Development of merosporangia in *Linderina pennispora* (**Kickxellales, Kickxellaceae**)

Mohamed E. Zain¹², Steve T. Moss¹, and Hussein H. El-Sheikh²

¹Center of Excellence in Biotechnology Research, King Saud University, Riyadh, Saudi Arabia; corresponding author e-mail: Mohamed E. Zain, mzain@ksu.edu.sa
²Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt

³[† Deceased 2001] School of Biological Sciences, University of Portsmouth, King Henry Building, King Henry 1 Street, Portsmouth PO1 2DY, UK

Abstract: The vegetative and sporulating structures of *Linderina pennispora* are described using scanning and transmission electron microscopy. The vegetative hyphae and sporangiophores were regularly septate, possessed a two-layered wall, and coated with rod-shaped, 0.2–0.7 µm long, 0.15–0.25 µm wide ornamentations. The sporangiophore was erect, cylindrical, and narrower (4–8 µm) than the vegetative mycelium (8–12 µm). The mature sporocladium was ovoid to dome-shaped, sessile, non-septate, 18–24 µm diam, possessed a two-layered wall, and coated with rod-shaped ornamentations. Mature pseudophialides were ellipsoid, 2.0–2.5 µm wide, 4–7 µm long, possessed a two-layered wall, and formed in a series of concentric groups radiating from the “apex” of the sporocladium. The pseudophialides had a round, ca. 1.5 µm diam, base with a narrower, 0.7–0.8 µm diam lobed, cylindrical neck structure in the distal region which extended to the pseudophialide neck. The merosporangia were obovate, 3–4 µm wide near the base, and narrowed distally to 2.0–2.5 µm wide, 18–23 µm long, possessed a three-layered wall, with regular surface annulation with interconnecting ridges, but lacked rod-shaped ornamentations. The merosporangia contained a single, obovate, 2.3–2.5 µm diam merosporangiospore, with a ca. 1 µm diam papilla-like base, that possessed a four-layered wall. Detached merosporangia had a single, acicular, unbranched, 3–5 µm long, ca. 0.1 µm diam “appendage” that was attached to the merosporangiospore inner cell wall layer and passed through the septum plug to the pseudophialide.

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INTRODUCTION

The order **Kickxellales** was established by Kreisel (1969) to accommodate the families **Kickxellaceae** and **Dimargaritaceae**. Benjamin (1979) suggested the separation of the **Dimargaritaceae** and the **Kickxellaceae** at the ordinal level, within the Zygomycetes. He established the order **Dimargaritales** for the **Dimargaritaceae**, and retained the order **Kickxellales** for the family **Kickxellaceae** (Benjamin 1979). Although the **Kickxellales** had been classified traditionally within the class Zygomycetes, recently it was segregated from other orders of this class to establish the subphylum **Kickxellomycotina** with **Harpellales** and **Asellariales**, and **Dimargaritales** (Hibbett et al. 2007, Kurihara et al. 2008).

**Kickxellales** are phylogenetically closest to the harpellalean genus **Orphella** (White 2006). Most species of **Kickxellaceae** are saprobess and are commonly isolated from soil, dung, humus, dead insects, or other organic debris. However, **Martensella pectinata** and **M. corticii** are obligate mycoparasites (Kurihara et al. 2008). The classification of **Kickxellaceae** and its genera has undergone several changes (Moss & Young 1978, Young 1985, Benny 1995, O’Donnell et al. 1998, Hibbett et al. 2007). The family currently contains 12 genera (Kirk et al. 2008, Benny 2012).

Young (1974) described a labyrinthiform organelle within the pseudophialides of *Kickxella alabastrina*, and a similar structure was demonstrated by Benny & Aldrich (1975) within the pseudophialides of *Linderina pennispora*. They coined the term “abscission vacuole” for that structure, and believed that this was responsible for the detachment of the merosporangia from their pseudophialides. Moss & Young (1978) demonstrated that *Kickxellaceae* (Zygomycetes) were are closely related to **Harpellales** and **Asellariales** (**Trichomycetes**) on the basis of the septa; they consist of a cross-wall with a central pore occluded by a biumbonate plug, the form of the asexual reproductive apparatus, and the similar wall structure. The labyrinthiform organelle in *Kickxellaceae* was speculated to be analogous to the trichospore appendage of *Harpellales* (Moss & Young 1978, Young 1985). This contribution describes the ontogeny of the sporulating structures of *Linderina pennispora*.
MATERIALS AND METHODS

