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BRIEF COMMUNICATION

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Endophytic bacteria discovered in oil body organelles of the liverworts *Marchantia polymorpha* and *Radula complanata*

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Abstract

Premise: Interactions between endophytic microbes and bryophytes have been understudied. The liverwort oil body has also remained poorly understood since its discovery, and modern studies have failed to ascertain its function and composition. Many liverwort species possess oil bodies with conspicuous granules of unknown structure. We surveyed these granular liverwort oil bodies for the presence of bacteria to improve upon the understanding of liverworts, their oil bodies, and bacterial endophytes in nonvascular land plants.

Methods: Wild-collected specimens from living samples of *Marchantia polymorpha* and *Radula complanata* were stained with SYTO-13 and RADA to determine the presence or absence of bacteria within their oil bodies. Samples stained with calcofluor white, SYTO-13, and RADA were observed with confocal fluorescent microscopy for presence of nucleic acids and bacterial peptidoglycan cell walls within oil bodies.

Results: We discovered large masses of bacteria within the oil bodies of *M. polymorpha* and *R. complanata* based on the presence of stained nucleic acids and peptidoglycans localized to the oil body "granules". Such bacteria were present in all oil bodies of the two species.

Conclusions: These newly discovered intraorganellar bacteria correspond to the previously described "granules" of oil bodies. The existence of granular oil bodies in many liverwort species implies that this endophytic association may not be isolated to species investigated here. Assessments of additional liverwort species for presence and identity of oil body bacteria are needed to understand this intriguing association in one of the oldest land plant lineages.

KEYWORDS

endophytic bacteria, endosymbiosis, intracellular bacteria, liverwort oil body, liverworts, Marchantiaceae, *Marchantia polymorpha*, Marchantiophyta, Radulaceae, *Radula complanate*

The cells of most liverwort species possess organelles termed oil bodies that are unique within terrestrial plants, having evolved only in the most recent common ancestor of all extant liverworts (Romani et al., 2022). Oil bodies were initially discovered almost 200 years ago, but their function has remained elusive (Hübener, 1834; He et al., 2013).

Oil body organelles are known to store many biologically unique compounds, primarily terpenoids and aromatics, but why liverworts commit resources to the synthesis of these compounds is not well understood (Asakawa et al., 1980). Some authors have suggested that oil bodies may be vestigial organelles that lack a function in extant lineages. However, secondary loss of oil bodies in liverworts is rare. Only ~10% of the approximately 7300 species of extant liverworts lack the organelle, with ~25% of the species that lack oil bodies occurring in just one genus (*Riccia*) (Müller, 1939; Crandall-Stotler and Stotler, 2000; Söderström et al., 2016; Romani et al., 2022). The rarity of secondary losses suggests there is a conserved and ancient function of the oil body organelle in liverworts, despite our

limited understanding of its function. Modern evidence has only resolved niche functions within specific species, such as desiccation tolerance in *Southbya nigrella* (De Not.) Henriq., UV protection in *Jungermannia exsertifolia* Steph., and herbivory defense in *Marchantia polymorpha* L. (Pressel et al., 2009; Fabón et al., 2012; Romani et al., 2020). Other studies have focused on the mechanisms of oil body function, such as the discovery that the oil body stroma is the site of synthesis for the various, often lineage-specific terpenoids and aromatic compounds these organelles sequester (Suire et al., 2000). Species-specific functions fail to answer why oil bodies are conserved in the vast majority of liverwort lineages, and elaboration on physiological mechanisms fails to explain why oil bodies sequester these compounds in the first place. We are left in the position of understanding some of the finer details of this organelle without the context of its wider function in this lineage of plants.

The apical growth of liverworts depends on a single meristematic cell (Ligrone et al., 2012). Oil bodies form early in cell development and begin to develop in the daughter cells of the apical meristematic cell during initial shoot growth (and later during leaf development) (Kanazawa et al., 2020). While oil bodies are visually absent, the specific genes necessary for oil body development are expressed in these young cells, indicating their initial formation (Kanazawa et al., 2020; Romani et al., 2022).

When young, oil bodies lack sequestered compounds and consist only of a shrunken membrane surrounding a stroma (Pihakaski, 1968; Kanazawa et al., 2020). The stroma primarily consists of carbohydrates and proteins that form a thin matrix between the oil and the outer membrane of mature oil bodies, and with electron microscopy, the stroma can be seen as a region of minute granules (Pihakaski, 1968; Kronestedt, 1983; Suire, 2000; Pressel et al., 2009). In many species, larger granules can be seen in the stroma with light microscopy, but these are unrelated to the minute granules seen with electron microscopy and have been attributed to miniscule oil globules or papillae in previous literature (Schuster, 1966; Pihakaski, 1968).

