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DECIPHERING INTERFUNGAL RELATIONSHIPS IN THE 410-MILLION-YR-OLD RHYNIE CHERT: *RHIZOPHYDITES SHUTEI* SP. NOV. (FOSSIL CHYTRIDIOMYCOTA) ON GLOMEROMYCOTAN ACAULOSPORES

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Abstract: The spores of arbuscular mycorrhizal fungi (Glomeromycota) in the Early Devonian Rhynie ecosystem served as a habitat for a diversity of other fungi, only a few of which have been studied in detail so far. *Rhizophydites shutei* nov. sp. occurs in planar assemblages and tuft-like clusters comprised of thalli in various stages of development on *Archaeosporites rhyniensis*-like acaulospores. The presence of multiple individuals on several closely spaced hosts allows a thorough depiction of this fungus. Thalli are monocentric and characterized by an ovoid, narrowly to broadly citriform, bulb-shaped, or globose inoperculate zoosporangium 10–35(–38) µm high and 7–33 µm wide, and an endobiotic rhizoidal system reaching into the host lumen. The sporangium can be epibiotic or interbiotic (stalked), or located between the wall layers of the host spore. Mature sporangia have a pronounced apical papilla. Similarities with the modern chytrid genera *Rhizophydium* and *Phlyctochytrium* are used to suggest that the fossil belongs to the Chytridiomycota, and to place it in the genus *Rhizophydites*, which accommodates chytrid-like fossils that are morphologically similar or even identical to present-day *Rhizophydium*. This discovery contributes to our understanding of the various roles mycorrhizal fungi had in early terrestrial ecosystems.

Key words: apophysis, discharge papilla, Glomeromycota, Early Devonian, rhizoidal system, *Rhizophydium*, zoosporangium

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Introduction

The Early Devonian Rhynie chert of Scotland is one of the most important fossil sites worldwide because it provides exceptional insights into the morphology, internal organization, and biology of plants, animals, and microorganisms that lived together in an early terrestrial ecosystem more than 400 million years ago (Trewin and Kerp 2017, Garwood et al. 2020). Moreover, parts of the Rhynie ecosystem are preserved in situ so that not only individual organisms, but also associations and interactions between different organisms can be directly studied.

The fungi described from the Rhynie chert constitute the largest body of structurally preserved evidence of fungi and fungal interactions collected to date from any fossil ecosystem (Krings et al. 2017a). Glomeromycota (arbuscular mycorrhizal fungi) and their close relationships with early land plants have always been at the center of research on Rhynie chert fungi because of their importance for our understanding of the early evolutionary history of mycorrhizal symbioses (e.g., Sharma et al. 1993, Remy et al. 1994, Taylor et al. 1995, 2005, Karatygin et al. 2006, Krings et al. 2007, Strullu-Derrien et al. 2014, 2018, Brundrett et al. 2018, Walker et al. 2018, 2021, Harper et al. 2020). Within the prostrate axes of their rootless plant hosts, these fungi produced intercellular hyphal systems, vesicles, and propagules (spores), and in some cases also intracellular arbuscules. Glomeromycotan spores are both abundant and morphologically diverse in the Rhynie chert, and a great many of them demonstrably have served as a habitat for other organisms, mainly other fungi (e.g., Hass et al. 1994, Krings and Harper 2018, Krings 2022), but also at least one alga (Krings et al. 2017b, Krings 2024b).

Chytrid-like morphologies are common among colonizers of glomeromycotan spores in the Rhynie chert (e.g., Kidston and Lang 1921, Taylor et al. 1992, 2004, Hass et al. 1994, Berbee et al. 2017, Krings 2022). They suggest that chytrids (i.e., members of the phylum Chytridiomycota)

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and chytrid-like organisms (i.e., forms that resemble chytrids morphologically, but cannot be safely assigned on the basis of morphological features; see Strullu-Derrien 2018) played important ecological roles in early continental environments. One major obstacle to a more comprehensive understanding of these relationships in the Rhynie ecosystem is that the thalli of the colonizers usually have very few, if any, diagnostic structural features that are also consistent among multiple specimens, thus making it difficult to delineate individual species and compare the fossils with present-day equivalents. Relatively few of the chytrid-like organisms associated with glomeromycotan spores in the Rhynie chert are morphologically distinct and have a high recognition value (e.g., Krings and Taylor 2014, Krings and Harper 2020, Krings in press).

Here, I present a portion of a land plant axis from the Rhynie chert that contains more than 30 glomeromycotan acaulospores, most of which bear multiple thalli of a chytrid-like colonizer. The overall appearance and basic morphology of the colonizer thalli are consistent on all host spores, and hence it is highly probable that the thalli all belong to the same species. The presence of more than 200 colonizer individuals of one species on host spores in the same plant axis is a fortunate circumstance as it allows a detailed characterization of the colonizer and provides specific details about its development and the biology of the association.

Geological setting

The Rhynie chert Lagerstätte (including the original Rhynie chert and the nearby, coeval Windyfield chert) is located in the Rhynie outlier of Lower Old Red Sandstone in Aberdeenshire, Scotland, within a sequence of sedimentary and volcanic rocks. The chert occurs in the Rhynie Block of the Dryden Flags Formation, northwest of the village of Rhynie. The Lagerstätte consists of fossiliferous beds containing lacustrine shales and chert that are viewed as a series of ephemeral pools within a geothermal wetland, with alkali-chloride hot springs as part of a complex hydrothermal system in a region affected by volcanic activity (Rice et al. 2002, Trewin and Fayers 2016, Channing 2017). Preserved in the chert are both aquatic facies and subaerial horizons with land plants. Preservation took place as a result of temporary flooding of silica-rich water, or by silica-rich groundwater percolating to the surface (Powell et al. 2000). The Rhynie chert biota has been regarded as early Pragian to earliest Emsian in age based on spore assemblages (Wellman 2006, 2017, Wellman et al. 2006); high-precision age constraints indicate absolute ages of 407.1 ± 2.2 Ma (Mark et al. 2011) and 411.5 ± 1.3 Ma (Parry et al. 2011), respectively. For details on the geology and paleontology of the Rhynie chert, refer to Trewin and Kerp (2017), Garwood et al. (2020), and the volume edited by Edwards et al. (2018).

