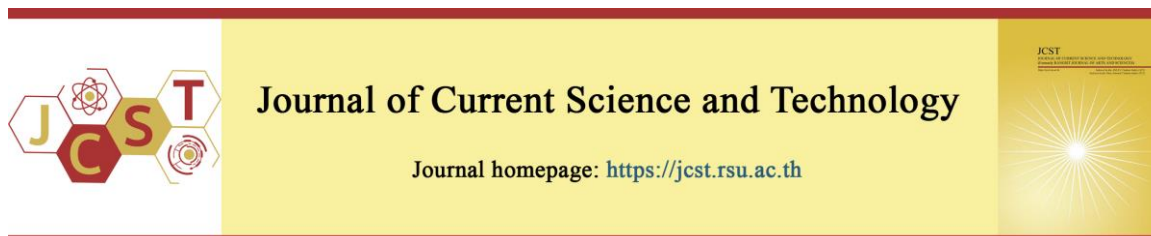


Cite this article: Jampathong, B., Rungkrajang, M., Chokchaichamnankit, P., & Rujeerapaiboon, A. (2026). Morphology and anisotomous branching of rhizoids in non-terminal cells of a *Spirogyra* sp. *Journal of Current Science and Technology*, 16(4), Article 224. <https://doi.org/10.59796/jcst.V16N4.2026.224>



Morphology and Anisotomous Branching of rhizoids in Non-terminal Cells of a *Spirogyra* sp.

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Received 7 May 2026; Revised 26 June 2026; Accepted 3 July 2026; Published 15 September 2026

Abstract

Spirogyra, a widespread filamentous green alga, typically anchors to substrates via rhizoids at the filament termini, an anchoring strategy that enhances their resilience to environmental disturbance. In this descriptive study, we examined rhizoid morphology in a *Spirogyra* unialgal culture using light and epifluorescence microscopy coupled with Calcofluor white and aniline blue staining. We observed rhizoid-like structures associated with non-terminal (intercalary) cells, suggesting that these cells may possess an inducible developmental potential beyond terminal cell identity. The observed rhizoids were consistently rod-shaped and dichotomously branched; however, rosette morphologies were not observed, possibly due to culture conditions or species-level variation. Notably, these single-celled rhizoids also exhibited anisotomous branching (unequal bifurcation). To our knowledge, this is a previously uncharacterized morphological feature in *Spirogyra* and a novel observation for unicellular rhizoids, raising important questions about developmental plasticity in streptophyte algae.

Keywords: aniline blue, anisotomous branching, calcofluor white, developmental plasticity, rhizoid, *spirogyra*

1. Introduction

Spirogyra is a widely distributed filamentous green alga, commonly found in freshwater habitats such as ponds, lakes, and slow-flowing streams. It is often recognized by its common names, "water silk" or "pond silk," due to its characteristic slimy, unbranched filamentous structure. The alga generally occurs as free-floating mats; however, it is sometimes anchored to a surface by rhizoids (e.g., Hodgetts, 1920; Wongsawad & Peerapompisal, 2015). *Spirogyra* rhizoids play a vital role in fixing *Spirogyra* filaments to the substratum. This attachment is crucial for the alga's survival, especially in environments where it

might otherwise be dislodged by currents or other physical forces. Additionally, the capacity for rhizoid production is considered a key parameter for quantifying the growth and overall survival of its filaments, as the presence of healthy rhizoids indicates algal vitality and the capacity to thrive (Gallego et al., 2013). The development of *Spirogyra* rhizoids is known to be influenced by external environmental factors. For instance, substratum properties (Ikegaya et al. 2008b), light irradiation (Nagata, 1973a), and severing (e.g., Inoue et al., 2002; Ikegaya et al., 2008a; Yoshida & Shimmen, 2009) can trigger

rhizoid development, highlighting their adaptive nature in response to environmental cues.

