulgaris*.

The initiation stage of sclerotia formation was visualized by SEM after 5 days of fungal incubation when small white, cotton-like hyphal clusters were observed on the nylon membrane. Cooke (1970) described this specific sclerotial differentiation as "age zero", which is characterized by the presence of hyphal masses that are difficult to remove from the culture medium. Microscopically, during this particular stage, the fungal surface looked like intricate mycelial masses that originate from a slight knob (Fig. 2), which will eventually mature to complete the formation of a sclerotium. In addition, Erental et al. (2008) indicated that this fungal process is accompanied by secretion of a mucilage-like substance, which may serve as a cementing agent for hyphae.

During the development stage, hyphae coalesced and became compacted, thus resulting in a slightly rounded structure (Fig. 3) and the mycelia covering its surface originated from a solid tissue that subsequently constituted the sclerotia rind layer. Once the sclerotium was formed, the increase in size was fast (24–48 h approximately) and, during this stage, small drops of water were observed on its surface. In this regard, Willetts and Wong (1980) indicated that these water drops of the sclerotia contain salts, carbohydrates, lipids, amino acids, proteins, and enzymes such as phenol-oxidases, peroxidases, catalases, and glucosidases.

For the maturation stage, the sclerotium was observed to increase in size and could be detached easily from the mycelium (Fig. 4). After 7 days, the sclerotium was surrounded by hyphae (Fig. 4b), and the apex of the hyphae of the inner part began to swell and protruded into the outer surface, to form the rind (Fig. 4d–e). In addition, a membranous material covering the cells was observed (Fig. 4f). Colotelo (1974) suggested that this membranous material corresponded to the collapsed remains of exudates released during sclerotium formation. In this regard, Willetts and Wong (1980) indicated that, at this stage, the sclerotia also pass through deposition processes such as structural and storage polysaccharides, pigmentation, and tissue desiccation.

After 9 days of fungal growth, the mature sclerotium had dark pigmentation with round shape, rough texture with the presence of several cavities (Fig. 5). Since the sclerotium was already dehydrated, it did not show water drops on the surface. This pattern of sclerotium formation concurs with the description provided by Colotelo (1974) and Erental et al. (2008) for *S. sclerotiorum*. During the maturation stage, the sclerotia surface becomes pigmented due to melanin production, which plays an important role in protecting the fungus against UV radiation or for exacerbated production of reactive oxygen species, and to avoid attack from certain antagonistic microorganisms (Bolton et al. 2006; Erental et al. 2008).

Overall, the proliferation of vegetative mycelium by *S. sclerotiorum* is the previous fungal stage that initiates the formation of sclerotia and, during this growth period (4 days under our experimental conditions), the mycelium can absorb essential nutrients required by the fungus for further development, as described by Fernando et al. (2004). Thus, structural and reserve components already incorporated into mycelial tissue are subjected to degradation processes mediated by the activity of specific enzymes (e.g. arylesterase or acid phosphatase) to provide energy and release of those nutrients required during sclerotia development (Willetts and Bullock 1992). Nevertheless, it should be borne in mind that the development of sclerotia, as well as the majority of fungal structures, depends on the growth and incubation conditions.

The present microscopy study of *S. sclerotiorum* provides information on the morphological stages of the sclerotia formation that plays a key role in the biological cycle of this important plant pathogen.

Acknowledgments This research was partially supported by the National Council for Science and Technology (CONACYT, Mexico) by the following grants: SEP-CONACYT 58594 and SEP-CONACYT 130262. Claudia Ordóñez-Valencia is grateful for financial support during her PhD studies.

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