Material and methods

The fossils were identified in a thin section from a teaching slide collection of the late Dr. Robert W. Baxter of the University of Kansas at Lawrence KS, USA, that is today kept in the Bayerische Staatssammlung für Paläontologie und Geologie in Munich (SNSB-BSPG), Germany, under accession prefix BAX. The slide (BAX-1) was prepared by cementing a wafer of the chert to a glass slide, and then grinding the rock slice until it was sufficiently thin to transmit light (for details on thin section preparation see Hass and Rowe 1999). All fossils were examined using normal transmitted light microscopy; digital images were captured with a Jenoptik Gryphax Naos camera, and processed for brightness and contrast in Adobe Photoshop CS5 (for details on microscopy and image optimization, refer to Krings et al. 2021).

Results

Context and host morphology

Rhynie chert thin section BAX-1 contains partially to largely degraded axes of the early land plants *Rhynia gwynne-vaughanii* Kidston et W.H.Lang and *Aglaophyton majus* (Kidston et W.H.Lang) D.Edwards in sections ranging from transverse to longitudinal, and surrounded by clear chert interspersed with scattered land plant and fungal spores, fungal hyphae, and organic debris. All *A. majus* axes appear to have been mycorrhizal based on the occurrence of hyphae, glomoid or acaulosporoid spores, and often also (remnants of) arbuscules in the partially intact or degraded cortical tissues (Krings 2024a).

One of the *Aglaophyton majus* axis portions, in a very oblique (slanted) cross section (Text-fig. 1a), shows a partly degraded and homogenized cortex (co in Text-fig. 1a) with a partially intact mycorrhizal arbuscule zone (maz in Text-fig. 1a), and a mostly intact cuticle (cut in Text-fig. 1a). A strand of water-conducting cells is located in the center of the axis (not shown in Text-fig. 1a). Extending lengthwise through what used to be the cortex are several tubular hyphae up to 12 µm wide (black arrows in Text-fig. 1a). Arising from these hyphae are narrower branch hyphae, some of which giving rise to spores (white arrows in Text-fig. 1a). The spores (Text-fig. 1b, c) are acaulospores borne laterally within the neck (sn in Text-fig. 1c) of a sporiferous saccule (sa in Text-fig. 1c); however, the neck and saccule are not recognizable in most spores, either due to the plane of the section through the specimen, or because they were already decayed at the time of fossilization. The spores range from globose to ovoid in shape and are up to 80 µm wide and 60 µm high; their composite wall consists of three components (c1, c2, and c3 in Text-fig. 1b). A translucent region (tr in Text-fig. 1b) up to 6(–9) µm wide occurs in many cases between the inner surface of wall component c2 and the outer boundary of the spore lumen (spl in Text-fig. 1b) formed by component c3. The spores are morphologically very similar to *Archaeosporites rhyniensis* C.Walker, C.J.Harper et M.Krings, a Rhynie chert member of the Glomeromycota family Archaeosporaceae (Harper et al. 2020).

Thirty-one acaulospores of the type described above are present in said axis portion, of which 25 are colonized by a chytrid-like organism, whose bipolar monocentric thalli grow singly or in clusters of up to 15 individuals on and in the spore wall, and also extend into the spore lumen. The

host spore wall is relevant with regard to understanding the morphology and development of this organism, which is formally described in the section below.

Systematic paleomycology

Phylum Chytridiomycota Doweld, 2001

Fossil-genus *Rhizophydites* M.Krings et C.J.Harper in Krings et al. 2021

Mycobank. MB 834563.

Type species. *Rhizophydites matryoshkae* M.Krings et C.J.Harper; Early Devonian, Rhynie chert, Scotland, United Kingdom.

***Rhizophydites shutei* nov. sp.**
Text-figs 1a, b, 2, 3

Holotype. Globose zoosporangium with a small subsporangial swelling shown in Text-fig. 2i; slide BAX-1 (the exact position of the specimen is indicated by an arrow on the slide).

Mycobank. MB 855708.

Repository. SNSB-Bayerische Staatssammlung für Paläontologie und Geologie, Munich, Germany (Robert W. Baxter Rhynie chert slide collection).

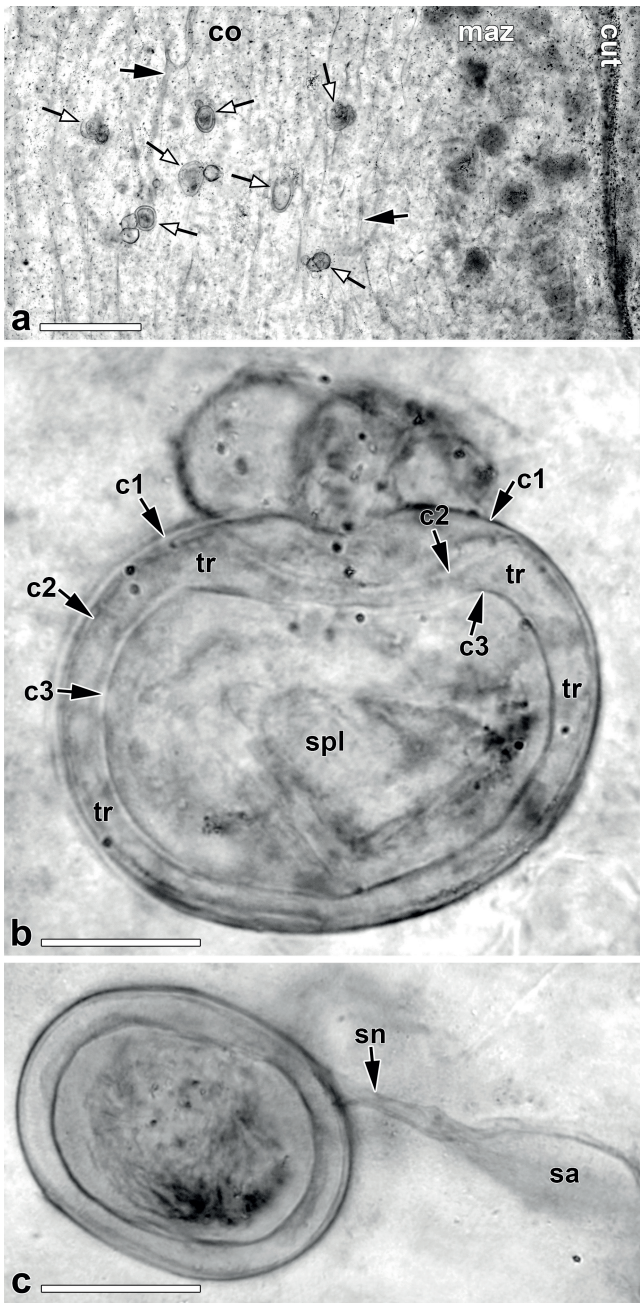
Etymology. The epithet honors the late Cedric H. Shute (1937–2019) and his important contributions to our understanding of Paleozoic plants and floras.

