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SETA STRUCTURE IN MEMBERS OF THE COLEOCHAETALES
(STREPTOPHYTA)

Timothy R. Rockwell

28 Pages

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The charophycean green algae, which include the Coleochaetales, together with land plants form a monophyletic group, the streptophytes. The order Coleochaetales, a possible sister taxon to land plants, is defined by its distinguishing setae (hairs), which are encompassed by a collar. Previous studies of these setae yielded conflicting results and were confined to one species, *Coleochaet. scutata*. In order to interpret these results and learn about the evolution of this character, setae of four species of *Coleochaete* and the genus *Chaetosphaeridium* within the Coleochaetales were studied to determine whether cellulose, callose, and phenolic compounds contribute to the chemical composition of the setae. Two major differences were found in the complex hairs of the two genera. In *Chaetosphaeridium* the entire seta collar stained with calcofluor indicating the presence of cellulose, while in the four species of *Coleochaete* only the base of the seta collar stained with calcofluor. In *Chaetosphaeridium* the seta proper exhibited no internal structures that were detectable with differential interference contrast microscopy or with calcofluor, but did have variable portions that stained with aniline blue indicating the presence of callose. In contrast, setae of the four species of *Coleochaete* had readily

observable internal structures that stained brightly with both calcofluor and aniline blue. More research on *Chaetosphaeridium* and *Coleochaete* is necessary to determine if the extensive work on the cell walls of *Coleochaete* can be generalized to *Chaetosphaeridium* and whether one genus might be a more appropriate model organism for studying the evolution of plant cell walls.

SETA STRUCTURE IN MEMBERS OF THE COLEOCHAETALES
(STREPTOPHYTA)

TIMOTHY R. ROCKWELL

A Thesis Submitted in Partial
Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

School of Biological Sciences

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SETA STRUCTURE IN MEMBERS OF THE COLEOCHAETALES
(STREPTOPHYTA)

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T.R.

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CHAPTER I

INTRODUCTION

The charophycean green algae (streptophyte algae) and land plants together form a monophyletic group called the streptophytes (Timme et al, 2012). The Coleochaetales, a group of algae within the charophycean green algae, have distinguishing setae (hairs) that mark their clade. The seta is encompassed by a collar (often also referred to as a sheath), which is about as long as the diameter of the cell, though its size can vary among species. The seta itself grows many times longer than the cell, up to and even greater than 100 times the diameter of the cell. Marchant (1977) proposed that the seta cells are secretory in nature based on the large surface area and vesicular activity, but no hard evidence of seta function has been documented.

The Coleochaetales include two monophyletic genera, *Chaetosphaeridium* and *Coleochaete* (Delwiche et al. 2002). The genus *Coleochaete* can be further grouped into two clades: the *C. scutata* clade and the *C. irregularis* clade (Cimino & Delwiche, 2002; Fig. 1). Although several representatives of these clades are known, only the setae of *Coleochaete scutata* have been studied in detail. Therefore the data collected on the setae of this species might not be readily generalized to the whole genus. More important, the genus *Chaetosphaeridium* has been completely left out of these studies.

The Coleochaetales

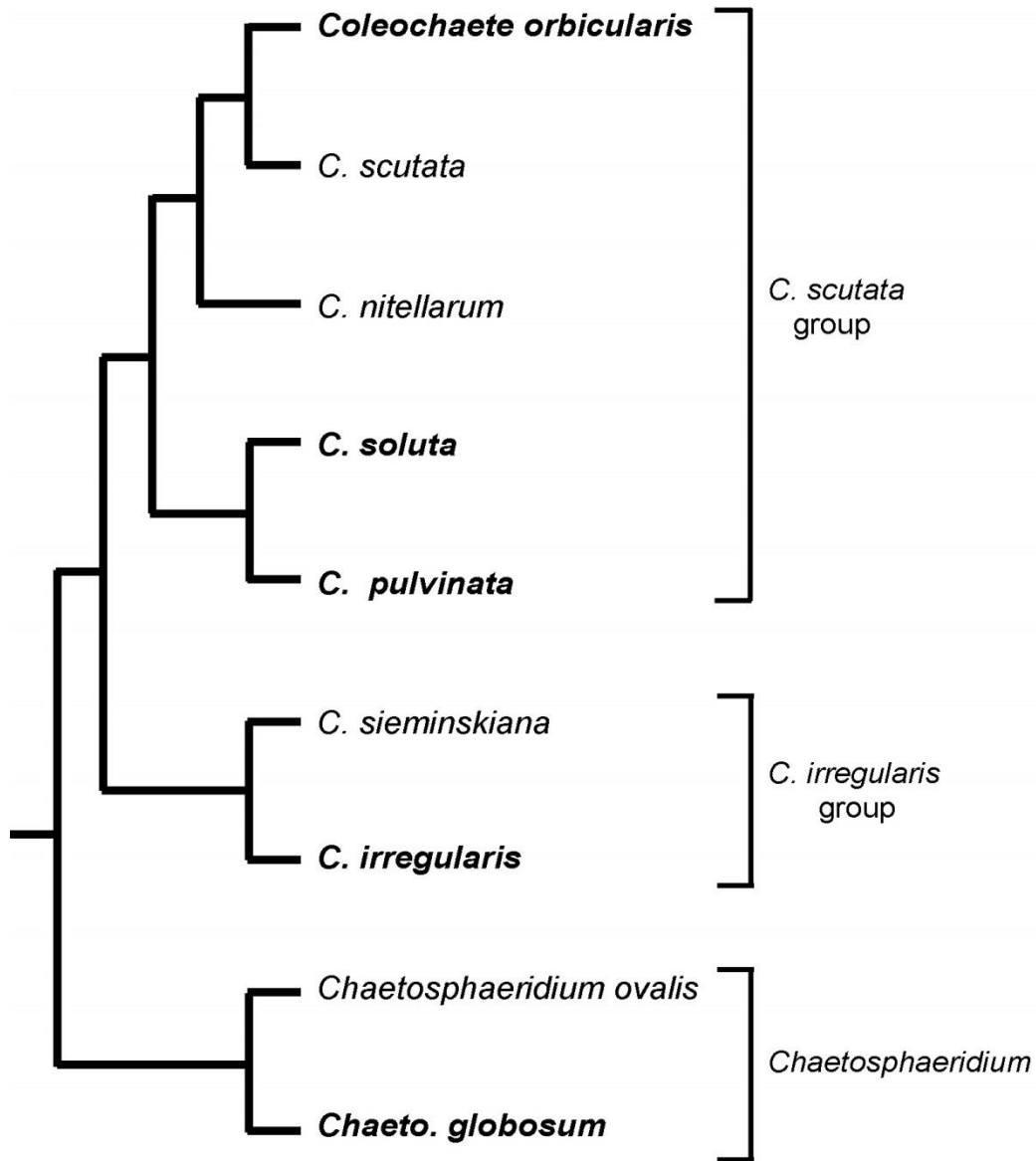


Fig. 1 Coleochaetales Phylogeny. After Cimino & Delwiche, 2002

Taxa included in the present study are indicated with boldface type.