As oil bodies develop, they sequester terpenoids and aromatics within droplets called oil globules inside the stroma. Depending on the species, these compounds accumulate as either a singular large oil globule or several small oil globules, causing each oil body to swell inside the plant cell (Pihakaski, 1972). Whether or not the oil globules inside each oil body each possess a membrane is not known, but globules do prevent the diverse terpenoids (lipids) and aromatic compounds that the oil bodies synthesize from coming into contact with the stroma and the contents of other oil globules within an oil body.

Endophytes are microorganisms that colonize plant tissues with no noticeable deleterious effects on the host. They are usually categorized as being commensal, mutualistic, or exhibiting circumstantial ecological interactions (Stone et al., 2000; White et al., 2019; Verma et al., 2021; Chang et al., 2023). Many endophytic bacteria are intercellular, meaning they inhabit the periplasmic spaces between plant cells (Sattelmacher, 2001; Thomas and Reddy, 2013). The route of colonization for intercellular endophytes is primarily from the environment through plant roots, but also through seeds colonized while attached to a mother plant (Kandel et al., 2017). Colonization of periplasmic spaces has until recently been seen as the only type of endophytic colonization of aboveground structures in bacteria–vascular-plant associations outside of highly specialized symbioses, with intracellular bacteria only known from xylem cells, which are dead at maturity (Hurek et al., 1994). However, more recent studies have shown the existence of intracellular endophytic bacteria in living, photosynthetic plant cells (Pirttilä et al., 2000; Thomas and Sekhar, 2014; Chang et al., 2021; Kurumisawa et al., 2021). These findings broaden the scope of endophyte–plant associations and raise the possibility of intracellular bacteria also occurring within liverworts, which primarily consist of photosynthetic tissue.

We present here new and detailed anatomical investigations of the oil bodies in the thalli of *Marchantia polymorpha* (Marchantiaceae) and leaves of *Radula complanata* (Radulaceae), representing two of the three classes of liverworts (Marchantiopsida and Jungermanniopsida, respectively; Bechteler et al., 2023). We used light microscopy, confocal microscopy, and chemical/fluorescent staining of live material to reach the novel conclusion that liverworts possess masses of intracellular, endophytic bacteria embedded within the oil body stroma.

MATERIALS AND METHODS

Collection, storage, and preparation of specimens

Specimens of *Marchantia polymorpha* subsp. *polymorpha* and *Radula complanata* were collected in New Jersey, USA (see Appendix 1 for voucher information). Specimens were wild-collected from nearby populations, placed in plastic bags, and brought to the lab. Specimens were either prepared immediately or kept alive in bright, humid conditions at 21°C in sterilized petri dishes with 12 h of fluorescent grow lights for no more than 24 h before preparation. Transverse cross sections of the thalli of *M. polymorpha* were cut and leaves of *R. complanata* dissected by hand with single-edged sterile razor blades in a laminar flow hood in preparation for staining and microscopy. Axenic culture of both species was attempted to allow for a robust control group for comparison but were discontinued after it became clear that the oil bodies of these plants could not be properly sterilized without destroying the organelle.

Staining

For light microscopy, bacterial cells were stained within live tissues using 0.1% w/v gentian violet solution (Gram's stain, Thermo Fisher Scientific, Waltham, MA) in distilled water for 5 s, then washed in a decolorizer of 25% v/v acetone in

distilled water for 60 s. Gentian-violet-stained material was washed in acetone rather than ethanol to prevent oil bodies from rupturing before observations. For fluorescent microscopy, plant material was stained with a 0.1% w/v calcoflour white aqueous solution (Sigma-Aldrich, St. Louis, MO, USA) for 1 min before being washed with distilled water, then 5 mM SYTO-13 (a nucleic acid stain; AAT Bioquest, Pleasanton, CA, USA) in 20% v/v DMSO (Bio-Techne, Minneapolis, MN, USA) for 5 s. Separate plant material was stained with a 500 μM RADA solution in 10% v/v aqueous DMSO for 15 min before being washed with distilled water. RADA was chosen for bacterial labelling because it is a fluorescent D-amino acid stain that is selectively incorporated into bacterial cell walls via peptidoglycan biosynthetic pathways and so is able to confirm both the presence of peptidoglycans, which are unique to bacterial cell walls, and the viability of the bacteria during staining, because the RADA stain cannot be incorporated without ongoing cell wall maintenance (Hsu et al., 2017). Staining of plant material with both SYTO-13 and RADA was attempted to better visualize oil body bacteria, but all attempts caused the RADA stain to bind to the SYTO-13, which interfered with imaging and prevented bacterial cell walls from staining appropriately with RADA, so these stains were used separately.