Isolate
The isolate used in this study was *Linderina pennispora* (IMI 174729) provided as a culture from CABl Bioscience (Egham, UK). Malt extract agar (20 g Difco malt extract, 20 g dextrose, 1 g peptone, 20 g agar, 1 L distilled water) was used for experimental studies and maintenance of a stock culture during the investigation.

Scanning Electron Microscopy
Colonized agar squares of 6–8 mm with sporulating material were fixed in 2 % (w/v) aqueous osmium tetroxide (OsO₄) at 4 °C for 12 h in the dark, and then washed in distilled water. Fixed and washed material was dehydrated through a graded (10 % steps) ethanol series from 10–90 %, and finally absolute ethanol. The absolute alcohol was replaced with acetone via a stepwise series (ethanol: acetone 3:1, 1:1, 1:3), and then finally maintained in water-free acetone for 1 h with three changes. Specimens dehydrated to acetone were critical-point dried using a Polaron E3000 apparatus with liquefied carbon dioxide as the drying agent. Using a stereomicroscope, the critical-point dried specimens were orientated and then attached to 2.5 cm diam aluminium stubs with carbon adhesive, and allowed to dry in a desiccator for at least 12 h. Specimens were coated with gold-palladium with carbon adhesive. Using a JEOL T20 scanning electron microscope at 20 KV. Specimens were used for experimental studies and maintenance of a stock culture during the investigation.

Transmission Electron Microscopy
Fungal material (ca. 2 mm³) was fixed in 1 % (w/v) aqueous potassium permanganate for 5 min at 20 ± 2 °C. Fixed material was then washed in distilled water for 15 min. Some fungal materials were fixed in 4 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, two changes each of 15 min, and then post-fixed in 2 % (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer, 2 changes each of 15 min. Fixed material was dehydrated through a graded ethanol series following the procedure described for SEM. Dehydrated specimens were embedded in an epoxy resin. For material fixed with potassium permanganate, agar 100 resin, 31 mL, DDSA (hardener) (dodecenyl succinic anhydride), 50 mL and DMP-30 (accelerator; 2.4 mL) were used. For material fixed with glutaraldehyde-osmium tetroxide, mixture A [agar 100 resin (62 mL) and DDSA (100 mL)], mixture B [agar 100 resin (100 mL) and NMA (89 mL)], and BDMA (benzyl-dimethyl amine) were mixed in the ration (3:7:0.15). Dehydrated specimens were infiltrated with the resin through a graded series (Resin : acetone (1:3), (1:1), (3:1) for 24 h with rotation at room temperature for each grade).

The resin was polymerised at 60 °C for 72 h and then allowed to cool to room temperature in a desiccator for 24 h. Flat embedded material was examined with a light microscope and different stages of fungal development identified. Selected specimens were cut from the flat blocks, glued to resin stubs in the desired orientation, and placed in the oven at 60 °C for 24 h to allow polymerization of the glue. Using a stereomicroscope, the mounted blocks were trimmed with razor blades to give a trapezoid-shaped block face, less than 1 mm in width and height with, when possible, most of the block faces comprising the embedded fungal material. Surface resin was trimmed off the block face using a Cambridge Instruments Huxley MK2 ultramicrotome and glass knives.

Ultrathin sections were cut with a LKB MRIII ultratome and the sections floated onto water. The knife had a clearance angle of 4° and the block a cutting speed of 1 mm/s; for routine work, silver-grey or gold (30–60 nm) sections were cut. Sections were then flattened with chloroform vapour and, using an eye-lash, sections were manipulated to the centre of the boat prior to picking up on hexagonal copper mesh (3.05 mm pores), coated with Parlodion (2 % Parlodion in amyl acetate) grids. Sections were double stained in a carbon dioxide-free atmosphere with lead citrate (Pb.[C₆H₅O₇]₂) followed by uranyl acetate. The lead citrate solution was centrifuged for 15 min to remove any precipitate and then single drops of the supernatant were transferred onto dental wax. A single grid was floated on to each drop of the stain, with sections facing the stain, for 15 min. Stained sections on grids were washed with 0.02 N NaOH followed by distilled water and then stained with uranyl acetate for 30 min in the dark. Stained sections were examined with a JEOL 100S transmission electron microscope at 80 KV.