Wesley (1928) used light microscopy to describe the development of the seta in *C. scutata*. She observed that the seta grows within the seta collar, which then ruptures, allowing the seta to continue growth long after the seta collar has stopped growing.

McBride (1974) used a combination of light and electron microscopy to describe seta

structure in *C. scutata*. Based on transmission electron microscopy (TEM) sections McBride found that the seta had a plasma membrane but not a cell wall. McBride used several assays to test the chemical composition of the seta collar including testing for cellulose (chloroiodide of zinc and potassium iodide-sulphuric acid), pectin (ruthenium red or ferric chloride-hydroxyl amine), lignin (Schiff's reagent, phloroglucinol-HCl, and chlorine sulphate), cuticular waxes (Schiff's reagent), and lipids (Sudan III and IV). The only test that yielded positive results was Schiff's reagent, which could have indicated cuticular waxes or lignin. Marchant (1977) also used light and electron microscopy to study seta structure in *C. scutata*. Marchant observed a seta cell wall with TEM and found that staining the seta with calcofluor indicated the presence of cellulose in cell walls. These results conflict significantly with McBride who suggested that the seta did not have a cell wall. Marchant did not report any calcofluor work on the seta collar, leaving it still unknown what the chemical composition of the collar might be.

By staining the seta cellulose with calcofluor, rinsing out the stain, and examining the setae after they had time to grow, Marchant documented regions of new growth identified by the absence of staining. Using this technique Marchant observed new growth in the basal region of the seta as opposed to the tip. From TEM work, Marchant reported annular wall thickenings inside the seta, which were regions of thick internal seta cell wall with only a narrowed strand of cytoplasm passing through. In his light microscopy studies, these thickenings fluoresced yellow when stained with aniline blue, indicating that callose was present. Marchant (1977) observed that broken setae appeared to be rapidly plugged with material that stained with aniline blue, implying that callose was involved in a wound healing mechanism such as that in plants and other green algae

(Chen & Kim, 2009, Scherp et al. 2001). Similarly callose was found in the complex charophycean green alga *Nitella flexilis* when wound healing plugs formed in response to stress (Foissner, 1988), suggesting that callose-related wound healing mechanisms evolved within the streptophytes prior to the evolution of land plants.

The first objective of this project was to expand knowledge of seta structure in the Coleochaetales to more species than *C. scutata*. Expanding the research to more species within the *C. scutata* clade and also to the other major clade in the genus *Coleochaete*, the *C. irregularis* clade, and also to the genus *Chaetosphaeridium* would make it possible to determine how similar the character is across taxa. Because these setae are a synapomorphy of the clade, refining our knowledge of how all of the representatives express the character may lead to an understanding of how this character evolved.

The second aspect of the project was to resolve the conflicts between the McBride (1974) and Marchant (1977) papers. The presence of cellulose in these setae dramatically changes our understanding of them, so this is not a trivial conflict. Also an understanding of collar composition is of importance since it is unclear if Marchant observed staining for cellulose in the seta collars of *C. scutata*.

Because the Coleochaetales have such a close evolutionary relationship to land plants, it is important to understand the structural components that make up these organisms. Like land plants, charophycean green algae have been shown to produce hemicelluloses, pectins, and arabinogalactan proteins in their cell walls (Sorensen et al, 2011). *Coleochaete* is especially interesting in this regard because it can express lignin-like substances (Delwiche et al, 1989, Sorensen et al, 2011). In addition, the rosette pattern of cellulose microfibril synthesizing complexes in land plants has been reported in

some of the streptophyte algae (reviewed by Tsekos, 1999), although *Coleochaete* has an extra and larger subunit in the middle of the rosette (Okuda, 1992). Consistent with the shared ancestry of the cellulose microfibril synthesizing complex, two cellulose synthase (*CesA*) genes of the zygnematalean streptophyte alga *Mesotaenium caldariorum* share significant similarity to the *CesA* genes of land plants, indicating that these genes arose within the streptophyte green algae prior to land plant evolution (Roberts, 2004). Therefore knowledge of diverse cell wall structure in the setae and seta collars of members of the Coleochaetales may lead to a greater understanding of cell wall diversity in the streptophytes.

CHAPTER II

METHODS

Taxa

The five species studied were *Coleochaete orbicularis*, *C. soluta*, *C. pulvinata*, *C. irregularis*, and *Chaetosphaeridium globosum*. These taxa are representative of several clades within the order, providing a broad sampling that includes four major subclades (Fig. 1 in bold). All species were grown in a 16° C culture chamber with a 16h light 8h dark cycle, with the exception of *C. pulvinata* which was collected from Moon Lake, two miles west of Montello, in Marquette County, Wisconsin (43°48'17.61"N 89°22'12.75"W) and maintained in a 10° C culture chamber with a 12h light 12h dark light cycle. The species grown in culture were grown on coverslips by leaving a coverslip in the bottom of a petri dish so new thalli grown from zoospores could form on top of the coverslip. *C. irregularis* and *C. orbicularis* were grown in C-Medium (Andersen, 2005), *C. soluta* in Bold's Basal Medium (Nichols, 1973), and *Chaetosphaeridium* in DYIII (Lehman, 1976) or in MES-*Volvox* Medium (Andersen, 2005). Thalli of *C. pulvinata* were removed with a razor blade or tweezers from the epidermis of the wetland plant *Scirpus* (bulrush).

Microscopy

To test for cellulose in setae and seta collars, specimens were stained with a solution of 0.1% calcofluor (Fluorescent Brightner 28, Sigma F-3397) (Marchant, 1977), a stain that binds to cellulose with hydrogen bonding (Haigler et al, 1980). Calcofluor fluoresces blue-white in UV light.

To test for growth patterns the specimens were stained for twenty minutes with calcofluor, rinsed thoroughly to remove unbound calcofluor, and left to grow for one to two days before being observed. The resulting new growth would lack staining whereas old growth would glow blue-white with calcofluor.

To confirm that the calcofluor wasn't labeling other beta glucans, a carbohydrate-binding module (CBM3a, Plant Probes) specific for crystalline cellulose was used (McCartney et al, 2004). The protocol used was adapted from Matos et al (2013). The organisms were grown on coverslips, then fixed on the coverslip in 4% formaldehyde in phosphate buffer (PB) solution for 30 minutes. They were blocked in 5% milk solution in phosphate buffer saline (PBS) for fifteen minutes and put directly into a 1:100 dilution of CBM3a in blocking solution for forty-five minutes to an hour. They were then put in a 1:1000 dilution of mouse Anti-polyHistidine antibody (Sigma-Aldrich H1029) solution made with blocking solution for forty-five minutes to an hour. Lastly, specimens were placed in a 1:100 dilution of Anti-Mouse AlexaFluor488 (Life Technologies, Molecular Probes) with the blocking solution for forty five minutes to an hour in the dark. Controls were prepared without the primary antibody.