Based on the available research, rhizoid formation in *Spirogyra* is primarily and almost exclusively a phenomenon of the terminal cells of a filament (Nagata, 1973a; Nagata 1973b; Inoue et al., 2002; Yamada et al., 2003; Yoshida et al., 2003; Ikegaya et al., 2008a; Ikegaya et al., 2008b; Yoshida & Shimmen, 2009). It has been suggested that these terminal cells are sensitive to a decrease in intracellular osmolarity caused by K⁺ efflux, which in turn triggers rhizoid formation (Yoshida et al., 2008). However, the presence of rhizoids in these studies was induced by cutting the algal filaments into fragments shorter than one centimeter. After culturing the cut fragments in suitable media, the cut cells detached from the algal fragments and the newly exposed end cells subsequently developed single-celled rhizoids as rod- and rosette-shaped structures (Nagata 1973a; Nagata 1973b; Inoue et al., 2002; Yamada et al., 2003; Yoshida et al., 2003; Ikegaya et al., 2008a; Ikegaya et al., 2008b; Yoshida et al., 2008; Yoshida & Shimmen, 2009). This concept of terminal cell position was supported by the capacity of terminal cells to shift from diffuse growth, which lengthens the algal filament, to localized tip growth associated with rhizoid development. However, this idea was inferred from experiments on cut algal filaments consisting of only a few cells (e.g., Nagata 1973a; Nagata 1973b; Inoue et al., 2002).

Filament fragmentation is a common occurrence in *Spirogyra* that instantly transforms an intercalary cell into a terminal one. This process demonstrates that the structural capacity for rhizoid formation is not restricted to a predetermined cell lineage, but rather exists throughout the broader cell population. However, while recent research (2010–present) has significantly advanced the genomic and ecological understanding of the Zygnematophyceae, investigations into its morphological plasticity have remained largely dormant since foundational studies on terminal cells (e.g., Yoshida & Shimmen, 2009). To our knowledge, no contemporary studies have addressed the natural occurrence of rhizoids on intact filaments or in association with non-terminal cells, leaving a critical gap in the structural record of this lineage.

Previous studies have largely described rhizoid formation in *Spirogyra* as a terminal-cell phenomenon, primarily observed after filament severing. To address this gap, and in keeping with the observational nature of early phycological records, this study aims to

document the occurrence and morphological characteristics of rhizoids associated with non-terminal cells in a cultured *Spirogyra* sp., rather than investigating their underlying physiological triggers. In doing so, we also document a branching architecture that has not, to our knowledge, been described previously for single-celled rhizoids in this genus.

2. Objectives

The objective of this study is to investigate whether rhizoids are present in association with non-terminal cells of a cultured *Spirogyra* sp., and to morphologically characterize any that are observed.

3. Materials and Methods

Study Organism, Collection Site, and Voucher Specimen

Samples of *Spirogyra* sp. were collected from the greenhouse of the Department of Botany, Faculty of Science, Chulalongkorn University [13°44'07.1"N, 100°31'49.6"E]. Morphological identification to the genus *Spirogyra* was based on the presence of unbranched filaments, cylindrical cells, and characteristic helical parietal chloroplasts. Although voucher specimens were deposited at The Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University under accession number 00634, species-level identification remains limited to *Spirogyra* sp. pending future molecular phylogenetic characterization.

Aseptic Isolation and Stock Culture Maintenance

Field samples were rinsed repeatedly with sterile distilled water. Single filaments were aseptically isolated under a dissecting microscope and transferred to solid 5% Bold's Basal Medium (BBM) agar plates to establish unialgal cultures. Stock cultures were maintained in liquid BBM medium under standard white fluorescent lighting and subcultured every two weeks to ensure vigorous vegetative growth.

Pre-experimental Subculture

Prior to the experiment, actively growing filaments from the stock cultures were transferred to fresh liquid media for a 7-day acclimation period. This procedure ensured that all filaments used in the experimental replicates were in a uniform, healthy vegetative phase without pre-existing stress-induced morphological changes.