Type locality. Rhynie chert site, Aberdeenshire, Scotland, UK National Grid Reference NJ 494276 (57° 20′ 09.97″ N, 02° 50′ 31.83″ W).

Type stratum and age. Rhynie Block of the Dryden Flags Formation; Early Devonian; early (but not earliest) Pragian to earliest Emsian (Wellman 2006, 2017), 411.5 ± 1.3 Ma (Parry et al. 2011), 407.1 ± 2.2 Ma (Mark et al. 2011).

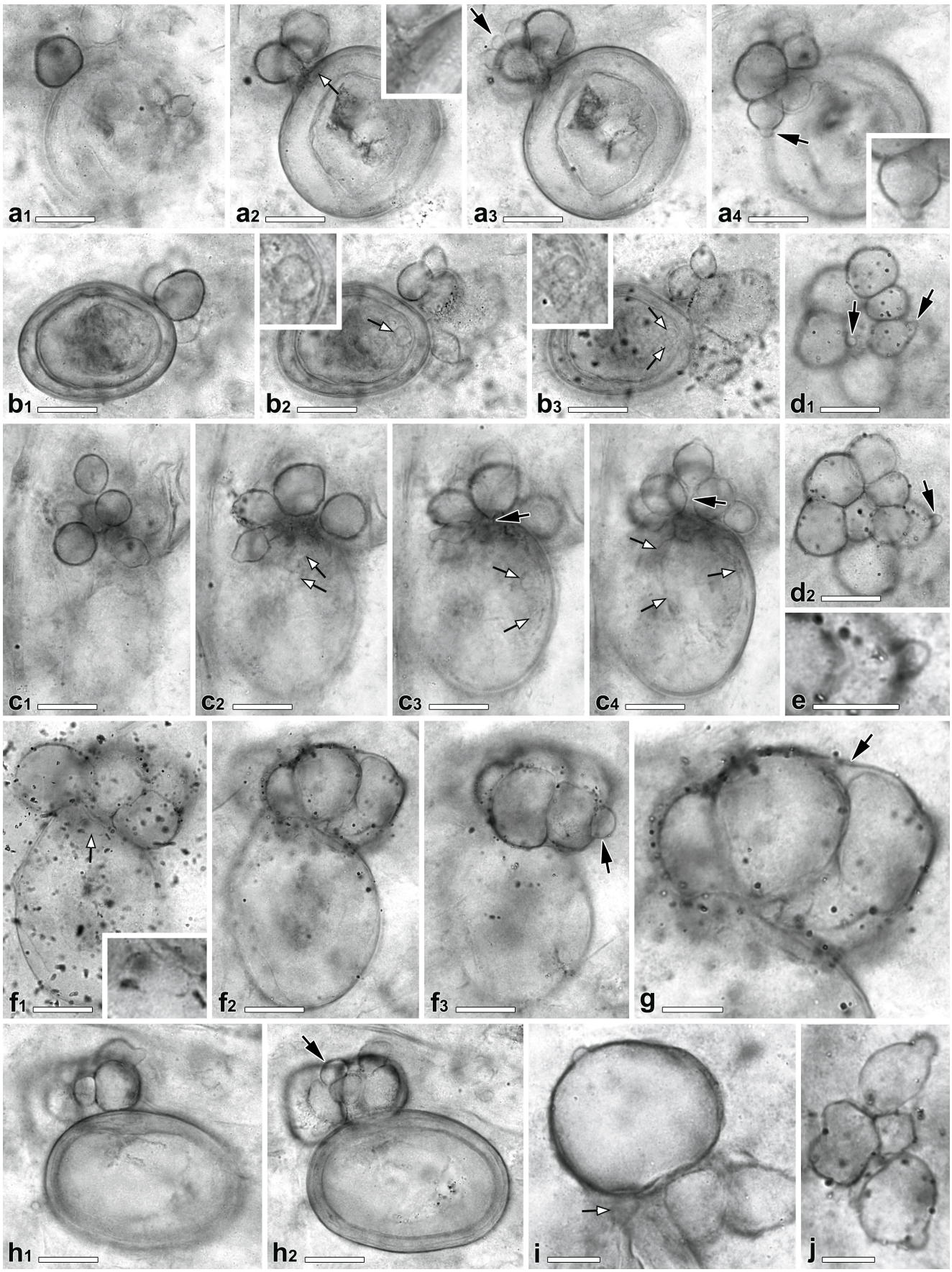
Diagnosis. Rhizoidal system composed of branched rhizoids arising from rhizoidal axis or (subsporangial) apophysis; apophysis, if present, rather small; zoosporangia very different in size and shape, due in part to availability of space in place of growth, ovoid, narrowly to broadly citriform, bulb-shaped, or globose, epibiotic, interbiotic (stalked), or located between host wall components, less than 40 µm high (without stalk) and up to 35 µm at widest point, wall smooth; stalk, if present, erect, short to nearly as long as zoosporangium; mature zoosporangia with a prominent, usually thin-walled apical papilla; post-discharge zoosporangia with a circular apical orifice with slightly raised borders; occurring singly (rarely) or in tuft-like clusters (in most cases) or planar assemblages comprised of multiple individuals at different stages of development. **Host**: Glomeromycotan acaulospores morphologically similar to *Archaeosporites rhyniensis*.