To test for callose in the setae annular wall thickenings as described by Marchant (1977), the specimens were stained with a freshly made 0.01% aniline blue solution made with a pH 8 phosphate buffer which would stain callose a yellow-green color (Currier and Strugger, 1956). Because callose is known to form as a wound response, *Chaetosphaeridium* and one species from the *Coleochaete* clade were also tested in aniline blue with parafilm placed between the coverslip and the slide to protect the organisms from the weight of the coverslip. To confirm results obtained with aniline blue, specimens were treated with 400-2 (BioSupplies, Australia), an antibody specific for (1→3)-beta glucans (callose) (Keikle et al, 1991). The cells were fixed in a 4% formaldehyde and PB solution for 30 minutes. They were blocked in a 5% milk solution made with PBS for at least 15 minutes and then directly moved into a 1:100 dilution of mouse 400-2 antibody made with blocking solution for forty-five minutes to an hour. Lastly they were put in a 1:100 dilution of Anti-Mouse AlexaFluor488 (Life Technologies, Molecular Probes) made with the blocking solution for forty-five minutes to an hour. Controls were prepared without the primary antibody.

To test for phenolic compounds organisms were observed with blue violet (BV) light using filter set (BP400-440, DM455, BA475), which makes phenolic compounds autofluoresce yellow (Kroken et al, 1996). Calcofluor and Aniline blue were observed using fluorescence microscopy with a UV light filter set (BP360-370, DM400, BA420) and antibodies were observed with a WIBA filter set (BP460-490, DM505, BA515-550). Matching differential interference contrast (DIC) images and autofluorescence controls were obtained. Specimens were examined with a 20x, 40x, 60x, or 100x planfluorite lens on a BX-60 microscope (Olympus America Inc., Melville, NY, USA).

CHAPTER III

RESULTS

Chaetosphaeridiumn globosum

When observed with UV light the chloroplasts of *Chaetosphaeridium* glowed a dark red (Fig. 2B). Under UV light dead cells often autofluoresced a light blue (Fig. 2B), but the walls of the living cells including the seta collar (Fig. 2A, arrowhead) and the seta (Fig. 2A, double arrowhead) did not autofluoresce. When specimens were observed under UV light after treatment with the beta glucan stain calcofluor, the walls of the cell proper, the entire seta collar and the seta glowed brightly blue (not shown). When exposed to calcofluor for a brief period, rinsed to remove unbound calcofluor, then observed after a day or two, the basal portion of the seta (Fig. 2C, arrowhead) had no blue color present whereas the apical portion of the seta (Fig. 2D, arrow) and the entire seta collar (Fig. 2D, asterisk) glowed with the characteristic calcofluor blue. When treated with the more specific CBM3a carbohydrate-binding module which binds to crystalline cellulose, the cell proper labeled, with progressively heavier labeling observed in older cells of the thallus (Fig. 2G). Neither the seta collars (Fig. 2F, arrowhead) nor the setae (Fig. 2F, double arrowhead) showed any labeling. When the primary antibody was removed there was no labeling of the organism at all (not shown).

Under the same UV light when treated with aniline blue, a stain used to test for the beta 1,3 glucan callose, the cells of the thallus sometimes displayed a bright yellow. The seta proper did show some bright yellow staining, but this staining was highly variable (Fig. 2H and I). No pattern in the spacing or length of stained portions of the seta was recognized. When compared to the DIC images there was no obvious difference between stained and unstained portions of the seta. Because callose is known to be produced in response to wounding in plants, specimens were also examined with coverslips supported by parafilm to make sure that pressure from the coverslip was not damaging the cells. Under these conditions the cells proper (Fig. 2J and K) and setae (not shown) still exhibited bright yellow staining when treated with aniline blue. Often the brightest staining was centralized between the cells of the thallus (Fig. 2J and K). To confirm that what was being seen with the aniline blue was indeed callose, an antibody specific for callose, 400-2, was used. When labeled with this antibody, the cell proper showed a similar labeling pattern to what was seen with aniline blue. Where the cells of the thallus were connected, there was bright labeling. The entire setae, including the collars (Fig. 2L, arrowhead), showed no labeling. When the primary antibody was omitted, none of the thalli exhibited any labeling (not shown).

Lastly, to test for phenolic compounds *Chaetosphaeridium* was exposed to blue violet light, under which phenolic compounds may fluoresce yellow. Although some dead cells and small portions of the cells proper did fluoresce yellow, the setae and setae collars (Fig. 2N, arrowhead) did not exhibit any yellow fluorescence.