Microscopy

Unstained and gentian violet-stained live plant material was observed using a trinocular Zeiss Axioskop 20 microscope with a Plan-Neofluar 100× objective lens and photographed with an Infinity3 digital camera using Infinity Analyze and Capture imaging software (Teledyne Lumenera, Ottawa, ON, Canada). Tissues stained with calcofluor white, SYTO-13, and RADA were observed using a Zeiss LSM710 Confocal Microscope with 405 nm, 488 nm, and 561 nm lasers, respectively, and photographed using ZEN microscopy software (Zeiss, Oberkochen, Germany).

RESULTS

The bacterial cell walls inside the oil bodies stained purple with gentian violet and remained stained after a decolorizing wash in acetone, which indicated that these are likely Gram-positive bacteria (Figure 1B, J). The bacteria fluoresced green when viewed with confocal microscopy using the 405- and 488-nm lasers after being stained with SYTO-13, indicating the presence of nucleic acids (Figure 1E, F, M, N). The bacteria also fluoresced red using the 561-nm laser when stained with RADA. indicating the presence of peptidoglycans, which are present in the cell walls of the bacteria only (Figure 1G, H, O, P). Only the plant cell walls fluoresced in control images using the 405-, 488-, and 561-nm lasers when stained with calcofluor white, showing that the observed oil body fluorescence was not caused by autofluorescence (Figure 1D, L). Both the nucleic acid fluorescence and peptidoglycan fluorescence were localized within the stroma of the oil body, i.e., in between the internal oil globule and the external organelle membrane (Figure 1F, N, H, P). Additionally, both were restricted to the bacterial cells, which corresponded directly with the previously mentioned "granules" (Figure 1F, N, H, P).

The bacteria in oil bodies of *M. polymorpha* and *R. complanata* were approximately 1–3 μm in diameter and coccoid to ovoid (Figure 1A, C, E, F, K, M, O, P). When observed with the microscope, the bacteria usually remained still, but movement occurred when oil globules ruptured inside the oil body (Appendices S1, S2). The bacteria then moved in directional cyclic arcs within the oil body, and once the contents of the oil body voided into the plant cell (i.e., the oil body ruptured) or was exposed to ethanol, their movement ceased (Appendices S1, S3). Both situations were consistent with the death of motile bacteria. Intracellular particles of similar size and shape to the observed oil body bacteria were also present in low numbers in young daughter cells near the meristem that had not yet formed oil bodies; however, the identity of these particles as bacteria has not yet been investigated (Appendix S4).

DISCUSSION

The large granules in oil bodies that have been interpreted as small oil globules or papillae by previous bryologists are here interpreted as bacteria in the study species *R. complanata* and *M. polymorpha* based on our visualizations of shape, size, and presence of nucleic acids/peptidoglycans localized to the bacterial cells within the oil bodies. Localized fluorescence of the RADA stain also provides support for active growth because the D-amino acid linked to the fluorophore must be incorporated into bacterial peptidoglycan maintenance pathways to avoid being removed when washed (Figure 1G, H, O, P; Kuru et al., 2015).

We conclude that the size, shape, growth, motility, and mortality of the bacteria within the stroma of liverwort oil bodies in tandem with their reactions to gram, nucleic acid, and peptidoglycan stains is strong evidence that they are intracellular bacteria.

Previous authors mentioned seeing movement within oil bodies, most notably Schuster (1966), who described this phenomenon as Brownian motion of granules within the oil body. The fact that the movement ceased when exposed to harmful compounds (the oil body contents once voided or ethanol) makes Brownian motion an unlikely explanation, especially considering that the pattern of movement is consistent with independent motility in bacteria (Deng et al., 2020). The observed movement may still be caused by other abiotic factors because the rupturing of the oil body invariably leads to mixing of lipids with the cytoplasm, which may be responsible for the movement observed. However, movement caused by abiotic factors alone is not necessarily a valid argument for the interpretation of these bacteria as nonliving because many bacteria are nonmotile.