Distinguishing features. *Rhizophydites shutei* differs from all chytrid-like fossils previously described from the Rhynie chert. However, there are some illustrations in the literature of hitherto undescribed and unnamed chytrid-like thalli on Rhynie chert glomeromycotan spores,



Text-fig. 1. *Rhizophydites shutei* sp. nov., a colonizer of glomeromycotan spores from the Early Devonian Rhynie chert. a: Portion of *Aglaophyton majus* axis (very oblique cross section) containing hyphae (black arrows) and *Archaeosporites rhyniensis*-like glomeromycotan acaulospores (white arrows), many of the latter colonized by *R. shutei*; cut = cuticle, co = cortex (largely degraded), maz = mycorrhizal arbuscule zone (partially degraded). b: Acaulospore in near-median sectional view, with a tuft-like cluster of *R. shutei*; c1, c2, c3 = spore wall components, tr = translucent region, spl = spore lumen. c: Uninfected acaulospore with intact saccule (sa); sn = saccule neck. Scale bars = 200 µm (a), 20 µm (b), 25 µm (c).

which could belong to *R. shutei* on the basis of their overall appearance (Krings and Harper 2019: fig. 4b, c, Krings 2022: figs 24, 25). *Rhizophydites shutei* is similar morphologically to *Stillula hyphicola* M.Krings, a monocentric chytrid from the Rhynie chert that colonizes glomeromycotan hyphae (Krings 2024a). Both forms have epibiotic sessile or stalked zoosporangia with a single apical papilla. However, the



Text-fig. 2. ***Rhizophydites shutei*** **sp. nov., a colonizer of glomeromycotan spores from the Early Devonian Rhynie chert. a: Spore with two clusters of** ***R. shutei*** **in four different focal planes; note apophysis in a_2 (white arrow + inset), small vesicles in a_3 (black arrow), and zoosporangium with prominent papilla in a_4 (black arrow + inset). b: Spore with large cluster of** ***R. shutei*** **in three different focal planes, illustrating differences in size and shape of the zoosporangia; note apophyses in spore lumen in b_2 and b_3 (arrows + insets). c: Tuft-like cluster of** ***R. shutei*** **in four different focal planes, showing intramatrical thallus portions still partly preserved (white arrows); note interbiotic zoosporangia with short (black arrow in c_3) and long (black arrow in c_4) stalk,**

papilla is less prominent and usually offset to the side in *S. hyphicola*. Moreover, *S. hyphicola* zoosporangia are on average much rounder and do not occur in tuft-like clusters. Two Rhynie chert fossils have previously been described in the genus *Rhizophydites*, namely *R. matryoshkae* thriving on land plant spores (Krings et al. 2021), and *R. bicornis* M.Krings on gomeromycotan spores (Krings in press). Both differ from *R. shutei* above all in regard to the number of discharge openings. There are 1–4 papillae or short tubes in *R. matryoshkae* and typically two (rarely one) in *R. bicornis*. Moreover, secondary sporangia have been reported in both *R. matryoshkae* and *R. bicornis*, but there is no evidence of them as yet in *R. shutei*. *Rhizophydites shutei* is also somewhat similar to *Illmanomyces corniger* M.Krings et T.N.Taylor, which occurs on certain thin-walled glomoid spores from the Rhynie chert (Krings and Taylor 2014). However, zoosporangia of the latter form are distinctly larger (greater than 60 µm in diameter) than *R. shutei* and have 4–5 prominent discharge tubes. Yet another Rhynie chert chytrid that is vaguely reminiscent morphologically, but is also on average bigger, is *Cultoraquaticus trewinii* Strullu-Derr., which usually has two discharge papillae (Strullu-Derrien et al. 2016).

Description. *Rhizophydites shutei* mostly occurs in tuft-like clusters arising from the wall of the host spore. Tufts consist of 2 to more than 10(–15) individual thalli in different stages of development (Text-figs 2, 3a, b, d–f). Thalli occurring in planar assemblages have also been observed, but are relatively rare (Text-fig. 3c). Tuft-like clusters usually occur singly on their substrate (= glomeromycotan spore), but there are also a few hosts with two tufts (Text-fig. 2a).

