# Observations on *Sphaeronaemella helvellae* in culture

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Dung, J., Rogers, J. D., Schroeder, Brenda, & Johnson, D. A. (2010).-Observations on *Sphaeronaemella helvellae* in culture. Sydowia 62 (1): 37–49.

*Sphaeronaemella helvellae* was isolated from sclerotia of *Colletotrichum coccodes* on potato. The fungus appears to parasitize the sclerotia of this potato pathogen. Observations on the fungus from pure culture show that ascospores germinate from one or both apices; no germ slits or germ pores are present. The *Gabarnaudia* anamorph produces conidia in chains from conidiogenous cells. Conidiogenesis appears to be phialidic with aspects of the holoblastic mode. No collarette was detected on the conidiogenous cell. A Gram-positive bacterium, determined by morphology and sequencing to be *Leifsonia aquatica*, was frequently associated with the fungus and appears to stimulate perithecial production.

Keywords:-Biological control, conidiogenesis, *Colletotrichum*, *Gabarnaudia*, perithecia, pyrenomycete, *Sphaeronaemella*

*Sphaeronaemella helvellae* (P. Karst.) P. Karst. (≡ *Sphaeria helvellae* P. Karst.) was described by Karsten (1884) from a decaying ascoma of *Gyromitra infula* (Schaeff.) Quel. Since that time it has been described from a number of fungal substrates (Hausner & Reid 2004, Malloch 1974). Hausner and Reid (2004) obtained SSrDNA phylogenetic data from *S. helvellae* isolated from a *Gyromitra* sp., *Gabarnaudia betae* (Delacr.) Samson & W. Gams and two isolates of *Sphaeronaemella fimicola* E. Marchal. These formed a monophyletic branch within order Microascales (Hausner & Reid 2004).

Although the molecular studies of Hausner and Reid (2004) clarified some aspects of the phylogenetic relationships of *Sphaeronaemella* and at least one isolate of *Gabarnaudia*, some nagging morphological problems remained. Cannon and Hawksworth (1982) defined *Sphaeronaemella* on the type species, *S. helvellae,* in part on its ascospores with what appeared to them to be germination slits as observed by scanning electron microscopy. In addition, they were not convinced that *Sphaeronaemella* possesses an anamorph, although such was reported by Seeler (1943) and Malloch (1974); it is now accepted that anamorphs of *Sphaeronaemella* are accommodated in *Gabarnaudia*. Moreover, the mode of conidiogenesis in the *Gabarnaudia* anamorphs

of *Sphaeronaemella* species, usually considered to be phialidic (Malloch, 1974; Samson, 1974), is not entirely clear. We obtained a pure culture of *S. helvellae* from sclerotia of *Colletotrichum coccodes* on potato collected in Montana. We were able to make critical observations on ascospore germination and conidiogenesis. We likewise identified a bacterium that is often associated with the fungus and appears to enhance perithecial production.

## Materials and Methods

*Sphaeronaemella helvellae* was isolated from potato grown in Montana. It was associated with *Colletotrichum coccodes* (Wallr.) S. J. Hughes, the cause of the black dot disease of potato. The fungus was isolated and maintained on the following Difco brand media: 2% Corn Meal Agar (CMA) and 2% Oat Meal Agar (OMA) and it also grew and fruited well on Bonar's modification of Leonian's agar ( Booth, 1971). Observations were made with an Olympus differential interference contrast (DIC) microscope, a Reichert brightfield microscope, and a Zeiss darkfield (DF) fluorescence microscope. For the latter procedure, fungal material was mounted in 0.2 % (W/V) aqueous Calcofluor White PMW and examined via exciter filter BG 12 and barrier filters 47 and 65 in tandem. Scanning electron microscopy (SEM) was done, as follows. Fungal material was examined without standard SEM preparation involving fixation, dehydration, critical point drying, and gold coating. Rather, 7 mm diameter discs were cut from colony margins and placed on Peltier cold stage sample holders and viewed with a FEI Quanta 200F Field Emission Electron Microscope at 0.5 ˚C, 45 % humidity and 15 KV or 20 KV accelerating voltage. Transmission electron microscopy (TEM) was done as described in Stahl *et al.* (1988).

The range of spore dimensions is given, rounded to the nearest full or half micrometer, with exceptional dimensions in parentheses. Means and standard deviations of length and width were computed from unrounded measurements. Cultures were deposited at Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan. Holotype and authentic material was examined (Figs. 24–28), thanks to a loan from the Botanical Museum, University of Helsinki, Finland (H) (Holmgren *et al.* 1990).