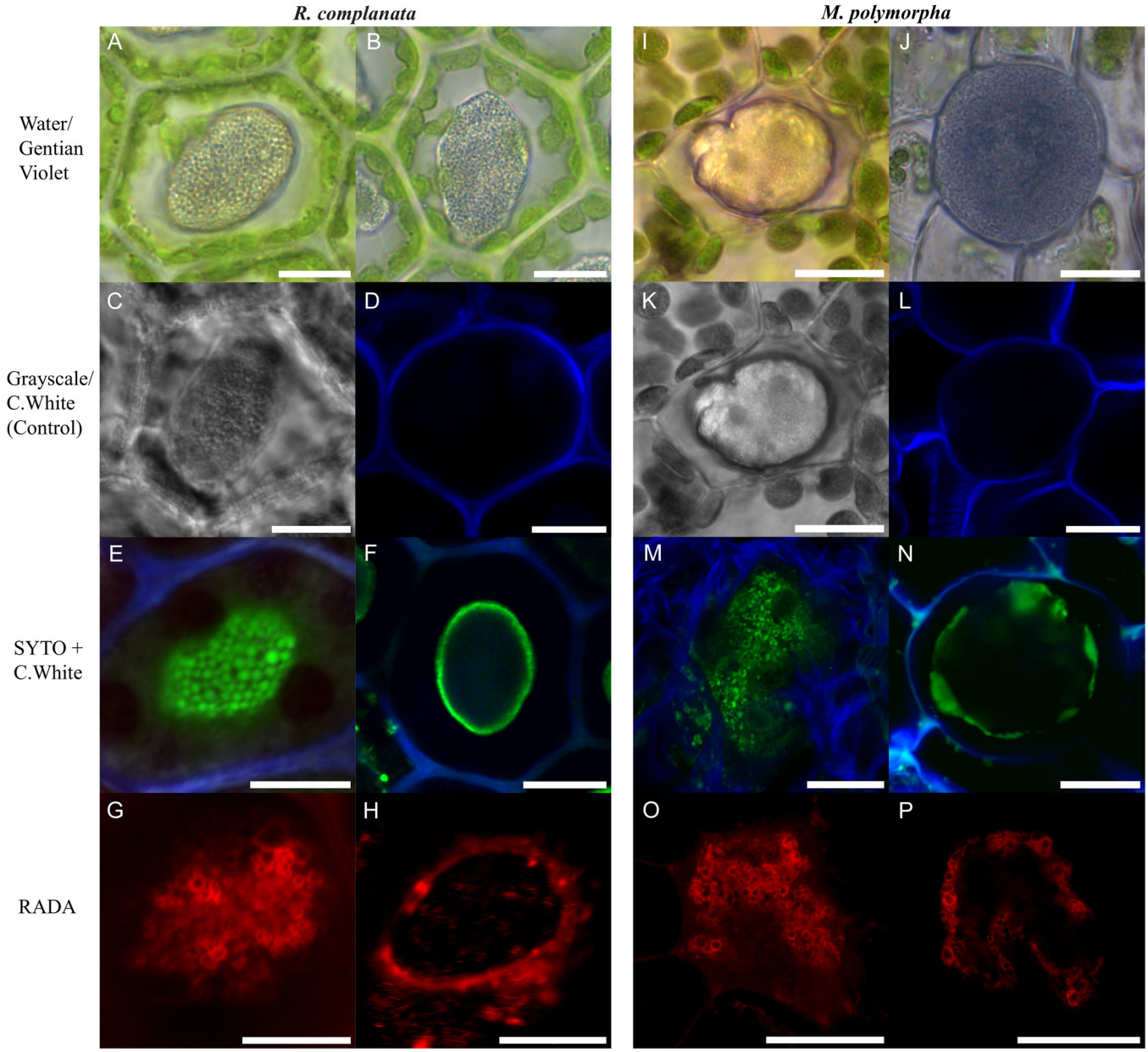

FIGURE 1 Light (A–C, I–K) and confocal (D–H, L–P) micrographs of oil bodies in living cells of *Radula complanata* (*Young* 568) (A–H, scale bars: 10 μm) and *Marchantia polymorpha* (*Young* 566) (I–P, scale bars: 20 μm). (A, I) Unstained tissue exhibiting typical color. (B, J) Blue to purple color in bacterial cell walls from Gentian violet staining. (C, K) Grayscale, *z*-stacks of oil bodies emphasizing bacterial morphology for comparison with confocal images D and L. (C) Calcoflour white fluorescing blue in plant cell walls after calcoflour white staining with no autofluorescence of oil body or bacteria. (E, F, M, N) Calcoflour white and SYTO-13 staining with blue fluorescence in plant cell walls and green fluorescence from nucleic acids (presumably bacterial DNA) from the oil body surface and in cross section. (G, H, O, P) RADA staining with red fluorescence of peptidoglycan in bacterial cell walls shown from the oil body surface and in cross section.