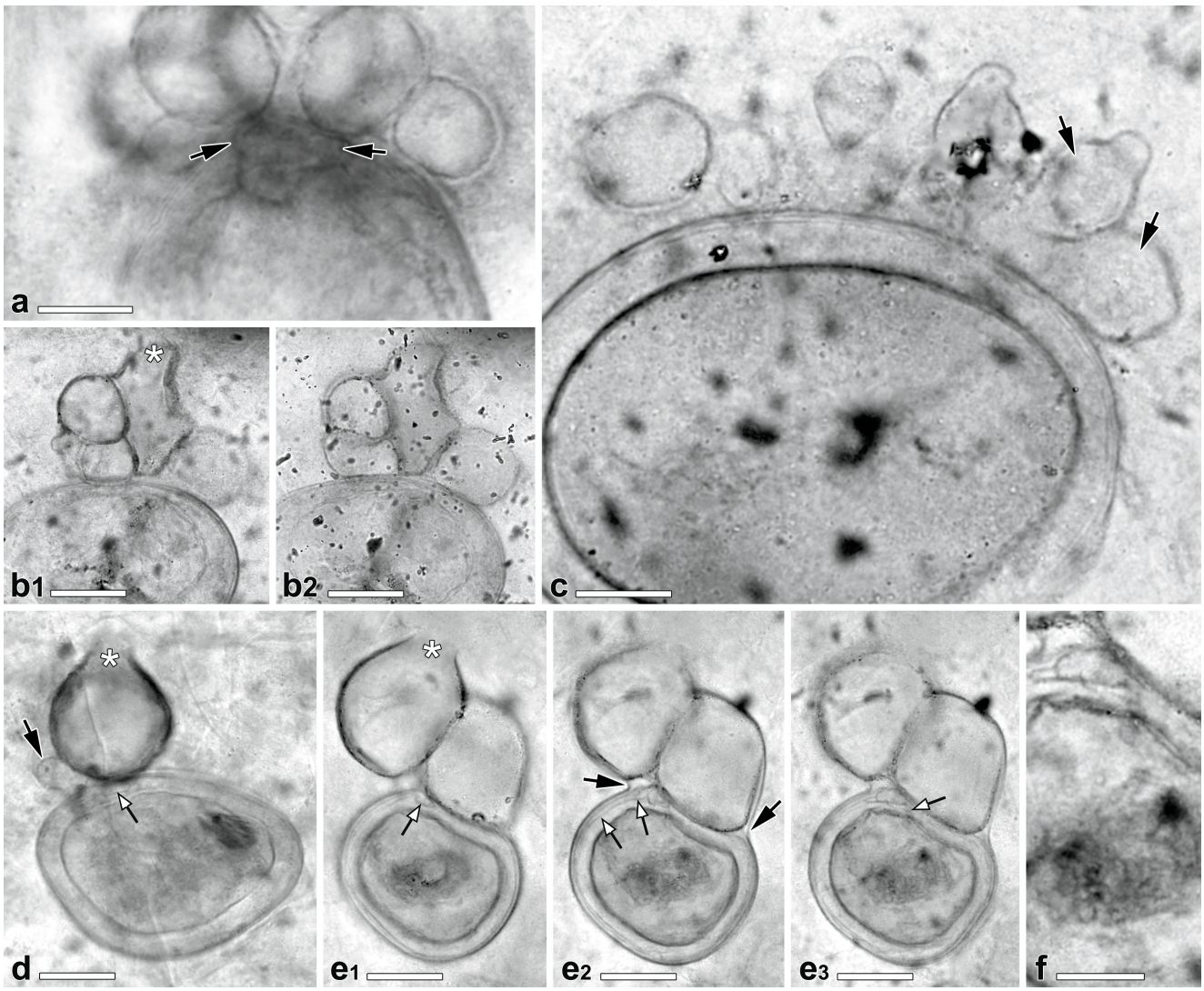
Thalli are bipolar and comprised of an epibiotic or interbiotic (but see below), smooth-walled, sac-like zoosporangium, and an endobiotic rhizoidal system reaching into the host lumen. The morphology of the extramatrical portion of the thallus appears, at least in part, as a function of the surrounding in which it developed. Zoosporangia differ significantly from each other in regard to size and shape; they vary between ovoid, narrowly to broadly citriform, bulb-shaped, and spheroidal, and 10–35(–38) µm high and 7–33 µm wide. Zoosporangia are mostly sessile, but can also be interbiotic and arise from an erect, tubular stalk, which is either short (black arrow in Text-fig. $2c_3$) or elongate and up to as long as the sporangium itself (e.g., black arrow in Text-fig. $2c_4$). A few very small spheroidal or drop-shaped vesicles are also present in many tuft-like clusters (arrows in Text-figs $2a_3$, f_3, h_2, 3d); as to whether these structures are mature but underdeveloped, or aborted, or still developing zoosporangia cannot be determined.

Most tuft-like clusters emerge from the outer surface of the host spore, but there are also several specimens in which zoosporangia have developed between the host wall components c1 and c2 (Text-figs 2f, g, 3e). Wall component c1 is greatly expanded in these specimens (e.g., black arrows in Text-figs 2g, $3e_2$), and the enclosed zoosporangia are densely packed and therefore in some cases asymmetrical or somewhat deformed (Text-fig. 2g [right-hand side of picture]). It remains uncertain what ultimately happened to the envelope when the enclosed zoosporangia matured and released their zoospores. It was presumably destroyed at some point, as there is no evidence in these specimens that the host wall component c1 covers post-discharge zoosporangia (asterisk in Text-fig. $3e_1$).

A prominent apical (in rare cases somewhat offset or slightly tilted) papilla, up to 4.5 µm high and ca. 4.6 µm wide, is visible in approximately 35 % of the zoosporangia (black arrows in Text-fig. $2a_4$ [+inset], d_1, d_2 [magnified in Text-fig. 2e]; Text-figs $2h_1$, i, j, 3c), while another ca. 5 % exhibit a distal opening with slightly raised borders that in most cases is more than 10 µm wide (asterisk in Text-fig. $3b_1$, d, e_1); no evidence of the presence of an operculum was found. The remaining ca. 60 % of zoosporangia lack any evidence of a papilla or other type of preformed discharge apparatus or opening, either due to the plane of the section through the sporangium, or because the sporangium was immature and the discharge apparatus not yet developed at the time of fossilization. The wall of the papilla appears in many cases to be thinner than the wall of the zoosporangium (e.g., Text-figs 2i, 3c). No evidence of the contents of the zoosporangia was found, with the exception of two specimens that contain a central, albeit only vaguely defined inclusion, which appears to consist of closely spaced individual bodies up to ca. 2(–2.5) µm in diameter (arrows in Text-fig. 3c).

Remains of the rhizoidal system (= intramatrical portion of the thallus) are recognizable in many specimens, but it is not really well preserved in any of them. The rhizoidal system consists of a tubular or unevenly slightly swollen rhizoidal axis (white arrows in Text-fig. $2c_{2–4}$ [one focal plane magnified in Text-fig. 3a]) that arises proximally on the zoosporangium (or from the proximal end of the stalk), passes through the outer two or all three host wall components, and gives off rhizoids (e.g., Text-fig. $2h_1$). The rhizoidal axis can also form a small (subsporangial) swelling or apophysis (white arrows in Text-figs $2a_2$ [+ inset], b_2 [+ inset], b_3 [+ inset], f_1 [+ inset], j, 3d, e_1), from which one or two main rhizoids are then given off that advance further into the host and, in some cases, branch profusely (e.g., Text-fig. 3f). The shape of the apophysis, and also its position within the host and the course of the main rhizoids, can vary. It may be oval or irregularly elongate in sectional view and located in the translucent region (tr in Text-fig. 1b) on the inner surface of the host wall component c2 (Text-figs $2a_2$, 3d, $e_{1–3}$), with the main rhizoids then initially extending

respectively. d: Tuft of zoosporangia seen from above in two different focal planes; black arrows indicate apical papillae. e: Close-up of (d_2), showing one of the papillae. f: Tuft of thalli with several zoosporangia located between wall components c1 and c2 of host in four different focal planes; note apophysis in f_1 (white arrow + inset) and small vesicle in f_3 (black arrow). g: Close-up of (f_2), showing host wall component c1 surrounding zoosporangia (arrow). h: Tuft-like cluster in two focal planes; arrow in h_2 shows small vesicles; note delicate rhizoidal system in spore lumen in h_1. i: Holotype specimen; globose zoosporangium with small subsporangial apophysis (arrow). j: Four zoosporangia seen from above; note two prominent papillae, the one in the lower part of the picture slightly tilted. Scale bars = 20 µm (a, b, c, d, f, h), 10 µm (e, g, i, j).