A yellow Gram-positive bacterium was often associated with cultures of *S. helvellae*. It was identified as a *Leifsonia* species based on 16S rDNA sequencing and physiological tests (Evtushenko *et al.* 2000, Holt *et al.* 1994, Sambrook *et al.* 1989, Suzuki *et al.* 1999) and a phylogenetic tree was constructed using neighbor joining analysis of the 16S rDNA sequence. Detailed procedures can be accessed at the following website: http://plantpath.wsu.edu/people/faculty/schroeder/supplemental.html

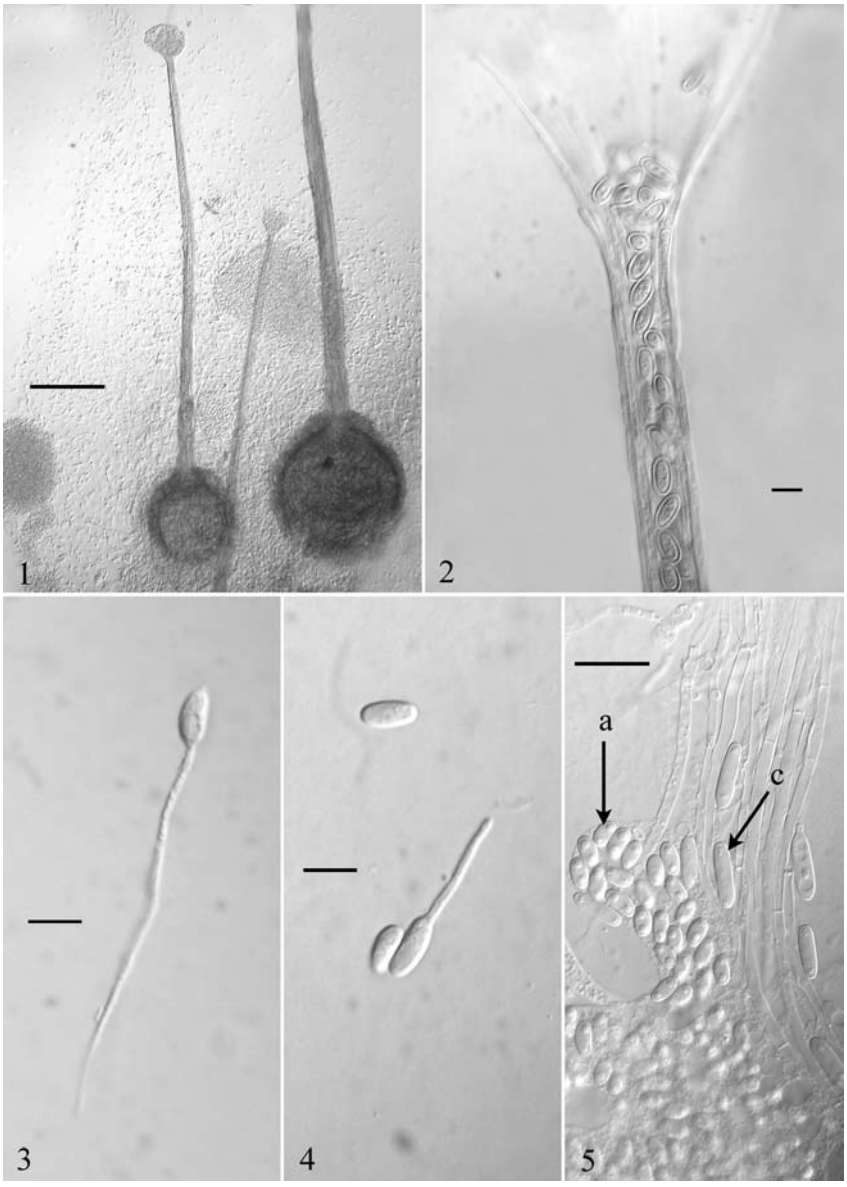
## Results

A brief description of *Sphaeronaemella helvellae* in culture follows. Perithecia yellow-orange, long-necked, 200–300 µm at base, necks 700–1300 µm long with flaring hyphae at apex. Ascospores accumulate at apices in a globular mass (Figs. 1, 2). Asci globoid to irregular, 11–22 × 8–16 µm, deliquescing immediately as ascospores mature. Ascospores hyaline or slightly subhyaline, ellipsoid to rotund, 6–9 × 4–5(–6) µm. Mean length (25 ascospores) = 7.25 ± 0.90 µm; mean width (25 ascospores) = 4.37 ± 0.68 µm. This compares favorably with measurements taken from authentic material, 7.5–9(–10.5) × 4.5–5 µm. FINNIA, Tavastia australis, Mustiala, ad *Helvellam infulam* , 25. IX. 1867, *leg*. P. A. Karsten, no. 1106 (Fig. 28). A note in the packet recognizes the fungus as *Sphaeronaemella helvellae* (Fig. 27). Lectotype material (P. A. Karsten, 1867, herb. H) (as *Sphaeria helvellae* Karst., Fig. 26) bore perithecia and conidia, but was not intensively examined owing to the fragility of the material.