Past studies of oil body structure have relied on preserved, fixed, and stained liverwort tissues using alcohols, fixatives such as osmium tetroxide, and stains such as glutaraldehyde under transmission electron microscopy (TEM) to describe the constituents of this organelle (Duckett, 1986). This approach likely concealed evidence of endophytic bacteria within the oil body, because bacteria would have appeared similarly electron-dense (and therefore dark) under TEM as proteinaceous deposits and lipid droplets, the primary constituents of oil bodies, especially in the context of the large-scale cross-sections used in these studies for proper observation of the organelle (Pihakaski, 1968; Galatis et al., 1978). Modern studies of liverwort microbiomes have focused on fungi rather than bacteria, with only a few bacterial studies having distinguished between epiphytic and endophytic communities or having attempted to segregate tissues that had endophytic bacteria (Alcaraz et al., 2018; Marks et al., 2018; Chen and Nelson, 2022). While some of the bacteria found belonged to lineages with endophytic life histories such as the Rhizobiales, these studies did not address what tissues these bacteria colonized.

The endophytic bacteria in oil bodies of the two study species were long assumed to be abiotic material, with their presence interpreted as small oil globules, papillae, or proteinaceous bodies. Our observations of nucleic acid and peptidoglycan cell wall fluorescence localized to cells of bacteria inside oil bodies contradicts such conclusions in the case of our two study species, because neither the oil globules, organelle membrane, or stroma matrix of the oil bodies reacted to the SYTO-13 or RADA stains (Figure 1E–H, M–P). Other possible interpretations of these "granules", such as biomineralization of minute crystals, oil bodies as modified plastids with plastid DNA, or the presence of mRNA within oil bodies, present other challenges. If these bacteria instead were biomineralized crystals, they would not react to either of the fluorescent stains, and their motility via Brownian motion would be constant.

As for the possibility of a plastid origin of oil bodies, the oil body is bounded by a single unit bilayer membrane unlike the double membrane of the chloroplast, and the membrane of this organelle has been shown to be under the maintenance of the same pathway that maintains the cell plasma membrane, which makes the possibility of a plastid origin for this organelle unlikely (Pihakaski, 1968; Duckett, 1986; Kanazawa et al., 2020). However, a plastid origin for oil bodies would not preclude the possibility of bacterial colonization because bacteria are known to colonize plant chloroplasts (Kurumisawa et al., 2021).

If the nucleic acids localized by the SYTO-13 staining were simply plant mRNA, the stain would have been weaker, and the peptidoglycan staining via RADA would have shown nothing, because peptidoglycans occur only in bacteria and plastids (the latter of which have a bacterial origin through ancestral endosymbiosis). The evidence presented here strongly suggests that the oil bodies of *R. complanata* and *M. polymorpha* possess endophytic bacteria, given that there is precedence for the use of SYTO dyes for the detection of localized endophytic bacteria within plant tissues, including intracellular bacteria (Thomas and Reddy, 2013; Thomas and Sekhar, 2014). The presence of nucleic acids alone could be explained by other hypotheses such as the previously mentioned plant mRNA or plastid DNA, but the presence of peptidoglycans localized to small coccoid cells within the oil body (Figure 1G, O) makes a plastid origin for the oil body unlikely. Plastids in *Marchantia polymorpha* are known to possess peptidoglycans, but the oil body membrane did not fluoresce during RADA staining (Figure 1G, H, O, P), making bacteria the most likely explanation for the "granules" within the oil bodies of these species (MacLeod et al., 2024).