Text-fig. 3. ***Rhizophydites shutei*** **sp. nov., a colonizer of glomeromycotan spores from the Early Devonian Rhynie chert. a: Close-up of Text-fig. 2c$_4$, showing rhizoidal axes arising proximally on zoosporangia (arrows). b: Cluster of zoosporangia in two focal planes; note large central sporangium with a distal opening (asterisk). c: Planar assemblage of** ***R. shutei*****, showing spheroidal inclusions in two zoosporangia (arrows). d: Zoosporangium with a distal opening (asterisk); note small vesicle (black arrow) and apophysis in translucent region (white arrow). e: Cluster of zoosporangia between wall components c1 and c2 of host in three different focal planes; note wall component c1 surrounding tuft of zoosporangia (black arrows in e$_2$), and small apophysis in translucent region in e$_1$ (white arrow) that gives off main rhizoids, which initially extend through the translucent region (white arrows in e$_2$), and of which one then advances into the spore lumen (white arrow in e$_3$), where it branches profusely (see (f)). f: Close-up of (e$_3$), showing rhizoid passing through host wall component c3, and forming multi-branched rhizoidal system in host lumen. Scale bars = 20 µm (b, d, e), 10 µm (a, c, f).**

through the translucent region (e.g., white arrows in Text-fig. 3e$_2$) and in some, but not all, cases later advancing into the host lumen (Text-fig. 3e$_3$ [magnified in Text-fig. 3f]). Conversely, the apophysis may also be more or less circular in sectional view and located in the spore lumen (spl in Text-fig. 1b) on the inner surface of the host wall component c3 (Text-fig. 2b$_2$, 3, f$_1$), in which case the rhizoids spread directly in the lumen. The rhizoidal systems in one tuft-like cluster have apparently developed largely between the host wall components c1 and c2, whereby the wall component c2 in this area has been pushed into the translucent region and the wall component c3 into the spore lumen (Text-fig. 1b). No further evidence of host wall deformation by the colonizer has been found, with the possible exception of a slightly more uneven course of the wall component c3 below the tuft of colonizers in the specimen shown in Text-fig. 3e. There is also no indication of a specific host response in any of the host spores.

Discussion

One conspicuity of the fossil record of fungi in the Rhynie chert is the abundance and morphological diversity of glomeromycotan spores, which are in many cases so structurally similar to present-day members of this fungal phylum that, were it not for the lack of molecular evidence, they could readily be assigned to extant taxa (Dotzler et al. 2006, 2009, Harper et al. 2020, Walker et al. 2021). The spores are evidence that Glomeromycota was a diverse lineage by Early Devonian times. Moreover, the spores, and

to a lesser extent also the vesicles and hyphae of these fungi, served as a place of growth and in most cases probably also as a carbon source for a whole range of other fungi (Hass et al. 1994, Krings et al. 2009, 2010, 2015, Harper et al. 2017, Krings and Harper 2018, 2020, Krings 2022, 2024a, in press), suggesting that Glomeromycota in the Rhynie ecosystem were important not only as partners in mycorrhizal symbioses, but also for the survival of a part of the microbial community (Brundrett et al. 2018).

A rare find, even by Rhynie chert standard

Several examples of chytrid-like colonizers of glomeromycotan spores with epibiotic sporangia are known from the Rhynie chert (e.g., Krings 2022: figs 11, 12, 18–25), and *Rhizophydites shutei* described above is another entry in this growing inventory. What is unusual about the latter form, however, is the fossil material that forms the basis for its description. Usually, only single or small sets of specimens of a particular colonizer morphology are available (e.g., *Illmanomyces corniger*, of which there are only seven thalli so far; see Krings and Taylor 2014), or there are many specimens of the colonizer, but only one or a few host spores on which they occur (e.g., *Brijax amictus* M.Krings et C.J.Harper, of which there are more than 70 thalli distributed over only three spores; see Krings and Harper 2020). Here, by contrast, we have an assemblage of about 200 colonizer thalli that are similar to each other in basic morphology and distributed over more than 20 closely spaced host spores, all of which are preserved in the same section of the cortex of the same plant axis (Text-fig. 1a). All thalli therefore almost certainly belong to the same species. This allows a broad characterization of *R. shutei*, as morphological differences among specimens can be identified as developmental stages, or as a function of either the surrounding in which the thallus developed or the condition of the host at the time of thallus development.