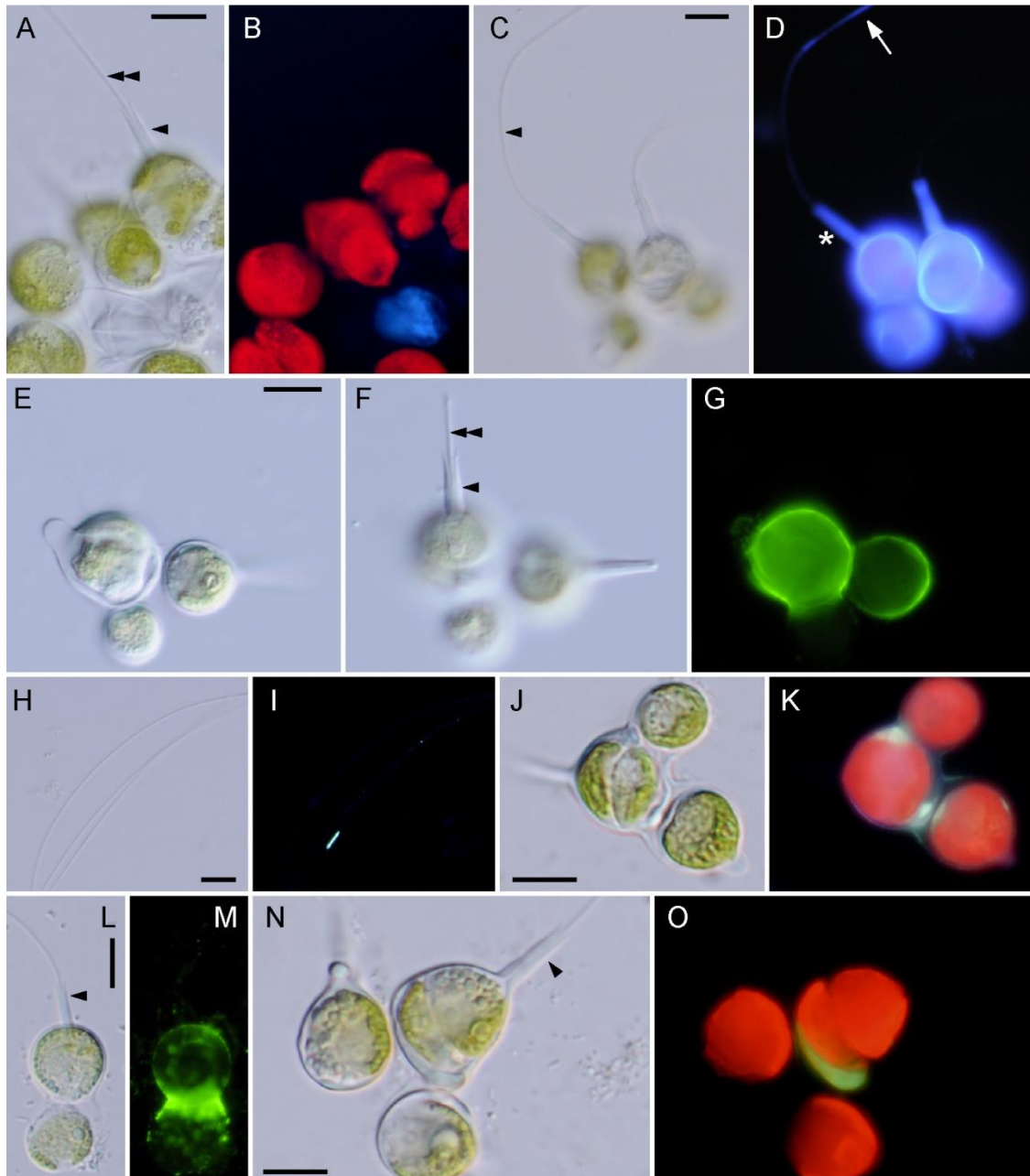


Fig. 2 *Chaetosphaeridium*

(A, B) Untreated thallus observed with DIC and UV light showing no autofluorescence in the seta collar (A, arrowhead) or seta (A, double arrowhead). (C, D) Calcofluor treated thallus after rinsing and 1-2 days of growth. New growth of the seta (C, arrowhead) with no calcofluor staining and bright by stained region (D, arrow) showing old growth. Entire collar (D, asterisk) labels with calcofluor. (E-G) Thallus labeled with carbohydrate-binding molecule in two focal planes with DIC and one in fluorescence. Seta collars (F, arrowhead) and setae (F, double arrowhead) do not label with carbohydrate-binding molecule. (H, I) Setae treated with aniline blue to show callose. (J,

K) Aniline blue treated thallus with parafilm spacers demonstrating callose present without weight of the coverslip. (L, M) Thallus labeled with 400-2 callose antibody demonstrating seta collar (L, arrowhead) and seta unlabeled. (N, O) Untreated thallus observed under blue violet light to show phenolic compounds seen as yellow autofluorescence. There is no collar autofluorescence (N, arrowhead). All scale bars are 10 μm .

Coleochaete irregularis

When *Coleochaete irregularis* was observed under UV light neither the walls of the cell proper, nor the seta collar or seta (Fig. 3A, arrowhead) exhibited any autofluorescence. After treatment with the calcofluor, *C. irregularis* exhibited a bright blue glow in the walls of the cell proper. However only the basal portion of seta collar stained (Fig. 3F, arrow) while the apical portion of the collar did not stain (Fig. 3E, arrowhead). The setae proper stained completely, however portions of the seta stained much brighter blue than the other portions. In some cases, these brighter spots matched portions of the wall that were thicker when observed in DIC (Fig. 3G and H). When treated with calcofluor and rinsed to remove unbound calcofluor after a day or two, the basal portion of the setae (Fig. 3I, arrowhead) showed no staining, indicating new growth, while the older apical portion of the setae (Fig. 3K, arrow) displayed the previously described blue staining.

Observation of *Coleochaete irregularis* under UV light after treatment with aniline blue revealed that the walls of the cell proper sometimes stained with aniline blue (not shown). The pattern of seta staining seen with calcofluor matched that with aniline blue, however the portions of the seta not associated with thickenings did not stain uniformly or consistently (Fig. 3L through N).