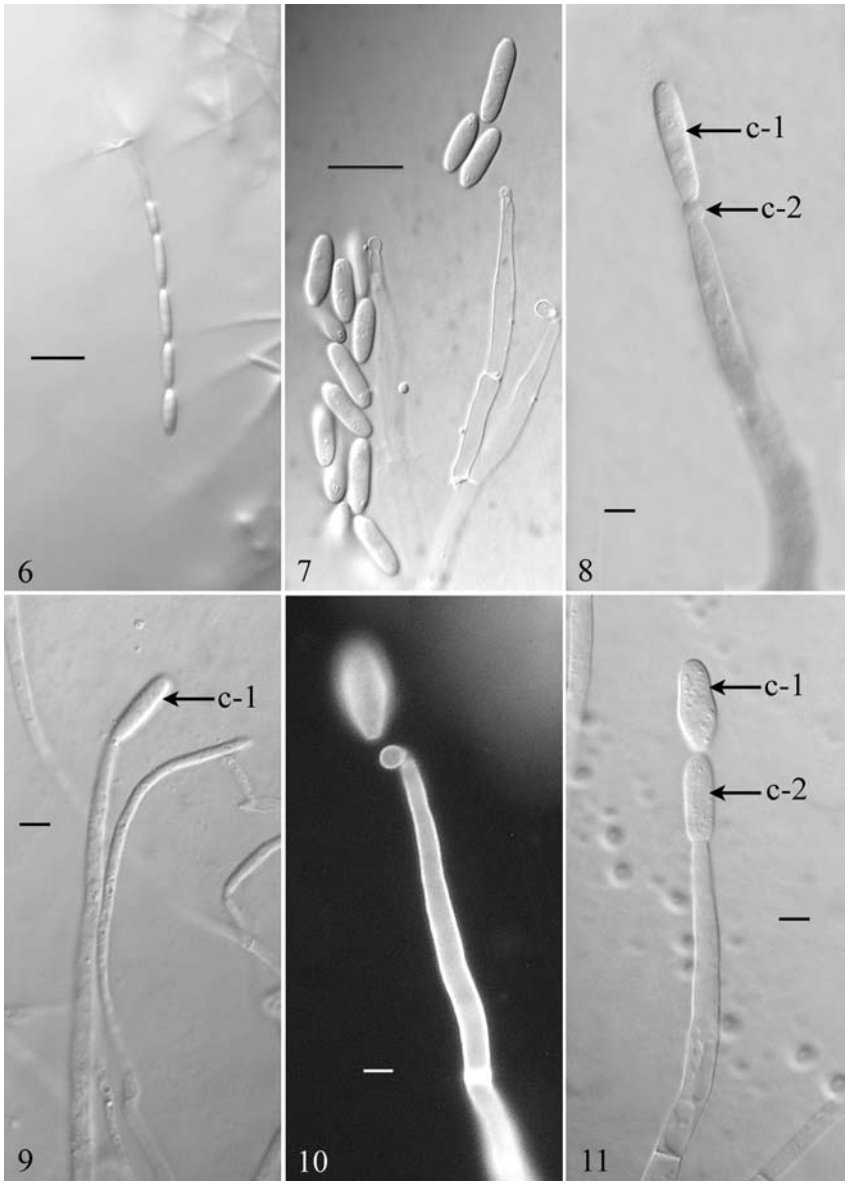
Ascospores from culture germinated readily on CMA and Leonian's media. A single germ tube usually issued from one end of the ascospore (Figs. 3, 4). Less frequently, a germ tube was produced from each end of the ascospore. No obvious germination site – germ slit or germ pore – was observed on ascospores. Observations by light microscopy and TEM preparations of ascospore longitudinal sections and cross-sections (Figs. 19 and 20) did not reveal breaks on ascospore walls. Occasionally an ascospore wall wrinkle or separation seen by SEM (Fig. 22) might be misinterpreted as a germination slit.

Conidiophores hyaline, smooth, upright or decumbent, simple to elaborately branched, up to 4 ranks, often in pseudowhorls, the entire structure 100–222 µm, or sometimes 400 µm, 4–6 µm diam. (Figs 16–18); individual branches 40–100 µm. Conidia produced in chains (Fig. 6, 11, 13, 15) that fall apart into individual conidia (Fig. 7) with the least disturbance. The first conidium is produced from the tapered apex of the conidiogenous cell (Figs. 8–10, 12). Initiation of a second conidium is indicated by a swelling or bulge at the apex of the conidiogenous cell (Figs. 8, 11, 14). The mechanics of conidiogenesis are discussed later herein. Conidia germinate readily by germ tube, produced from one or both ends, often swelling prior to germination, becoming conspicuously rotund or elongated. A TEM section of a conidium is shown in Fig. 21.