Comparisons and affinities

Based on morphology, I consider it very likely that *Rhizophydites shutei* is a eucarpic, monocentric chytrid (Chytridiomycota). I dismiss the possibility of rhizomycelial polycentricity, in which multiple zoosporangia are interconnected by a mycelium-like system (e.g., Powell et al. 2018b, Simmons et al. 2021), as there is no evidence of the formation of zoosporangia from different branches of the same rhizoid in any of the spores. Well-suited present-day morphological equivalents of the fossil seem to me to be certain species in the "traditional" genus *Rhizophydium* Schenk ex Rabenh., which is broadly defined morphologically by epibiotic, monocentric, eucarpic thalli with zoosporangia that discharge in an inoperculate manner, usually by the deliquescence of one or more papillae, and endobiotic nutrient-gathering systems composed of tapering rhizoids which arise from the base of the sporangium or from an isodiametric stalk (Barr 1973, Sparrow 1977). A high level of morphological congruence appears to exist between the fossil and *Rhizophydium mammillatum* (A.Braun) A.Fisch., a parasite of various aquatic organisms (Sparrow 1960), which is characterized by sessile, smooth-walled zoosporangia that are spheroidal when young and long-ovoid or narrowly to somewhat broadly citriform when mature, and up to 30 mm high and 22 µm in diameter (Braun 1856: pl. II, figs 9–12, Minden 1911, Letcher and Powell 2012: pl. 11, figs 1–6). Zoosporangia have a more or less prominent apical papilla and a rhizoidal system composed of branched rhizoids arising from a slender main axis. Similar in overall morphology is *R. asymmetricum* (P.A.Dang.) Minden, another parasite of aquatic organisms with sessile zoosporangia and an apical discharge papilla, which, however, is slightly tilted in most cases (Letcher and Powell 2012: pl. 2, figs 10–15). Further *Rhizophydium* species that resemble the fossils to varying degrees include *R. acuforme* (Zopf) A.Fisch. (Zopf 1884: pl. 10, figs 33–43, Letcher and Powell 2012: pl. 24, figs 1–7), *R. carpophilum* (Zopf) A.Fisch. (Karling 1958: fig. 8), and *R. granulosporum* Scherff. (Letcher and Powell 2012: pl. 27, figs 1–6), as well as an unnamed parasite of microscopic animals and pollen grains in soil described as *Rhizophydium* sp. by Karling (1946: figs 19–22). The latter form has broadly or narrowly pyriform sporangia which in most cases are stalked (interbiotic), but can also be sessile and rest directly on the host. Finally, Sparrow (1933: figs 15–22) reported on the behavior of the zoospores of a fungus which he had found on *Spirogyra* Link and identified as a member of *Rhizophydium*, whose zoosporangia are characterized by a single apical discharge papilla. The zoospores, upon being discharged from the sporangium, swarmed for several hours and then settled down on the host in compact groups of 4–10 or more and developed into thalli (Sparrow 1933: pl. 49, fig. 20). These groups of thalli are strongly reminiscent of the tuft-like clusters seen in the fossil.

The presence of an apophysis has historically not been considered a feature of *Rhizophydium*, but is rather a characteristic of several other taxa, for instance genera *Phlyctochytrium* J.Schröt., *Polyphlyctis* Karling (both Chytridiales; Barr 1969, Johnson 1969, Kazama 1972, Letcher and Powell 2005, 2018), *Gaertneriomyces* D.J.S.Barr, and *Spizellomyces* D.J.S.Barr (both Spizellomycetales; Barr 1980, 1984, Wakefield et al. 2010, Powell et al. 2018a). Support for a relationship of the fossil with one of these latter taxa is perhaps the notable similarity in the overall appearance between the apophysate fossil thalli on and in their host spores and specimens of the present-day *Phlyctochytrium synchytrii* E.Köhler on and in resting sporangia of *Synchytrium endobioticum* (Schilb.) Percival, as illustrated by Köhler (1925: pl. I, figs 1–4). Today, however, it is known that the apophysis is neither a stable nor a constant feature, and that several species in *Rhizophydium* can in fact also appear apophysate (Letcher and Powell 2005), whereas *Phlyctochytrium*, for example, can be both apophysate and non-apophysate (Letcher et al. 2012). For this reason, and given the considerable geologic age of the Rhynie chert, and also because molecular data and zoospore ultrastructure are required to safely assign chytrids to present-day genera and species (e.g., Powell and Letcher 2011, Powell 2017, Hurdeal et al. 2021, 2024, Voigt et al. 2021), I refrained from assigning the fossil to any modern taxon, but rather placed it in the fossil genus *Rhizophydites*, which is used for apophysate and non-apophysate chytrid-like fossils that are morphologically similar or even identical to present-day *Rhizophydium* (Krings et al. 2021). Finally,

however, it must not be forgotten that some members of the Hyphochytriales, which belong to a completely different lineage of organisms, the Stramenopiles, resemble chytrids in basic thallus organization (Beakes et al. 2014, Beakes and Thines 2017). Consequently, and despite the fact that the fossils are very similar morphologically to certain chytrids, I cannot completely rule out the possibility that *R. shutei* was a member of the Hyphochytriales.

Nutritional mode and aspects of development

One difficult question that invariably arises when interfungal associations are considered in the fossil record is the nature of the relationship between the partners. To determine this in the fossils described in this study, it is necessary to establish whether the host spores were viable or non-viable at the time of colonization by *Rhizophydites shutei* (see Krings and Harper 2020). If the spores were viable (i.e., capable of germinating and growing into a new mycelium), and if *R. shutei* lived and derived the majority of its nutrients at the expense of the spores, then this association would represent a form of parasitism. However, no immediate evidence of parasitism in the form of a host response has been found (see Hass et al. 1994, Krings and Harper 2018). In the absence of recognizable evidence of parasitism, fossil fungal parasites cannot normally be safely distinguished from saprotrophs (Harper and Krings 2021). Nevertheless, a few of the spores colonized by *R. shutei* show a peculiarity that allows some speculation on the state of the spores at the time of colonization, namely zoosporangia located between the wall components c1 and c2 of the host, rather than on its outer surface (Text-figs 2f, g, 3e). Glomeromycotan spores with chytrid-like colonizers, whose sporangia have developed between individual layers of the host wall, are not an absolute exception in the Rhynie chert and have been reported previously (e.g., Hass et al. 1994: figs 6, 11, Krings 2022: fig. 13). Based on a comparison with the morphologically similar *Archaeosporites rhyniensis* (see Harper et al. 2020), it can be concluded that the wall component c1 of the acaulospores colonized by *R. shutei* represents the saccule wall that continues around the spore. This wall was flexible and expanded considerably during the formation and massive increase in size of the spore within the saccule neck, and in the present case was apparently also able to continue to expand and remain intact until the enclosed *R. shutei* zoosporangia had reached a mature size. At some point, the tension may have become too great and the wall component c1 ruptured, thus allowing the zoosporangia to release their zoospores unhindered into the environment. It is difficult to imagine that the fossil saccule wall retained its flexibility after the spore had reached its final size or even lost its viability. In fact, colonization could have taken place at an early stage of spore development when the wall component c1 was still actively expanding. It remains unclear what caused the zoosporangia to form within the host wall, rather than on the surface, in these specimens, but this is probably related to rare deviations from the normal mode of thallus development (see Blackwell et al. 2006).