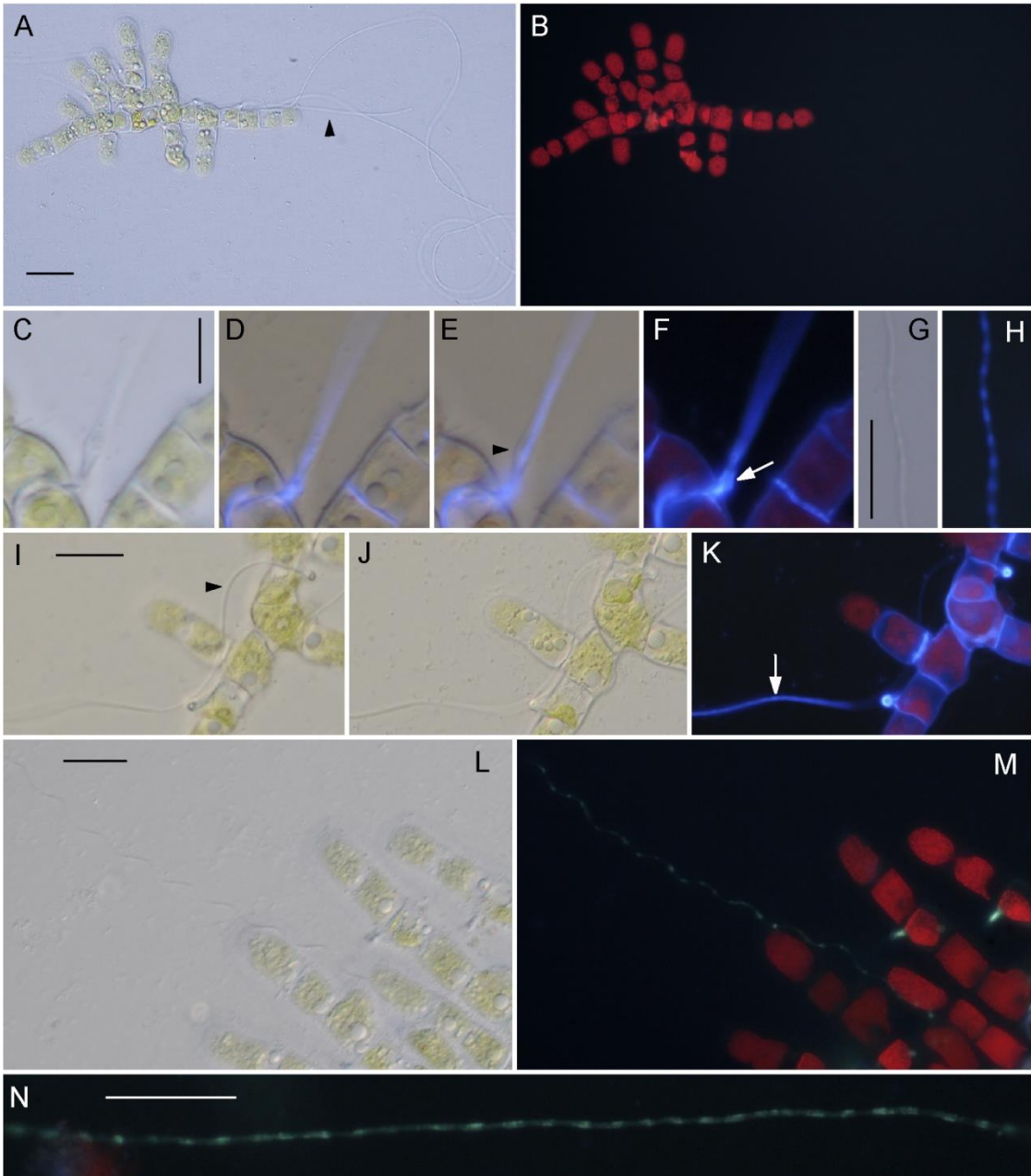


Fig. 3 *Coleochaete irregularis*

(A, B) Untreated thallus observed in DIC and UV light shows no autofluorescence in the setae (A, arrowhead). (C-F) Calcofluor-treated thallus with seta collar observed in DIC (C), UV light (F) and a combination of DIC and UV light in two planes of focus (D, E). Basal portion of the seta collar (F, arrow) stained though the apical region of the seta collar (E, arrowhead) did not stain. (G, H) Calcofluor treated seta with observable thickenings that corresponds to brighter staining pattern. (I-K) Calcofluor treated thallus after rinsing and 1-2 days of growth shown with two DIC focal planes (I–J). New growth shown with lack of stain (I, arrowhead) and old growth showing blue stain (K, arrow). Aniline blue-treated thallus with seta showing yellow staining pattern (L, M), and

shown at higher magnification in another seta (N). Scale bar for A and B is 40 μm , for C through E 10 μm , and for G through N 20 μm .

Coleochaete pulvinata

When observed with UV light, neither the walls of the cell proper, the seta collar (Fig 4A, arrowhead), nor the seta of *Coleochaete pulvinata* autofluoresced.

When treated with calcofluor and observed under the UV light, the walls of the cell proper, the basal portion of the seta collar (Fig. 4C and E, asterisk), and the seta proper (Fig. 4E, big arrow) glowed blue while the apical portion of the seta collar (Fig. 4D, arrowhead) lacked stain. The basal portion of the collar was also thinner apical portion of the collar. Thickenings found within the seta glowed a brighter color than the rest of the seta (Fig. 4E, small arrow). When exposed to calcofluor, rinsed to remove any unbound calcofluor, and left to grow for 1 to 2 days, the very basal portion of the seta did not fluoresce (Fig. 4G, arrowhead) whereas the apical portion of the seta glowed bright blue (Fig. 4G, arrow).

When observed with UV after treatment with aniline blue to indicate callose, the cell proper often stained bright yellow. The seta collar (Fig. 4H, arrowhead) did not glow yellow under these conditions (Fig. 4I), but the seta often did (Fig. 4K, big arrow). The brightest staining in the seta was associated with the thickenings that were distinguishable via DIC (Fig. 4J and K, small arrows). These wall thickenings still stained with aniline blue even when the pressure of the coverslip was removed by using parafilm as spacers (Fig. 4L and M, arrows)

Coleochaete pulvinata observed under blue violet (BV) light was found to have yellow autofluorescence in its cell proper and seta collar (Fig. 4O, arrow). However this was only observed when *C. pulvinata* was going through its sexual life cycle.

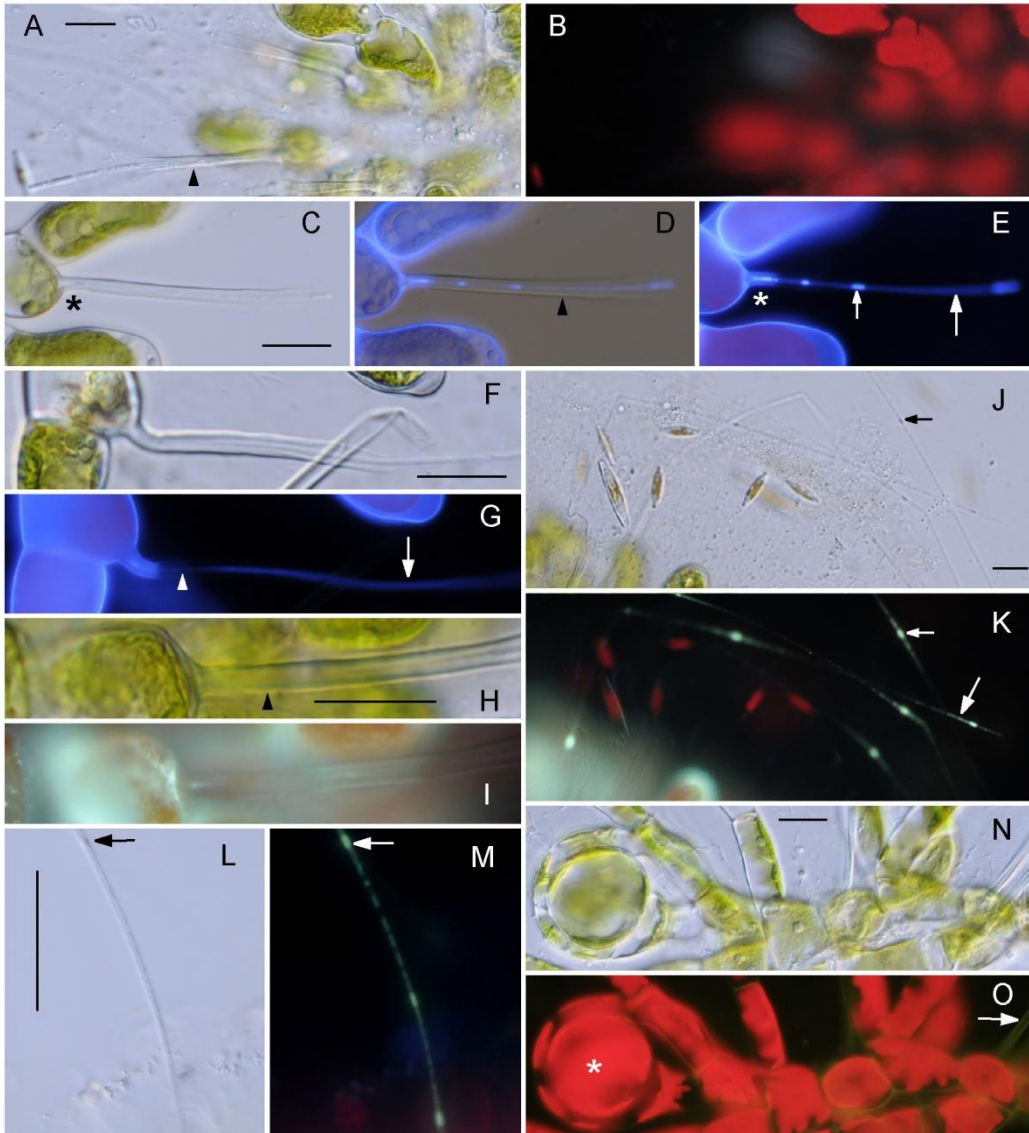


Fig. 4 *Coleochaete pulvinata*

(A, B) Untreated thallus observed in DIC and UV light showing no seta collar (A, arrowhead) or seta autofluorescence. (C-E) Calcofluor treated thallus observed with DIC (C), UV light (E) and a combination of DIC and UV light (D). Seta collar stained with Calcofluor at the base (C and E, asterisk) but not in the apical portion (D, arrowhead). Seta stained throughout (E, big arrow) and brighter at the thickenings (E, small arrow). (F, G) Calcofluor-treated thallus after rinsing and 1-2 days of growth showing new seta

growth unstained at seta base (G, arrowhead) and old growth staining brightly (G, arrow). (H, I) Aniline blue treated thallus, lacks staining along entire collar (H, arrowhead). (J, K) Several setae treated with aniline blue stain brightly at thickenings (J and K, small arrow) and less brightly at areas near the thickening (K, big arrow). (L, M) Aniline blue treated seta with spacers to show callose staining (L and M, arrows) not created by weight of the coverslip. (N, O) Untreated thallus with zygote (O, asterisk) observed under blue violet light reveals phenolic compounds yellow (N, O) in a seta collar (O, arrow). All scale bars are 20 μm .