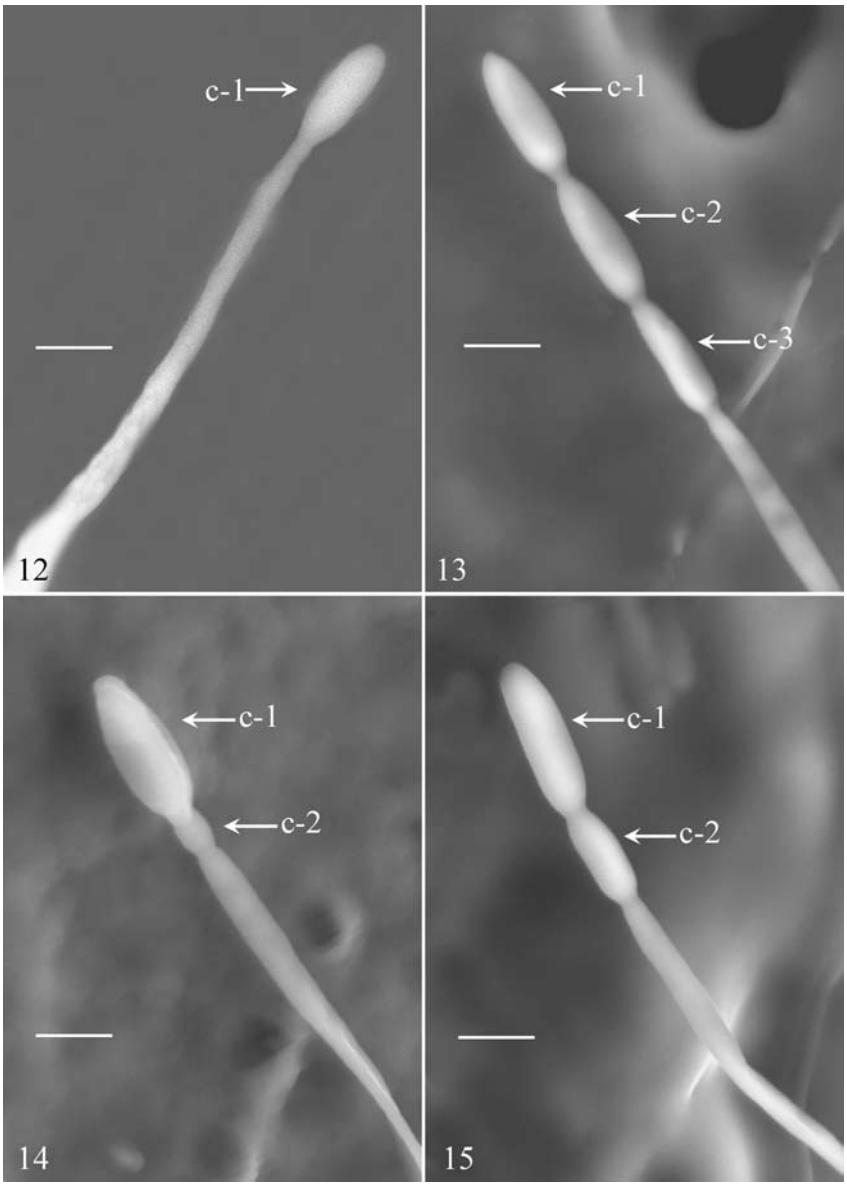
Conidia hyaline, subcylindrical to ellipsoid, (8–)12–15(–16) × (3–)4–5 µm, mean length (20 conidia) = 12.90 ± 1.43 µm, mean width (20 conidia) = 4.0 ± 0.64 µm. A contrast in sizes among conidia and ascospores is seen in Fig. 5. Conidia from authentic Karsten material (cited earlier herein) are similar to those discussed above. Conidial *Sphaeronaemella helvellae* is referable to *Gabarnaudia* Samson & W. Gams (Samson, 1974).