Experimental Design and Culture Conditions

The study was designed as a strictly descriptive and qualitative observational assessment of non-terminal rhizoid occurrence. Cultures in liquid and solid agar media were maintained in an incubator at 25°C. Light intensity was not quantified; cultures were grown under standard white fluorescent lighting with a 16:8 h light:dark photoperiod. Unmanipulated, intact filaments served as the primary observational group for non-terminal rhizoid development.

Microscopic Monitoring of Rhizoid Development

Observations were conducted weekly over a four-week period. To ensure methodological rigor, specific classification criteria were applied. Rhizoids were strictly defined as polarized, tube-like cellular outgrowths displaying localized cell wall deposition at the extended tip. This distinguished true rhizoids from non-specific cell wall bulges, damaged structures, or external mucilage artifacts. A cell was classified as occupying a non-terminal position only if it was visibly flanked by intact, living adjacent vegetative cells on both sides within a continuous filament at the time of observation.

In total, thirty independent culture plates were examined. At each of five sampling points (Weeks 0–4), three plates each of liquid and solid agar media were examined. Because this study utilized cross-sectional weekly monitoring rather than continuous live-cell tracking, the findings are presented strictly as documentation of the occurrence and final morphology of these structures on intact filaments, rather than demonstrating real-time *de novo* morphogenesis. Following the four-week incubation, filaments displaying non-terminal rhizoids were carefully harvested using sterile micropipettes. Samples were immediately placed in sterile Eppendorf centrifuge tubes for histochemical processing to prevent desiccation or mechanical distortion of the rhizoidal outgrowths.

Fluorescent Staining for Cellulose and Callose

To analyze cell wall composition, the algal samples were first washed with deionized water three times to remove culture media and stained with Calcofluor white and aniline blue to detect the

presence of cellulose and callose, respectively, using the methods described by Herburger & Holzinger (2016). The stained regions were examined under an Olympus BX51 Fluorescence Microscope, using an excitation wavelength of 365 nm for both Calcofluor white and aniline blue. This study was strictly qualitative and descriptive in nature, focusing on the occurrence and morphological characteristics of non-terminal rhizoids. Morphological observations were documented via digital micrography, and representative images capturing the most frequently observed unequal dichotomous branching phenotypes were selected for publication. No quantitative morphometric statistical analysis was performed.

4. Results

Morphological and Histochemical Characterization of Vegetative Filaments

A unialgal isolate of *Spirogyra* sp. was successfully established and maintained in liquid BBM medium. Under light microscopy, the vegetative filaments exhibited the characteristic diagnostic architecture of the genus, featuring an unbranched filamentous habit encased in a distinctive mucilaginous sheath. High-magnification analysis revealed helical chloroplasts containing prominent pyrenoids and a centrally suspended nucleus supported by an intricate network of cytoplasmic strands (Figure 1a). Although these features support the genus-level taxonomic assignment, future molecular phylogenetic characterization is recommended to confirm species identity.

Histochemical analysis via epifluorescence microscopy provided insights into the cell wall's macromolecular organization. Staining with Calcofluor white produced a uniform blue fluorescence along the longitudinal and transverse walls, indicative of a consistent and even distribution of cellulose throughout the algal cell wall (Figure 1b). Conversely, aniline blue staining revealed a heterogeneous distribution of callose. Although baseline fluorescence was detectable throughout the wall, a stronger fluorescence signal was localized at the cross septa, particularly in regions undergoing active wall biogenesis (Figures 1d, f).