RESULTS

*Linderina pennispora* grows on malt extract agar (MEA), with sporulation occurring at 20 °C within 6–8 d of inoculation. The axenic cultures were yellow, and having two types of hyphae occurred; submerged and aerial. Sporangiophores were erect, “zigzag”-shaped, and produced from submerged hyphae. The asexual apparatus was composed of a non-septate, dome-shaped sporocladium that produced numerous pseudophialides. Each pseudophialide produced a single merosporangium (Fig. 1F).

Sporocladias
The mature sporocladium was ovoid to dome-shaped, sessile, non-septate, 18–24 µm diam, and coated with rod-shaped ornamentation similar in shape but fewer in density per unit area than ones coating the sporangiophore (Fig. 1B). Five to eight sporocladia were arranged on alternate sides of the sporangiophore. The sporocladium initials were initially apical (Fig. 1A), but sympodial branching of the sporangiophore beneath the sporocladium later displaced the mature sporocladium laterally. The sporocladium initials were spherical, and coated with rod-shaped ornamentation. Each sporocladium produced several pseudophialide initials.

The wall was two-layered, similar in nature to the sporangiophore cell wall, with an outer electron-dense and an inner less electron-dense layer. The wall of the sporocladium was irregularly undulate, which may represent the early stages of pseudophialide formation (Fig. 1C).

Pseudophialides
Pseudophialides were initially spherical, but with maturity became ellipsoid, non-septate, 2.0–2.5 µm wide, 4–7 µm long, and possessed a two-layered wall. They formed in
Merosporangia of *Linderina pennispora*

A series of concentric groups radiating from the “apex” of the sporocladium. Pseudophialides at the “apex” of the sporocladium form first, and those at the periphery, last (Fig. 1D). The pseudophialides were produced holoblastically from the sporocladium. Only the peripheral pseudophialides possessed surface ornamentation and each arose approximately perpendicularly to the surface of the sporocladium. The distal region of the pseudophialides comprised a 1–1.5 µm diam neck region which lacked surface ornamentation (Fig. 1J). The necks were formed at the apex of the pseudophialides on those at the centre of the cluster, but subterminally and towards the inner pseudophialides on those at the periphery. Each pseudophialide produced a single merosporangium.

An different structure occurred in the distal region and extended to the pseudophialide neck (Fig. 1I). Here the pseudophialide had a round, ca. 1.5 µm diam base and a narrower, 0.7–0.8 µm diam, lobed, cylindrical neck.
The structural cell membrane was contiguous with the membrane of the septum, between the pseudophialide and merosporangium cross-walls. Here the pseudophialide necks were cylindrical, 1.2–1.4 μm long, ca. 1–1.1 μm diam, with a septum delimiting the merosporangium (Fig. 1E). Pseudophialides from which merosporangia had been released had a flared-shape structure from the merosporangium cell wall attached to the pseudophialide neck; the septum and septal plug, and structure within the pseudophialide, was shrivelled (Fig. 1J).

**Merosporangia and merosporangiospores**

Immature merosporangia were obovoid, whereas mature ones were obovate. Merosporangia matured first on those pseudophialides towards the “apex” of the sporocladium with those on the peripheral pseudophialides the last to mature. The merosporangia of *L. pennispora* were obovate, 3–4 μm wide near the base, narrowed distally to 2.0–2.5 μm wide, 18–23 μm long, and possessed regular surface annulation with interconnecting ridges, but lacked any rod-shaped ornamentation (Fig. 1G).

The merosporangia were produced terminally or subterminally and singly on the pseudophialides. A septum formed at the apex of the pseudophialide neck delimited the merosporangium from the pseudophialide. Merosporangia had a three-layered wall continuous with the pseudophialide wall. The merosporangium wall comprised an outer, 150–200 nm thick, electron-opaque layer; a middle, 200–250 nm thick, electron-opaque layer; and an inner, 40–50 nm thick, electron-dense layer (Fig. 1H). Each merosporangium contained a single merosporangiospore.