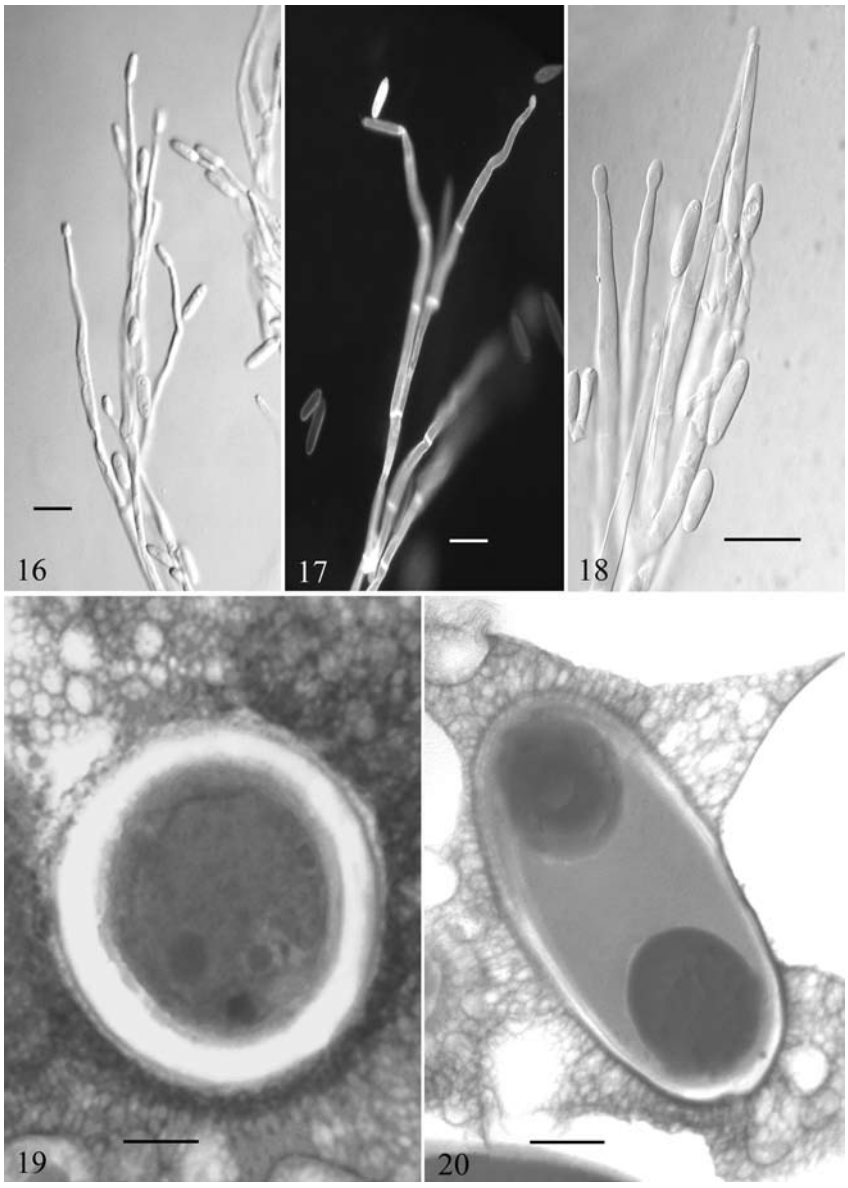

**Figs. 1–5. –** *Sphaeronaemella helvellae*. **1.** Perithecia. **2.** Perithecial neck showing ascospores within. **3.** Germinated ascospore. **4.** Germinated and ungerminated ascospores. **5.** Hyphae overlain with ascospores (a) and conidia (c). All Figs. by DIC. Scale bars: 1: 150 µm; 2: 8 µm; 3 and 4: 9 µm; 5: 1.4 µm.