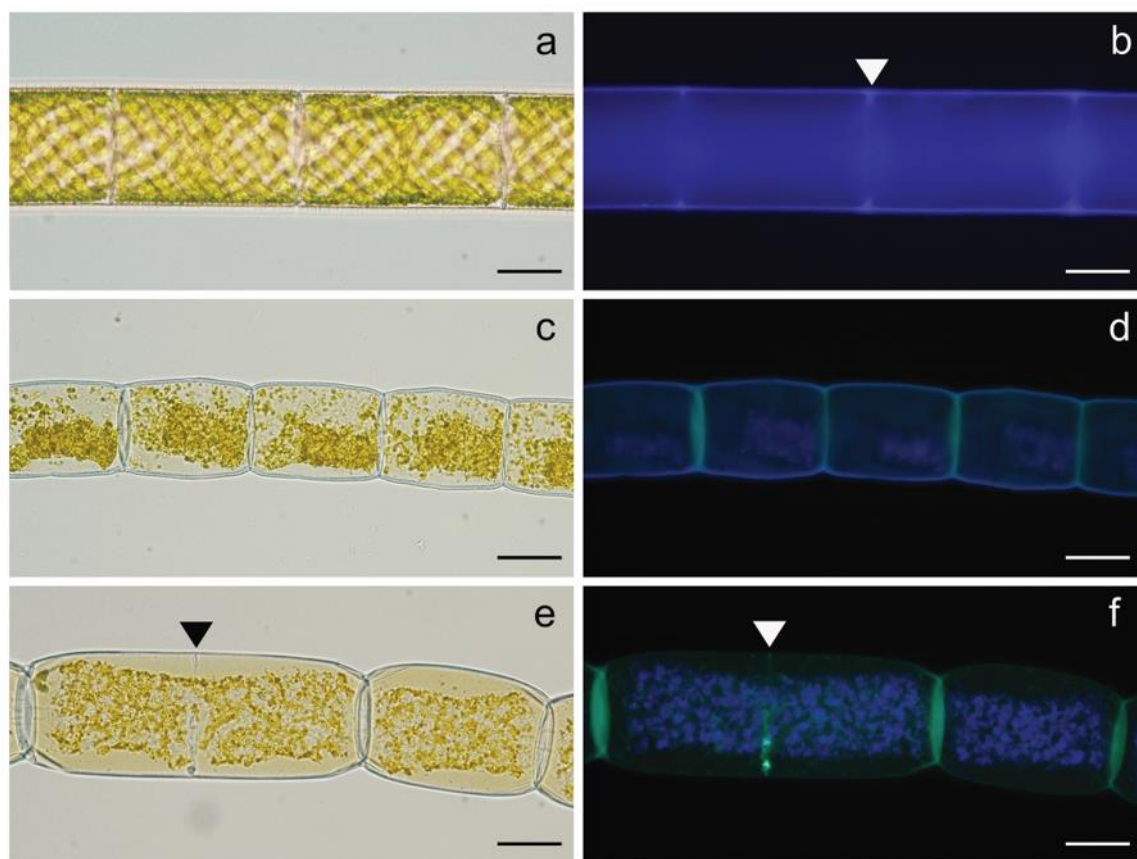


Figure 1 Pre-experimental characterization of *Spirogyra* filaments using light microscopy (a, c, e) and epifluorescence microscopy (b, d, f). A living *Spirogyra* filament illustrating distinctive morphological features, including unbranched filamentous habit, mucilaginous sheath, helical chloroplasts containing prominent pyrenoids, and a centrally suspended nucleus (a). The same filament stained with Calcofluor white, revealing uniform blue fluorescence throughout the cell wall, indicative of an even distribution of cellulose (b). Non-living *Spirogyra* filaments showing mature cells (c, d) and a dividing cell (e, f). Aniline blue staining revealed a heterogeneous distribution of callose, with increased fluorescence signal intensity notably localized at the cross septa and in wall-forming regions (d, f). Arrowheads indicate the algal cross septum. Scale bar = 50 μ m.

Occurrence of Non-terminal Rhizoid-like Structures

Rhizoid formation was observed in all liquid and solid agar media after one week of culture (Figure 2), with no qualitative morphological differences observed between the two media conditions. Under these conditions, typical terminal cells exhibited polarized tip growth, ultimately yielding rod-shaped rhizoids. Notably, the formation of rhizoid-like structures was documented in association with non-terminal (intercalary) cells. To ensure that these structures developed independently of micro-

fragmentation artifacts, they were scored only if the intercalary cell was visibly flanked by intact, living vegetative cells on both sides. Cell wall analysis of these developing rhizoids revealed the presence of both cellulose and callose. However, their distributions differed; while cellulose maintained a relatively uniform structural presence, specific regions of the protruding rhizoids displayed localized, increased fluorescence signal intensity of aniline blue, indicating regional callose deposition (Figures 2d, f).