The merosporangiospore was obovate, 2.3–2.5 μm diam, with a ca. 1 μm diam papilla-like base. The merosporangiospore had a four-layered wall: an outer, 2–5 nm thick, electron-dense layer; adpressed to the outer layer, a thick, 5–10 nm, electron-dense layer; and an innermost fourth, 90–100 nm thick, amorphous, electron-transparent layer. The merosporangiospore wall was contiguous with the merosporangium wall at the distal region, and separated by an electron-opaque layer at the base of the merosporangium. Detachment of the merosporangia from their pseudophialides occurred at the base of the merosporangium. Detached merosporangiospore possessed a single, 3–5 μm long, “appendage” that was attached to the base of the merosporangium (Fig. 1I). The appendage was acicular, unbranched, ca. 0.1 μm diam and attached to the merosporangiospore inner cell wall layer and passed through the septum plug to the pseudophialide. Pseudophialides from which merosporangia had been released possessed a flared collar-like distal region with the septum, which delimited the pseudophialide from the merosporangium, retained at the base of the collar (Fig. 1J).

The detachment of the merosporangium from the pseudophialide occurred by rupture of the merosporangium wall near the base, when the merosporangiospore wall becomes coated with the electron-opaque layer (Fig. 1K). When the merosporangium has been detached, a part of the merosporangium wall remained attached to the pseudophialide neck, as well as the septum between the pseudophialide and merosporangium. The cytoplasmic layer between the merosporangiospore base and the merosporangium wall appeared as a spherical-shape structure attached to the septal-plug.

**DISCUSSION**

Aerial hyphae of *Linderina pennispora* were investigated previously at the ultrastructure level (Young 1969, 1970b, Benny & Aldrich 1975). The hyphae had a two-layered wall which comprised an outer amorphous layer and an inner fibrillar layer (Young 1969, 1970b, Benny & Aldrich 1975). Numerous spines were described as attached to the outer layer of the hyphal wall (Young 1970b). Benny & Aldrich stated that the spines were attached to the inner layer of the wall and appeared to be covered by material from the outer wall layer (Benny & Aldrich 1975). The results presented here show that the surface ornamentations of the aerial hyphae of *Linderina pennispora* appears rod-shaped rather than spine-like. The ornamentation is attached to the outer layer of the hyphal wall. It is fibrillar, electron-dense, and seem to be derived from the same material as the outer layer. There was no definite description for the sporangiophore in all the previously published studies on the morphology of the species. This study revealed that the sporangiophore of *L. pennispora* arose as a lateral branch of the vegetative hyphae. The sporangiophores are narrower in diameter than the vegetative hyphae, and the ontogeny of the sporangiophore and its sympodial growth are described here for the first time, and explain the diagnostic “zigzag” form of the sporangiophore.

Benjamin (1966) described the sporocladia of *Kickxella-ceae* species as the most highly developed sporiferous branchlets in *Zygomycetes*. The present study provides details of the sporocladia and their ontogeny. The sporocladium initials are produced terminally by the sporangiophore, and, when the sporangiophore resumes its growth, the sporocladia are displaced laterally. The terminal sporocladium is displaced after the formation of the merosporangia, particularly at the late stages of merosporangia development. Benny & Aldrich (1975) observed the surface ornamentations of pseudophialides of *L. pennispora* and stated that they were coated with fewer rod-shape surface ornamentations than the sporocladium, and comprised a structure they termed an “abscession vacuole”. However, Young (1974) described a similar structure in the pseudophialides of *Kickxella alabastrina*, and then termed the structure a “labyrinthiform organelle” based on its morphology. A similar structure was also demonstrated in the pseudophialides of *Dipsacomycyes acuminosporus* and *Martensiomycyes pterosporus* (Young 1968). Benny & Aldrich (1975) suggested that this structure was related to the abscession and dispersal mechanism of the wet-spored species of *Kickxellaceae*. They believed that this structure was produced from the septum delimiting the merosporangium (Benny & Aldrich 1975).