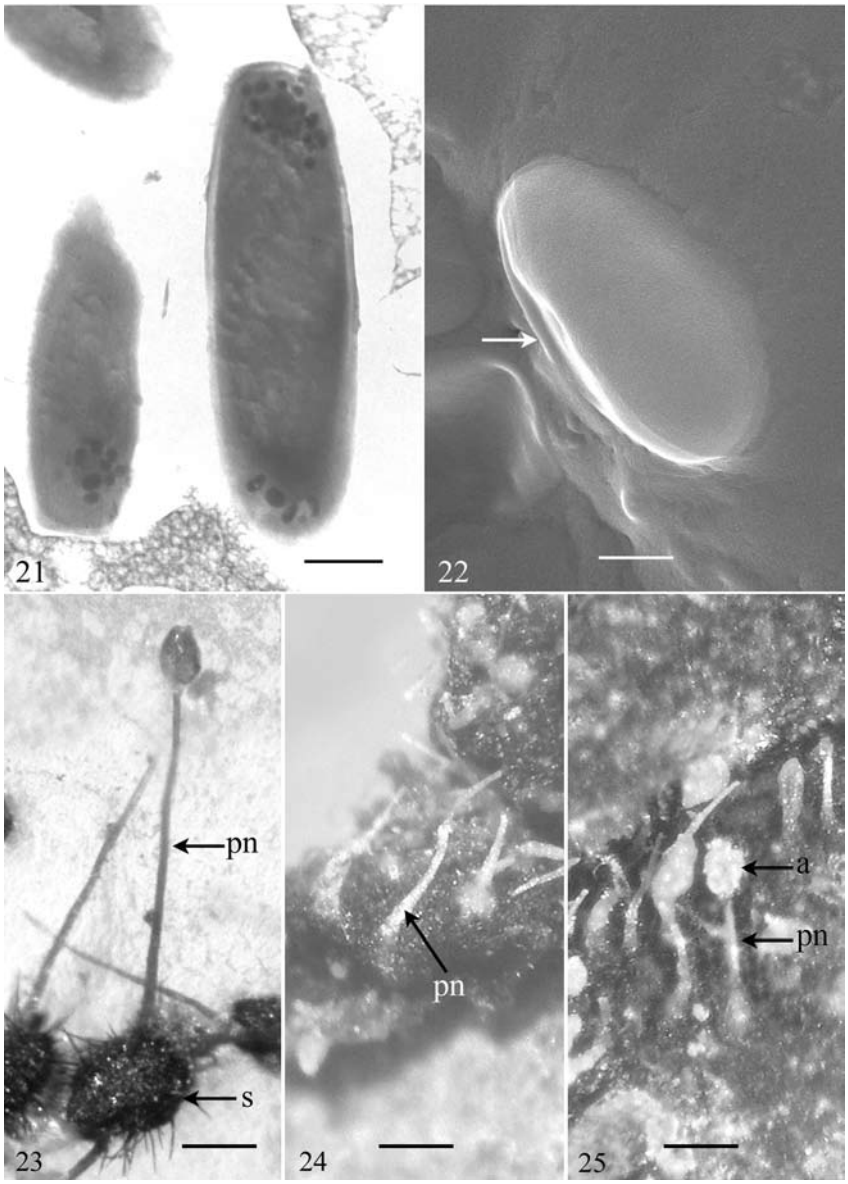

**Figs. 6–11. –** *Sphaeronaemella helvellae*. **6.** Chain of conidia. **7.** Conidia. **8.** Conidium (c-1) with bulge in conidiogenous cell indicating formation of a second conidium (c-2). **9.** First conidium from conidiogenous cell (c-1). **10.** Second immature conidium dislodged from open conidiogenous apex; initial mature conidium out of focus. **11.** Two conidia (c-1 and c-2). Fig. 10 by DF; others by DIC. Scale bars: 6: 14 µm; 7: 13 µm; 8–11: 4 µm.



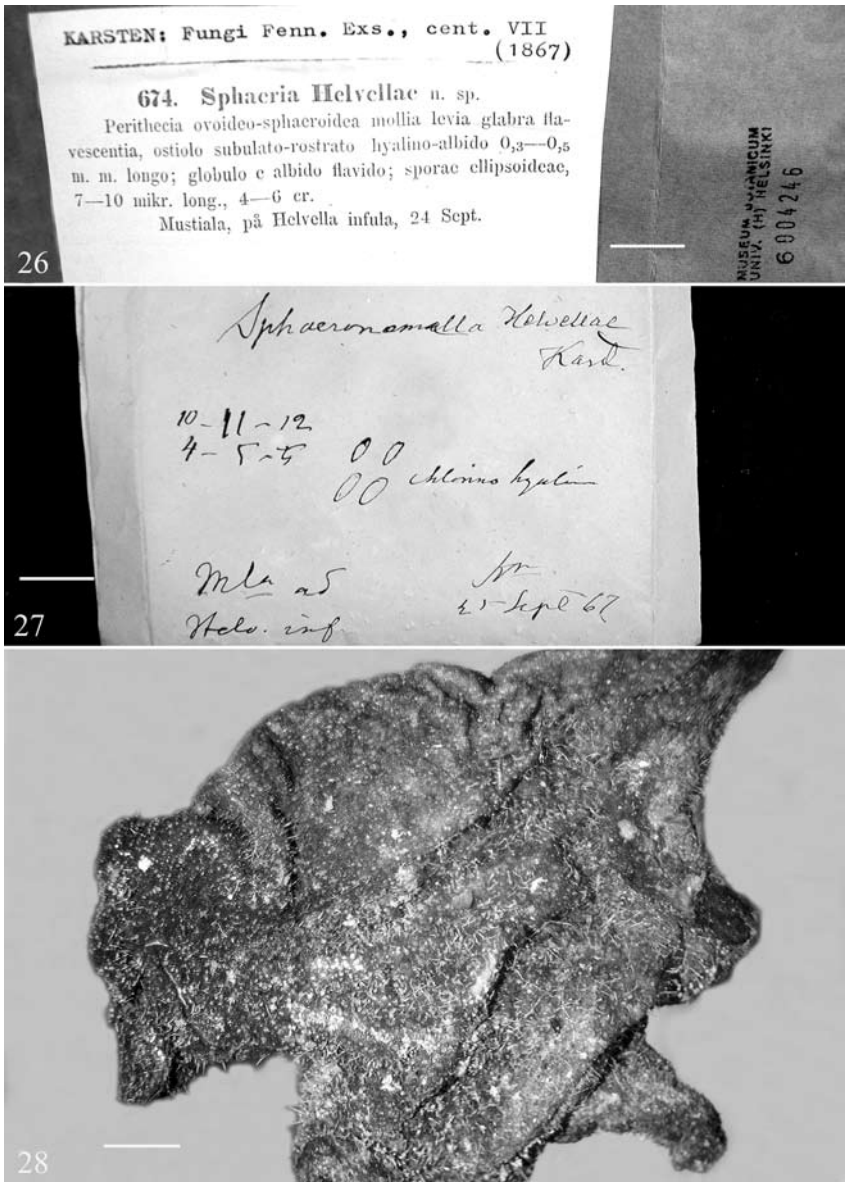

**Figs. 12–15. –** *Sphaeronaemella helvellae*. **12.** Initial conidium (c-1). **13.** Chain of three conidia (c-1, c-2, c-3). **14.** Conidium (c-1) and bulge in conidiogenous cell indicating formation of second conidium (c-2). **15.** Two conidia (c-1 and c-2). All Figs. by SEM. Scale bars: 12–15: 7.5 µm.