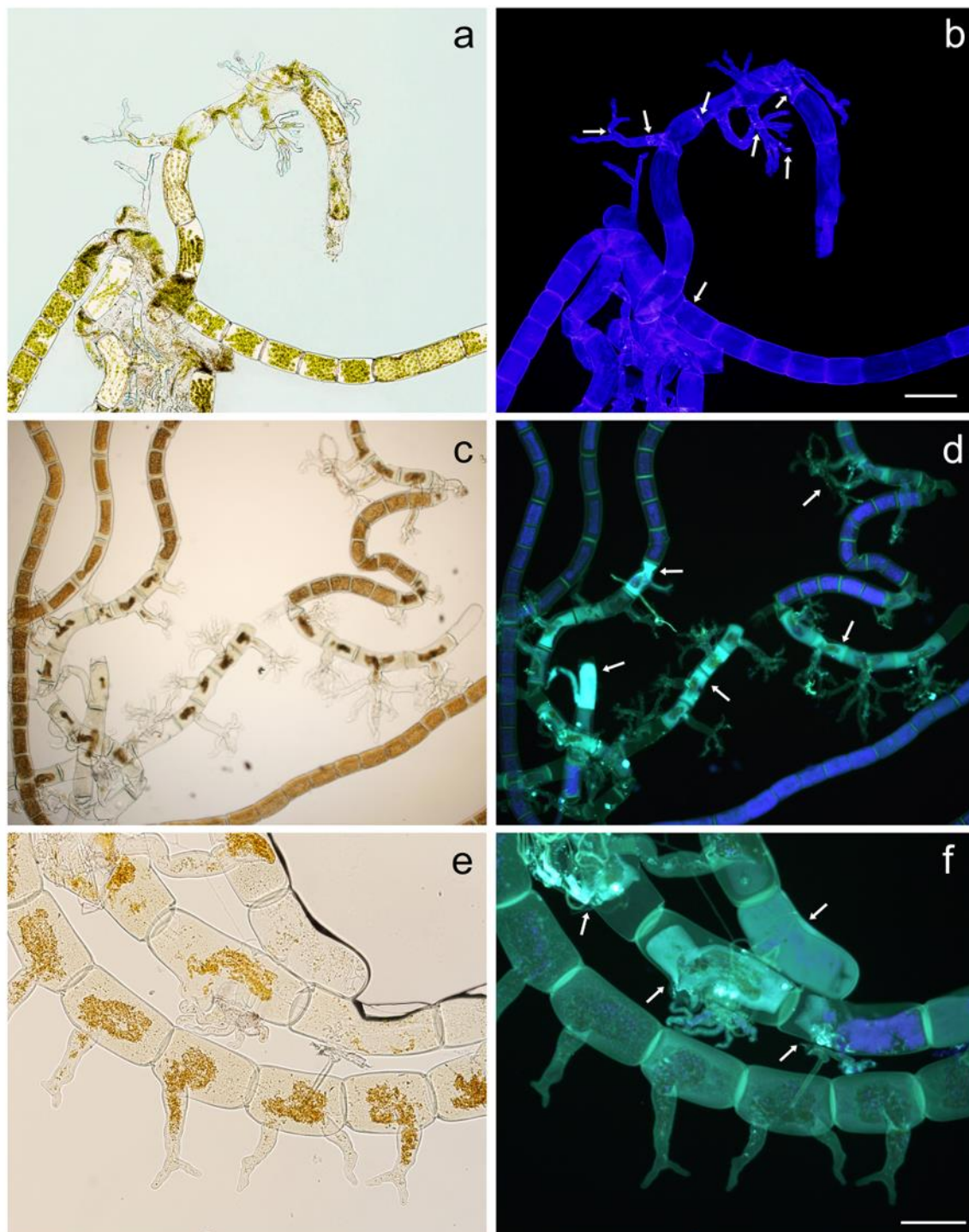


Figure 2 Characterization of rhizoid structures in *Spirogyra* filaments using light microscopy (a, c, e) and epifluorescence microscopy (b, d, f). Filaments stained with Calcofluor white (b) exhibited uniform fluorescence intensity across the cell walls, indicating a consistent distribution of cellulose. Conversely, filaments stained with aniline blue (d, f) revealed localized areas of increased fluorescence signal (arrows), indicating regional deposition of callose. Note that rhizoidal outgrowths emerge from all consecutive vegetative cells along the visible continuous filament, confirming the intercalary position of the internal rhizoid-bearing cells. Scale bar = 50 μ m.

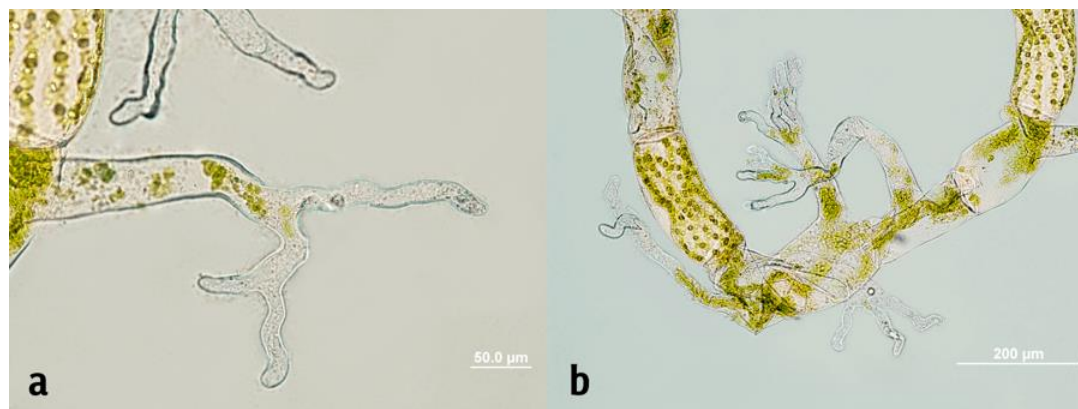


Figure 3. Morphological architectures of *Spirogyra* rhizoids observed via light microscopy. The structural differentiation occurring during rhizoid formation is illustrated by two distinct branching patterns: dichotomous branching (a), characterized by symmetrical bifurcations, and anisotomous branching (b), where the two daughter branches exhibit unequal growth and size.

Characterization of Branching Architectures

All rhizoid structures were evaluated to assess branching architecture (Figure 3). The rod-shaped rhizoids predominantly exhibited two distinct branching morphologies. First, dichotomous branching, characterized by repeated and symmetrical bifurcations of the rhizoid tip, was observed in most rhizoids. Second, and more significantly, the study identified anisotomous branching in some rhizoids, in which the bifurcating daughter branches exhibited unequal growth and size (Figure 3). Careful examination through multiple focal planes confirmed that these branches were continuous extensions of a single-celled rhizoid rather than overlapping adjacent filaments or imaging artifacts. Notably, no transformation into rosette-form rhizoids was observed in any sample, with all structures maintaining their rod-shaped morphology throughout the experimental period.

5. Discussion

The morphological observations of *Spirogyra* rhizoids in this study yielded three primary findings. First, the spatial distribution of callose appears strictly regulated during both cytokinesis and rhizoid development, with increased fluorescence signal localized primarily at the cross septa and the extending rhizoid tips. Second, rhizoid-like structures were observed in association with some intercalary cells, suggesting that the morphological capacity for polarized growth is not restricted to terminal cells. Third, and to our knowledge previously undocumented in this genus, single-celled *Spirogyra* rhizoids exhibited anisotomous branching.