Coleochaete soluta

When *Coleochaete soluta* was observed under UV light, neither the walls of the cell proper (Fig. 5A and B), nor the seta collar, nor the seta (Fig. 5C, arrowhead) revealed any autofluorescence. When treated with calcofluor and observed under UV light the walls of the cell proper, the base of the seta collar (Fig. 5G, asterisk) and the seta (Fig. 5H, arrow) stained bright blue. Only the apical portion of the seta collar (Fig. 5F, arrowhead) lacked staining.

After treatment with aniline blue, the walls of the seta proper sometimes glowed bright yellow (Fig. 5I and J). The seta exhibited some staining (Fig 5J, big arrow), the brightest portions of which (Fig. 5J, small arrow) were sometimes associated with thickenings that could be seen in DIC. Not all of the thickenings stained with aniline blue however (Fig. 5I, arrowheads).

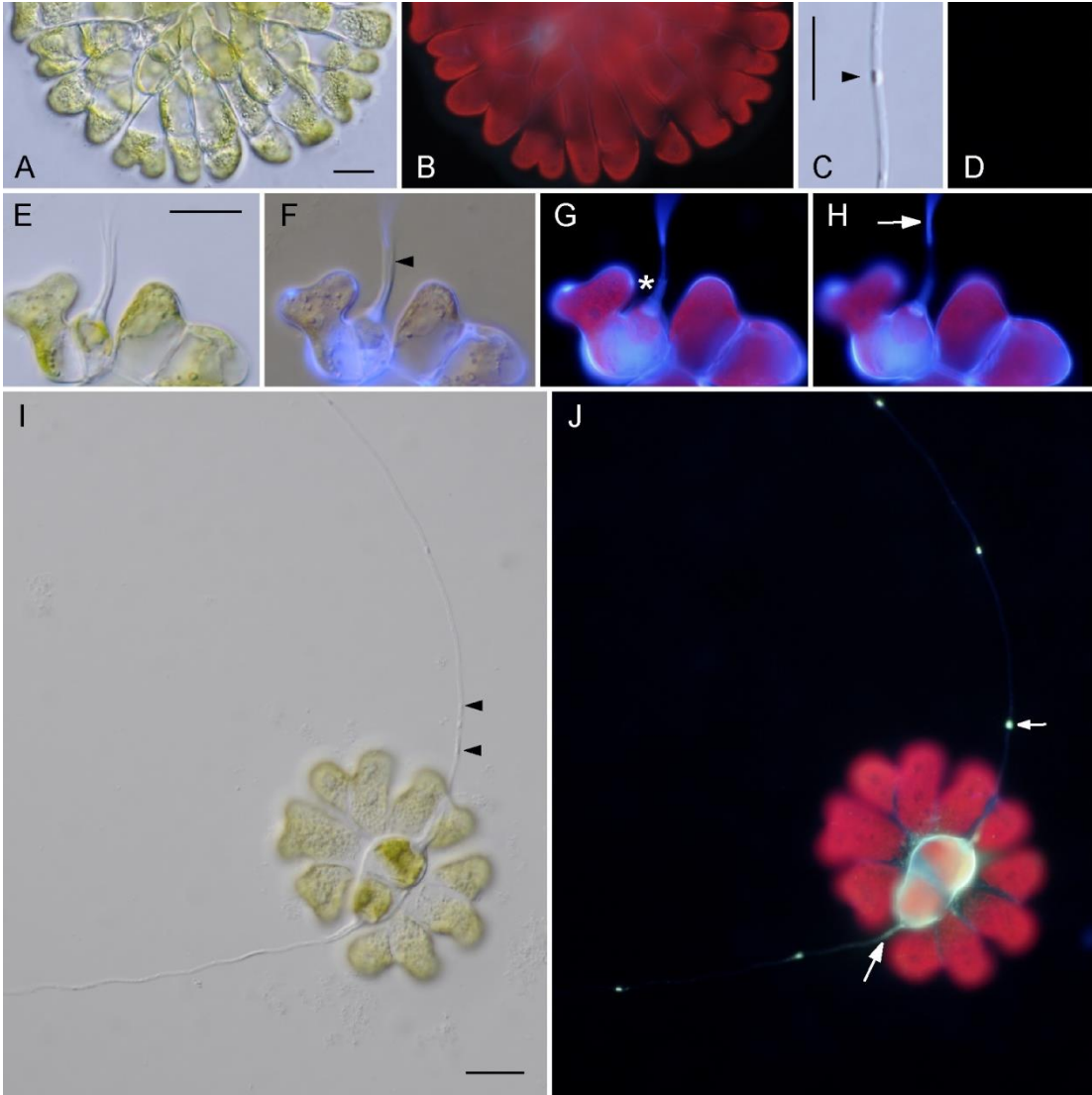


Fig. 5 *Coleochaete soluta*

(A, B) Untreated thallus observed in DIC and UV light shows no cell wall autofluorescence. (C, D) Untreated seta observed in DIC and UV light with no autofluorescence in either seta or thickening (C, arrowhead). (E-H) Calcofluor treated thallus observed with DIC (E), two focal planes of UV light (G, H), and a combination of DIC and UV light (F). Apical portion of the seta collar (F, arrowhead) shows no staining while basal portion of the collar (G, asterisk) and the seta (H, arrow) are stained. (I, J) Aniline blue treated thallus showing staining of some portions of the seta not associated with thickenings (J, big arrow) and staining of most seta thickenings (J, small arrow). A few thickenings (arrowheads) did not stain (I, arrowheads). All scale bars are 20 μm .

Coleochaete orbicularis

When *Coleochaete orbicularis* was observed under UV light, neither the walls of the cell proper, nor the seta collar, nor the seta (Fig. 6A, arrowhead) revealed any autofluorescence. When *C. orbicularis* was observed under UV light after treatment with calcofluor, the walls of the cell proper and the seta stained bright blue, while the seta collar lacked staining in the apical region (Fig. 6D, arrowhead). When treated with calcofluor, portions of the seta identified as thickenings (Fig. 6F, arrow) glowed brighter than the rest of the seta. When *C. orbicularis* was exposed to calcofluor, rinsed to remove unbound calcofluor, and left to grow for 1-2 days, the basal portion of the seta (Fig. 6G, arrowhead) showed no staining while the apical portion (Fig. 6H, arrow) glowed a bright blue.