**Figs. 16–20. –** *Sphaeronaemella helvellae*. **16–18.** Examples of conidiophore branching. **19.** Cross-section of an ascospore. **20.** Longitudinal section of ascospore. Figs. 16 and 18 by DIC; Fig. 17 by DF; Figs. 19 and 20 by TEM. Scale bars: 16 and 17: 14 µm; 18: 13µm; 19: 1 µm; 20: 1.3 µm.




**Figs. 21–25. –** *Sphaeronaemella helvellae*. **21.** Longitudinal section of conidium. **22.** Ascospore with loosened wall (arrow) that might be misinterpreted as a germination slit. **23.** Perithecial neck (pn) emerging from sclerotium of *Colletotrichum*. **24** and **25.** Perithecial necks (pn) emerging from *Helvella* (*Gyromitra*), the one in Fig. 25 bearing a head of ascospores (a). From Karsten 1884. Fig. 21 by TEM; Fig. 22 by SEM; Figs. 23–25 by macrophotography. Scale bars: 21 and 22: 2 µm; 23–25: 200 µm.




**Figs. 26–28. –** *Sphaeronaemella helvellae*. **26.** Karsten description of *Sphaeria helvella* on lectotype packet. **27.** Karsten note in packet of 25. IX. 1867, no. 1106 as *Sphaeronaemella helvellae* Karst. Packet label is also *Sphaeronaemella Helvellae,* probably attached prior to the formal description. **28.** Fragment of Karsten lectotype of *Helvella* bearing abundant perithecia, seen here as minute white "threads". All Figs. by macrophotography. Scale bars: 26: 6 mm; 27: 7.3 mm; 28: 2 mm.

## Discussion

Cannon and Hawksworth (1982) reported that *Sphaeronaemella helvellae* ascospores have a longitudinal germination slit. Hausner and Reid (2004) suspected that, in fact, the putative germination slit was the result of a preparative error. Our observations of ascospore germination and general morphology show definitively that a germ slit is not present. Cannon and Hawksworth (1982) were not convinced that *S. helvellae* has an anamorph. We show that an anamorph for *S. helvellae* is indeed extant, as reported by Seeler (1943) and, later, Malloch (1974). *Gabarnaudia* was erected to include the anamorphs of *Sphaeronaemella* species; *S. helvellae* was not among those represented (Samson 1974). The species considered were well-illustrated and conidiogenesis was considered to be phialidic (Samson 1974). The *Gabarnaudia* states of *S. fimicola* Marchal and *G. betae* (Delacr.) Samson & Gams discussed by Samson (1974) were shown by Hausner and Reid (2004), along with *S. helvellae*, to form a monophyletic group on an SSrDNA sequence-based phylogenetic tree.

Conidiogenesis in *S. helvellae* has been difficult to interpret. It has aspects of the holoblastic and phialidic modes. After much study we accept it as phialidic. The process seems essentially like the formation of secondary phialides in *Ceratocystis adiposa* (Butler) C. Moreau (Cole & Samson 1979). Our photographs of *S. helvellae* (Figs. 8-15) are highly reminiscent of Figs. 4.39 A-H of *C. adiposa*, especially in the swelling of the conidiogenous cell apex that is indicative of the formation of another conidium. We did not see evidence of scars or collarettes by either light microscopy or SEM as did Cole and Samson (1979) for *C. adiposa* (see their Figures 4.42-4.44). However, darkfield fluorescence microscopy reveals that the apex of the conidiogenous cell is open (Fig. 10). What might be a ring-building region fluoresces very close to the apex. The wall of the conidium does not appear to be continuous with the conidiogenous cell. In the shallowness of the active region of conidiogenesis *S. helvellae* resembles the process in *Penicillium* (Cole & Samson 1979). Connections between contiguous conidia do not seem as well-developed as in *Penicillium*.

It was not surprising to discover *S. helvellae* as a parasite on sclerotia of *Colletotrichum coccodes*, the cause of black dot of potato (Fig. 23). It possibly exerts some degree of biological control, an aspect that we intend to investigate further. In addition to *Helvella* (*Gyromitra*) spp. it has been reported on a variety of other fungi (see Hausner & Reid 2004 for refs.). These authors expressed the likelihood that *S. helvellae* is an obligate or facultative mycoparasite, an opinion with which we agree.