Another curious detail of *Rhizophydites shutei* are the differences in the location of the apophysis. This structure, if present, is located either in the translucent region (i.e., on the inner surface of the host wall component c2; see Text-figs $2a_2$, 3d) or in the host spore lumen (i.e., on the inner surface of the wall component c3; see Text-fig. $2b_2$, 3). A translucent region as part of the spore wall has previously been described in other types of glomeromycotan spores from the Rhynie chert (Dotzler et al. 2009, Krings et al. 2015), and is believed to be an artefact caused by the shrinkage of the innermost wall component, possibly as a result of plasmolysis caused by the assumed hypertonic nature of the infiltrating mineralized water (Dotzler et al. 2009). A similar effect was observed with present-day glomeromycotan spores in certain acidic mounting media (e.g., Gamper et al. 2009, Rosas-Moreno et al. 2023). If this also applies to the spores colonized by *R. shutei*, then the apophyses located on the inner surface of the wall component c3 have either already existed in this position when the shrinkage began, or developed from rhizoidal axes that grew into the spore lumen through a pre-existing translucent region. Conversely, the apophyses that occur in the translucent region appear to have formed when the components c2 and c3 of the host spore wall were still connected, based on the fact that they are typically elongate, flattened structures, while those in the spore lumen are all spheroidal.

Paleobiological considerations

A notable difference between present-day Glomeromycota and their fossil relatives in the Rhynie ecosystem is that the former sporulate mainly outside their host plants in the substrate (extramatrical sporulation), whereas the latter formed their spores mainly inside their plant hosts (intramatrical sporulation) (Souza 2015, Walker et al. 2018). One could assume that intramatrical sporulation offers protection against colonization by other fungi, as the spores develop shielded by the surrounding (living) plant tissues, while spores that form extramatrically can be colonized directly from the ambient substrate. However, this does not appear to have been the case in the Rhynie ecosystem, as there are countless glomeromycotan spores that indicate fungal colonization within intact and decaying land plant axes (e.g., Krings and Harper 2018, Krings 2022). This raises the question of why *Rhizophydites shutei*, and other fungi as well, targeted the glomeromycotan spores in the plant axes and not the plants directly, which were a much larger and easier to reach carbon source? There are many fossils of fungi that thrived in the tissues of the land plants in the Rhynie chert (e.g., Taylor et al. 1992, Strullu-Derrien et al. 2015, Krings et al. 2017a), but these are not the same morphologies as on the glomeromycotan spores. Unfortunately, we know little about the degradation enzymes, mechanical processes, and physiology of nutrient assimilation in chytrids (Laundon and Cunliffe 2021). However, the preference for glomeromycotan spores by *R. shutei* and other fungi in the Rhynie ecosystem was probably linked to the biochemical makeup of the available carbon sources, along with the ability of the fungi to degrade and digest them (Gleason et al. 2011).

A question directly following this is how *Rhizophydites shutei* entered *Aglaophyton majus* and found its way to the spores? Chytrids today have rarely been reported as endophytes, and thus little is known about their behavior

inside plants (Rungjindamai and Jones 2024). Nevertheless, chytrids produce motile zoospores which enable the targeting of trophic substrates and hosts, and dissolved molecules can act as chemo-attractants for the zoospores (Laundon and Cunliffe 2021, Laundon et al. 2022, Hanrahan-Tan et al. 2023). Chemotaxis assays have shown that the zoospores can detect chemical gradients, and will swim towards specific nutrients (e.g. sugars, proteins, fatty acids, amino acids) usually associated with target hosts or substrates (Muehlstein et al. 1988, Medina and Buchler 2020). Because of their small size, they can swim through even the smallest water-filled interstices (Gleason et al. 2012). It is therefore quite conceivable that individual or small numbers of zoospores entered plant axes that resided on or in the substrate or in water, either through stomata or surface injures, and advanced through the intercellular system to the spores in the cortex. The large number of *R. shutei* thalli on the spores could have resulted either from the continuous immigration of new zoospores from the outside or, more likely, from the reproduction of *R. shutei* within the plant axis.

Conclusions

The spores of arbuscular mycorrhizal fungi today are intimately associated with a wide range of other fungi (Muthukumar and Udaiyan 1999), some of which are parasites believed to have the potential to decrease the number of viable spores, leading to a delay and reduced incidence of plant colonization and mycorrhiza formation, which might affect plant diversity and fitness (Paulitz and Menge 1986, Purin and Rillig 2008). Other fungi invade Glomeromycota to use the hyphae of their host as a pathway for the entry into the plant (De Jaeger et al. 2010), and still others use dead glomeromycotan spores as a convenient site for sporulation (Muthukumar and Udaiyan 1999, Krings et al. 2015). On the other hand, there is also some evidence to suggest a possible mycoparasitic capability of some arbuscular mycorrhizal fungi (Wang et al. 2009). In the fossil record, where there is virtually no possibility of observing organisms over a longer period of time, interfungal relationships are generally not well understood (Harper and Krings 2021). Nevertheless, it is believed that they have existed since the early days of life on land (Krings et al. 2017c). While we will likely never be able to fully decipher the effects of fungal colonization of mycorrhizal fungi in past ecosystems, *Rhizophydites shutei* from the Rhynie chert exemplifies how we can at least approach the individual associations, preservation permitting, by analyzing and documenting the partners and their spatial distribution in as much detail as possible. This information can then be used to formulate ideas about the biology and ecology of the relationship based on comparisons with present-day equivalents. Unfortunately, most fungal colonizers of mycorrhizal fungi in the Rhynie chert provide too few characters to recognize species and estimate diversity. *Rhizophydites shutei* underlines the value of large sample sets in tracing the range of structural features that are necessary to safely define a fossil taxon. I hope that this study will further stimulate interest in the fungal colonizers of mycorrhizal fungi in the Rhynie ecosystem and in the complex world of fossil microbial interactions as a whole.

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