In *Coleochaete orbicularis* some walls of the cell proper glowed a bright yellow when exposed to aniline blue and viewed under UV light (Fig. 6I and J). The seta showed regions that glowed yellow (Fig. 6I through L), the brightest and most consistent areas of the seta that glowed yellow were associated with thickenings observed with DIC (Fig 6I through L, small arrows) or areas near the thickenings (Fig. 6L, arrow); not all thickenings glowed (Fig. 6I, arrowhead).

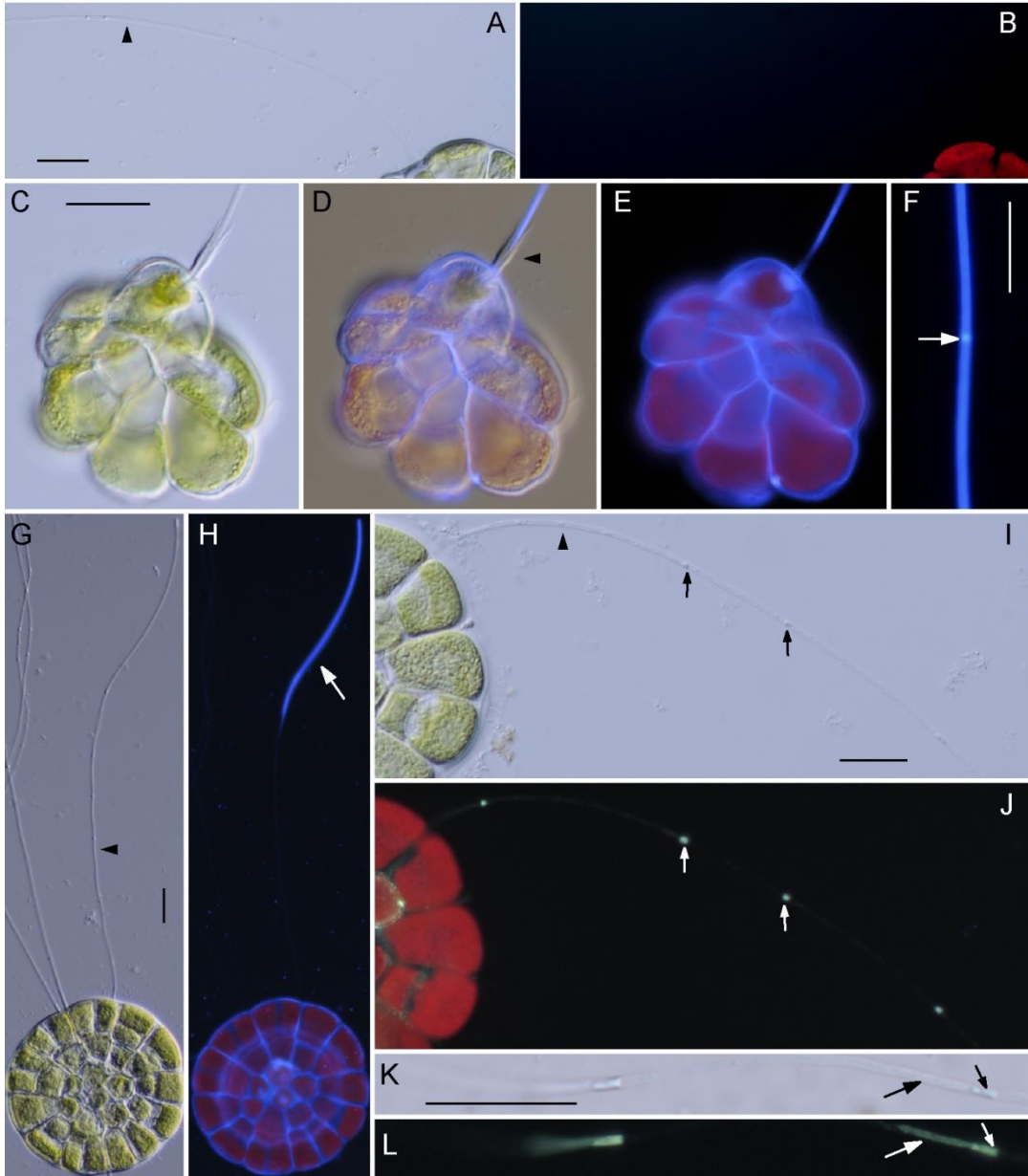


Fig. 6 *Coleochaete orbicularis*

(A, B) Untreated thallus observed in DIC and UV light showing no autofluorescence in the seta (A, arrowhead). (C-E) Calcofluor treated thallus observed with DIC (C), UV light (E), and a combination of DIC and UV light (D) showing the apical portion of collar not staining (D, arrowhead). (F) Calcofluor treated seta showing entire seta staining and bright spot associated with thickening. (G, H) Thallus treated with calcofluor, rinsed, and grown for 1-2 days. New growth not stained (G, arrowhead) and old growth seen with bright blue staining (H, arrow). (I, J) Aniline blue treated thallus (I, J) showing bright labeling of annular thickenings (I and J, small arrows) and an unlabeled thickening (I, arrowhead). Aniline blue stained seta with thickenings labeling bright yellow (K, small arrow) portion next to thickenings also staining (L arrow). All scale bars are 20 μ m.

CHAPTER IV

DISCUSSION

Calcofluor stained the setae consistently among all five species of the Coleochaetales studied, demonstrating that the seta does have a layer of beta glucans surrounding it, which indicates presence of a cell wall. Thus the data presented here resolve the conflicting results of McBride (1974) and Marchant (1977) as to whether the seta proper has a cell wall. Because calcofluor is a general beta glucan stain there is some question as to whether it can be confidently said that the seta wall is composed of cellulose. However, cellulose is one of the most basic components of cell walls of both plants and the streptophyte algae (reviewed by Tsekos, 1999), and given that the Coleochaetales do have the machinery to make cellulose (Okuda and Brown, 1992), the most likely explanation for staining of the seta and the seta collar with a general beta-glucan stain like calcofluor is that these structures are made of cellulose. Callose can be excluded as the beta-glucan being stained by calcofluor along the length of the seta because the majority of the aniline blue staining there was isolated in discrete areas. The carbohydrate-binding module specific for crystalline cellulose (CBM3a) labeled the cell proper but not the seta collar or the seta of *Chaetosphaeridium*. This raises the possibility that the seta collar and the seta proper are made of amorphous cellulose rather than crystalline cellulose. CMB3a appears to be a good test for crystalline cellulose in *Chaetosphaeridium* because it labeled the more mature cells of the thallus more brightly

than the younger cells. Because the seta collar appears to be the extension of the wall of the cell proper, it is surprising that these structures are not made of the same components. On the other hand it is easy to see that the cell walls of the seta proper are remarkably thinner than those of the seta collar or the cell proper. Moreover the setae are able to flex with water movement, hence the cell walls of the setae are probably not as rigid as those of the cell proper. It has been demonstrated that crystalline cellulose is facilitated by the COBRA protein and not accomplished by cellulose synthesizing rosettes in *Arabidopsis* (Sorek et al, 2014). A simple hypothesis is that this protein is absent in the process of seta formation in the taxa studied here.