Our electron microscopic studies of the pseudophialides of *Linderina pennispora* show, for the first time, a concentric arrangement of the pseudophialides on the sporocladium, and that only the peripheral pseudophialides are coated with a rod-shaped ornamentation. Ultra-thin sections
showed this structure was in the distal, neck region of the pseudophialides. The structure has a round base located in the distal part of the pseudophialide, with a cylindrical neck occupying the whole of the remaining pseudophialide neck. The structure is covered with a membrane-like layer contiguous to both the septum cross walls and the inner wall of the neck. The electron microscopy results of the merosporangia ontogeny and its detachment confirms that this structure has no role in its release, in addition this confirmed that the merosporangiospore appendage contains the appendage. Consequently, in future this structure would be better termed “appendage sac” rather than abscission vacuole or labyrinthiform organelle.

The ultrastructure of the merosporangia of *Linderina pennispora* has been the subject of many previous studies (Young 1968, 1970a, 1971, Benny & Aldrich 1975, Moss & Young 1978, McKeeown et al. 1996). However, no obvious differentiation was made between the merosporangium and the merosporangiospore since some of these studies used the term “spore” (Young 1968, 1970a, 1971) without any reference to the merosporangium or merosporangiospore. Differentiation between merosporangium and merosporangiospore has been made in the present study. The merosporangium prior to release is characterised by a surface ornamentations comprising annular rings with interconnecting ridges. The merosporangiospore is included within the merosporangium, and has a papilla-like base lies above the septum delimiting the merosporangium from the pseudophialide.

The detached spore of *L. pennispora* was found to be the merosporangiospore covered with the merosporangium wall, except at the base where that remained attached to the pseudophialide neck. On the other hand, the surface ornamentation that characterises the morphological maturity of the merosporangium is caused by the formation of a dentate-like surface ornamentation by the merosporangiospore wall. Young (1971) and Benny & Aldrich (1975) described this dentate-like ornamentation as spines regularly arranged on the surface of the merosporangiospore, which they believed to be the liberated spore. This situation is now established, and in addition to the new interpretation of the liberated spore of *L. pennispora*, explains the results of McKeeown et al. (1996) who described two different regions of microfibrils in the arrangement of the merosporangium wall of this fungus. It is conceivable that the surface ornamentations are involved in the merosporangium detachment by pushing out the cell wall.

The two-layered nature of the wall of the aerial hyphae of *L. pennispora* was confirmed. However, the results of the transmission electron microscopy revealed that the merosporangium had a three-layered wall: an outer, an electron-dense, and a thinner layer. The wall at the merosporangium base was similar and continuous with the two-layered wall of the pseudophialides that comprised an outer, electron-dense, thinner layer, and an inner, electron-opaque, thicker layer. The rupture of the merosporangium wall appears to occur at the point where the two-layered pseudophialide wall is contiguous with the three-layered merosporangium wall. On the other hand, the merosporangiospore possessed a four-layered wall: an outer, 2–5 nm thick, electron-dense layer; adpressed to the outer layer a thick, 5–10 nm, electron-dense layer; and an innermost fourth, 90–100 nm thick, amorphous, electron-transparent layer. Young (1970a) described the merosporangiospore wall of *Linderina pennispora* as an outer and inner complex, but it is possible that he actually described the wall of the detached merosporangiospore within the merosporangium wall.

Our results reveal that the merosporangiospore of *L. pennispora* possess an “appendage”. This is the first such observation not only for the species, but also within the family. The formation of the merosporangiospore appendage proceeds at a late stage of merosporangia development, almost prior to merosporangium detachment. The appendage is attached to the collar-like base, particularly to the inner layer of the merosporangiospore. It is ca. 3–5 µm long and formed inside the “appendage sac” in the pseudophialides. The function of this appendage is unknown and necessitates more work in order to be resolved.

The septum that comprises a cross-wall, a central pore occluded by a biumbonate plug, the coenmansoid form of the asexual reproductive apparatus, the similar wall structure, and the serological affinity indicate that Kickxellaceae are closely related to Harpellales and Asellariales (Moss & Young 1978). The demonstration of the merosporangiospore “appendage” strongly supports this hypothesis.

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**REFERENCES**