These observations are consistent with the hypothesis that *Spirogyra* maintains a high degree of intracellular spatial control. The observation of increased aniline blue fluorescence at wall-forming regions (Figure 1f) and rhizoid protrusions (Figure 2f) suggests that callose deposition may be involved in localized wall modification and subsequent expansion, a function analogous to its role in other streptophyte algae (Davis et al., 2020; Herburger & Holzinger, 2015). Furthermore, the observation of anisotomous branching in a single-celled, septate filament provides a novel morphological data point. Current models of polarized tip growth in the Zygnematophyceae primarily focus on simple longitudinal expansion. The occurrence of complex, unequal branching patterns in a unicellular context suggests that these models may need to be expanded to account for the establishment of secondary growth axes within a single cell.

As a purely morphological observation, the underlying mechanisms of this anisotomous pattern remain unknown. We cautiously hypothesize that it may represent an early structural form of resource partitioning, establishing a "dominant axis" compared to the symmetrical doubling seen in dichotomous branching (Figure 3a). Future experimental work is required to determine if this morphology relies on non-symmetrical distributions of regulatory molecules, such as localized gradients of calcium or differential actin-microtubule stabilization, as seen in other polarized plant cells (Hepler et al., 2001). Ultimately, documenting these complex axial structures in *Spirogyra* raises the possibility that this genus could serve as an important model for

understanding the evolution of complex growth forms from simpler filamentous ancestors.

Although previous evidence has established that mechanical stress or substrate contact can induce rhizoid formation (Ikegaya et al., 2008b), the lack of rosette-form rhizoids in our samples might simply reflect specific culture conditions. Furthermore, the presence of rhizoid-like structures on intercalary cells leads us to propose a "community-response" hypothesis for future investigation: if terminal cells fail to secure the filament, internal cells might be recruited to ensure survival in high-energy aquatic environments. This remains entirely speculative and requires dedicated experimental validation.

Importantly, the present study has several limitations. Rhizoid occurrence was described qualitatively, and future work should rigorously quantify rhizoid incidence by cell position across independent biological replicates. In addition, because filament handling can impose mechanical stress, time-resolved imaging and fragmentation controls would be valuable to definitively confirm de novo rhizoid initiation from intact intercalary cells without undetected micro-fragmentation. Furthermore, although we used morphological markers (e.g., plastid presence) to distinguish algal rhizoids from fungal or epiphytic structures, our cultures were unialgal rather than axenic; future studies employing axenic cultures would definitively eliminate any potential confounding effects of microbial contaminants. Finally, our histochemical observations lack molecular or cytoskeletal confirmation; future fluorescence observations should be complemented by standardized imaging settings and quantitative image analysis to strengthen inferences regarding callose dynamics. Addressing these limitations will be essential before the developmental significance of non-terminal rhizoid formation can be firmly established.

6. Conclusion

In summary, this descriptive investigation expands the known morphological boundaries of a *Spirogyra* sp. by documenting the occurrence of rhizoid-like structures associated with non-terminal cells and identifying a novel anisotomous branching architecture. Our histochemical observations are consistent with the localized deposition of callose at cross septa and advancing rhizoid tips, suggesting that callose is involved in cell wall biogenesis and polarized expansion. By documenting complex, asymmetrical growth patterns within single-celled

rhizoids, this study provides new observational data on the architectural diversity of the Zygnematophyceae. To build upon these morphological findings, future experimental directions—such as the molecular characterization of intercalary rhizoid induction signals and continuous live-cell imaging—are highly recommended to elucidate the underlying mechanisms of polarized growth in this evolutionary lineage.

7. Abbreviations

Abbreviation	Full term
BBM	Bold's Basal Medium
ISSR	Inter simple sequence repeat

8. Acknowledgements

We gratefully acknowledge financial support from the Science Senior Project fund and the use of Google's Gemini large language model for assistance with language refinement and minor grammatical corrections during manuscript preparation.

9. CRediT Statement

Buntariya Jampathong: Conceptualization, methodology, investigation, visualization, and writing – original draft

Mahsarahka Rungkrajang: Investigation

Ploenpit Chokchaichamnankit: Methodology, visualization, supervision, validation, and writing – review & editing

Anchittha Rujeerapaiboon: Conceptualization, methodology, investigation, supervision, validation, and writing – review & editing

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