When comparing the two genera, differences in two major structural characteristics were found. The first is the difference in staining of the seta collar with calcofluor white. In *Chaetosphaeridium* all portions of the collar stained, whereas in *Coleochaete* only the basal portion of the collar stained. Furthermore, within the genus *Coleochaete* the amount of the basal portion of the collar that stained was not consistent among the different species. In *Coleochaete pulvinata* a very similar portion of the basal collar stained consistently, and the basal portion was also narrower than the rest of the collar, but for other species it was more difficult to determine how consistent the amount of basal collar staining is within the species because the collars are not as readily seen due to the form of the thallus. In *Coleochaete irregularis*, for instance, the collars often turned upward towards the microscope objective, making it difficult to find a collar that faced the right direction for observing its entirety in one focal plane. Similarly the collars of *Coleochaete soluta* and *Coleochaete orbicularis* were often hidden behind the thallus

itself and only on rare occasions could one be found facing the right direction for observation in one focal plane.

Perhaps the unstained apical portion of the collar is not composed of beta glucans. A more attractive hypothesis however is that the apical portion of the collar is made at least partially of cellulose and that there is some other component either within the wall or outside it that is blocking the binding of calcofluor to it. Yellow autofluorescence under UV light in the seta collars of sexually active *Coleochaete pulvinata* indicated the presence of phenolic compounds all along the collar. The presence of differentially expressed wall compounds emphasizes that the cell wall of the seta collar is most likely very complex and raises the possibility that other cell wall components could prevent the beta glucan stains, carbohydrate-binding molecule, and callose antibody from attaching. The conflicting staining results of McBride (1974) in his work on *Coleochaete scutata*, the species most similar to *Coleochaete orbicularis* in form and phylogenetic relationship, could possibly be explained if the basal portion of the collar was not visible or if his material was sexual with phenolic compounds expressed in the collars. Furthermore the positive results of the Schiff's reagent could have indicated either cuticular wax or lignin while other tests for lipids (Sudan III and IV) and for lignin (phloroglucinol-HCl and chlorine sulphate) were negative. Perhaps cuticular waxes were present and prevented the stains from binding to the collar. Whatever the reason that the apical portion of the seta collar did not stain with calcofluor in the four species of *Coleochaete*, this character does mark a striking difference between the two genera, demonstrating that these characters may not be identical in the entire Coleochaetales clade.

The second difference between the two genera studied was the presence of thickenings found only in the seta proper of *Coleochaete*. To confirm that these thickenings are indeed annular as Marchant (1977) reported in *Coleochaete scutata*, would require that TEM work (underway) be associated with the current light microscopy techniques. However because of the similarities in thickening size and staining pattern with aniline blue, most likely this is the same characteristic that Marchant described and these thickenings are indeed annular like those of *Coleochaete scutata*. In the setae of *Chaetosphaeridium* there was some staining with aniline blue, but the stained regions were not associated with thickenings. Although most certainly internal structures in the seta of *Chaetosphaeridium* exists, none were identified with DIC microscopy. If seta thickenings in *Chaetosphaeridium* are similar to those observed in setae of *Coleochaete*, it could be difficult to detect because in *Chaetosphaeridium* setae are noticeably narrower than those of *Coleochaete scutata* clade. *Coleochaete irregularis* has narrower setae as well, and the thickenings were much harder to detect with DIC microscopy than those of the *Coleochaete scutata* clade. However compared to that in setae of *Coleochaete* that it seems the aniline blue staining of *Chaetosphaeridium* is so inconsistent in frequency and size are most likely that there are no thickenings in its setae. Perhaps the production or use of callose evolved before the thickenings evolved. It has been shown in pollen tubes that similar plugs have been made with callose, staining brightly with aniline blue (Quin et al, 2012). Marchant (1977) hypothesized that the thickenings could be possible breaking points for the setae and that the annular nature of the thickenings provided an open center that could be plugged by callose to minimize cytoplasmic loss. The staining with aniline blue in *Chaetosphaeridium* could be marking the evolution of callose plugs

as the ancestral character, before the evolution of seta wall thickenings but at the moment this is just a hypothesis. The lack of any labeling at all with the anti-callose antibody 400-2, in the setae of *Chaetosphaeridium* is difficult to explain. More TEM and light microscopy work on the internal structure of the hairs and differences between the two genera may lead to understanding of the evolution of this character and whether the annular nature of thickenings is consistent among the other species of *Coleochaete*.

Speculation about how these characters arose may provide insight into the function of these setae, which often grow several hundred times the length of the cells proper indicating that they likely serve an important function for the organism. Marchant (1977) and McBride (1974) both posited that the seta might function to make mucilage. Another hypothesis Marchant posited was that setae defend against predators, similar to the function of many trichomes on plants. The setae may have a function similar to root hairs, by extending surface area for the cell to collect nutrients. A further hypothesis could be made: perhaps the seta collar and the seta proper are actually separate, independently evolving characters. The seta collar could have evolved to make mucilage as previously proposed and the seta proper simply could have taken advantage of the seta collar as a way to exit the cell to then function as either a defense against predators or for nutrient acquisition. Despite much speculation, the function of this character has not yet been confidently demonstrated. With help from the new found insight that the setae of *Coleochaete* are much more complex than those of its sister taxon it might be possible to unravel this mystery.

Since differences have been found between the two genera, an evaluation of how these differences influence our understanding of the character is in order. One of the most

interesting questions is whether or not *Chaetosphaeridium* has a cellulose rosette more similar to that of plants or to that of *Coleochaete*. Since *Coleochaete scutata* has been shown to have extra units in its rosette compared to that of plants or other derived streptophyte algae (Tsekos, 1999) and has been also shown through x-ray diffraction to have only a low degree of crystallinity of its cellulose (Okuda and Brown, 1992), perhaps these two characters are linked. The slightly different rosettes of *Coleochaete scutata* might be a derived character linked to the *Coleochaete* clade that prevents it from making cellulose with high crystallinity. *Chaetosphaeridium* may have a cell wall much more similar to plants requiring more research on the synthesis and composition of the cell walls of *Chaetosphaeridium* for comparison to plants, despite *Chaetosphaeridium* having less a plant-like growth form than does *Coleochaete*. Perhaps *Coleochaete scutata* has a divergent cellulose synthesizing complex bringing into question further differences between the species within the *Coleochaete* genus itself. The carbohydrate-binding module labeling of crystalline cellulose should be conducted on *Coleochaete* to give insight about the question of the crystallinity of the cell walls. More callose antibody (400-2) work also be conducted on *Coleochaete* to further characterize the thickenings that stained with aniline blue. Tests on other common cell wall components including pectins and arabinogalactan proteins could be illuminating. The setae are not only complicated in their morphology but also in their molecular composition requiring many tests to help unravel their function.

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