A yellow, mucoid, Gram-positive bacterium was initially associated with cultures of *S. helvellae* and appeared to induce increased perithecial production. Because of its association with *S. helvellae* and

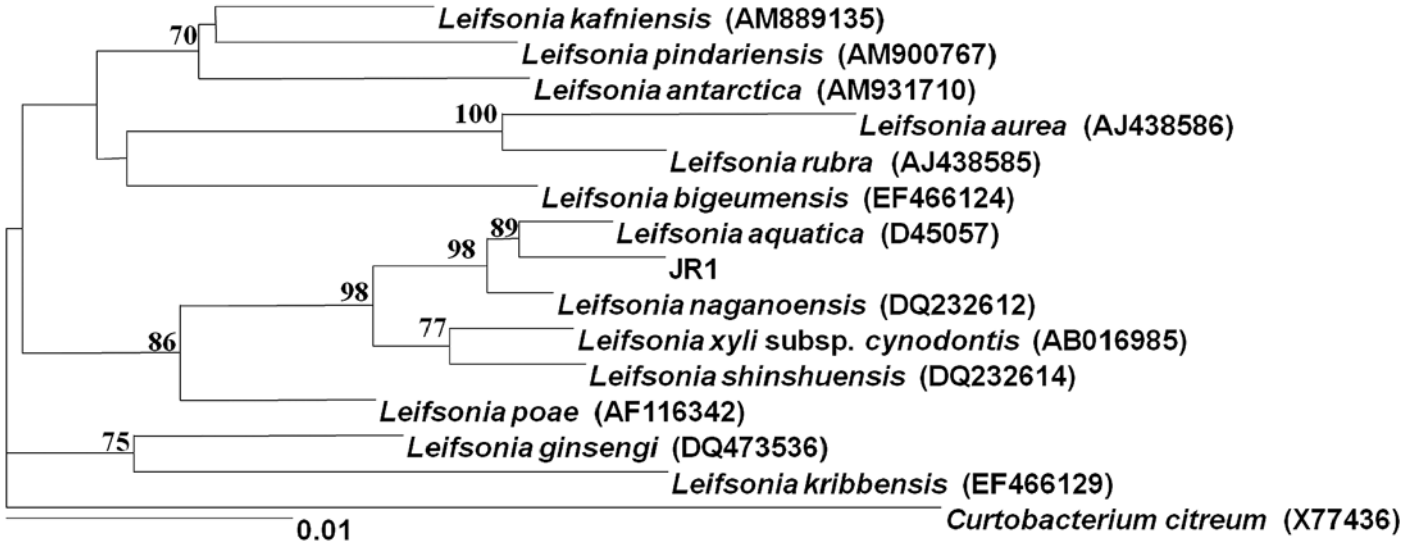



**Fig. 29**. – Phylogenetic tree showing the position of strain JR1 relative to other *Leifsonia* species based on 16S rDNA and neighbor joining analysis. The sequence of *Curtobacterium citreum* (X77436) served as the outgroup sequence. Numbers within the dendrogram indicate bootstrap values out of 1000 bootstrapped trees. Bar, 0.01 substitutions per nucleotide position.

its apparent influence on the fungus it was identified using a combination of traditional and contemporary protocols and designated as strain JR-1. Strain JR-1 was placed in a well-supported clade (98 %) with *Leifsonia aquatica* and *L. naganoensis* based on neighbor joining analysis of the 16S rDNA sequence and 1000 bootstrap replicates (Fig. 29). Bacterial strains classified as *Leifsonia* species have been reported from diverse environments and substrates ranging from soil (Dastager *et al*. 2008, 2009; Pindi *et al*. 2009, Suzuki *et al*. 1999), water (Davis *et al*. 1984), the Antarctic (Pindi *et al*. 2009, Reddy *et al*. 2003) and Himalayan glaciers (Reddy *et al*. 2008) to ginseng root (Qiu *et al*. 2007) and nematode galls formed on grasses (Evtushenko *et al*. 2000); others are known to be plant pathogens (Davis *et al*. 1984). It is not certain if the association observed between strain JR-1 and *S. helvellae* was casual or specific, but it seems possible that the two organisms could co-occur in a commensalistic or mutualistic relationship.

## Acknowledgments

PPNS 0533 Department of Plant Pathology, College of Agricultural, Human, and Natural Resources Sciences Research Center, Projects 3061, 0565, 0652, Washington State University. We thank Michael J. Adams for electron microscopy and assembling the plates. We thank Jane Stewart for aid in constructing the phylogenetic tree for *Leifsonia aquatica*.

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*(Manuscript accepted 24 March 2010; Corresponding Editor: R. Pöder)*