It is unlikely that the bacteria within oil bodies are parasites or pathogens because the liverworts used in this study (*M. polymorpha* and *R. complanata*) harbored bacteria within every individual plant and within each oil body of each plant to no detrimental effect. Therefore, these oil body bacteria are most likely endophytes, which are common in tissues of land plants (Stone et al., 2000). The oil body bacteria are also in a specific "compartment" similar to other endophytes with close associations with their hosts, such as the relationships between mycorrhizal fungi and land plants, *Rhizobium* and Fabaceae, *Nostoc* and hornworts, and *Anabaena* and *Azolla* (Ho and Trappe, 1973; Stewart and Rodgers, 1977; Ronson et al., 1981; Abd El-Aal, 2022). One bacterial endophyte, *Azorhizobium caulinodans* Dreyfus, has been shown to colonize the stroma of chloroplasts in *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae; Kurumisawa et al., 2021) in a strikingly similar way to the oil body bacteria we present here. It is not yet known whether the bacteria inhabiting oil bodies have a mutualistic association with their liverwort hosts, but the similarity of this system to other known mutualisms makes mutualism an intriguing possibility.

Much of the studies on the function of liverwort oil bodies have explored possible functions for this organelle that mirror common functions of plant endophytes, such as assisting metabolism or providing defense against abiotic stressors, pathogens, or herbivores (Suire et al., 2000; Pressel et al., 2009; Bacon and White, 2016; Romani et al., 2020). While some of these hypotheses have fallen out of favor, oil bodies provide defense against herbivores in *M. polymorpha* (Romani et al., 2020). Liverworts also synthesize terpenoids in the stroma of oil bodies, the same site where bacteria are found (Suire et al., 2000; Romani et al., 2020).

The cytotoxic and antimicrobial effects of many of the compounds within oil bodies seem unfavorable for bacterial colonization. However, it has been shown in analogous plant secretory structures such as glandular trichomes that bacteria are capable of colonizing environments with similar volatile organic compounds present. Bacterial isolates from the leaves of oregano [*Origanum vulgare* subsp. *hirtum* (Link) J.H. Ietswaart] have been shown to tolerate the aromatic compounds in the plant's glandular trichomes and can grow in trichome-derived extracts (Karamanoli et al., 2012). In cultivated tomatoes (*Solanum lycopersicum* L.) and another wild tomato species (*Solanum habrochaites* S. Knapp & D.M. Spooner), bacterial colonies are present in the glandular trichomes, which contain some of the same types of compounds as those in liverwort oil bodies, including sesquiterpenes (Kusstatscher et al., 2020). While many oil body compounds are biologically unique, they nonetheless belong to the same classes of secondary metabolites that are produced by other plants, which have been shown to support colonization by bacteria.

The discovery of endophytic bacteria within liverwort oil bodies cannot explain oil body function without further studies using genomics and metabolomics, and a much broader sampling of liverwort species, but it is a significant step toward a better understanding of the function of the liverwort oil body.

CONCLUSIONS

We demonstrated here that the liverworts *Radula complanata* and *Marchantia polymorpha* possess colonies of intracellular bacteria within the stroma of their oil body organelles, with evidence from bright-field and confocal fluorescence microscopy. The observations and arguments

presented here explain how previous assumptions and explanations as to the identity of oil body granules are no longer supported in these two species. Oil body organelles are present in most liverwort species, and their function in these plants has remained elusive, but this novel discovery of bacteria within the oil bodies of the study species *Radula complanata* and *Marchantia polymorpha* presents many new and exciting future research endeavors into symbiosis, physiology, land plant evolution, phytochemistry, horizontal gene transfer, plant stress, and liverwort macroevolution.

AUTHOR CONTRIBUTIONS

B.Y., J.W., L.S.: substantial contributions to conception and design; B.Y.: acquisition of data; B.Y., J.W., L.S., B.T.: analysis and interpretation of data; B.Y., L.S., B.T., J.W.: involved in drafting the manuscript or revising it critically for important intellectual content. All authors approve the final version to be published. Each author should have participated sufficiently in the work to take public responsibility for appropriate portions of the content; B.Y., B.T., J.W., L.S. agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Bacteria moving in cyclic arcs characteristic of bacterial motility in an oil body of *Marchantia polymorpha* whose internal oil globule was ruptured by applying pressure on the sample under a microscope slide, causing the bacteria, stroma, and oil to mix. Light microscopy, 100×, cropped.

Appendix S2. A 10-min time-lapse video of cells of *Radula complanata* with stationary bacteria beginning to move after an addition of 30% v/v ethanol penetrates the oil bodies and ruptures their internal oil globules, causing the bacteria, stroma, and oil to mix. Light microscopy, 100×.

Appendix S3. A 10-min time-lapse video of a cell of *Radula complanata* with the movement of bacteria ceasing after dying from exposure to 50% v/v ethanol and oil globule compounds as the oil body swells with ethanol and collapses, leaving only oil droplets. Light microscopy, 100×, cropped.

Appendix S4. Video of bacteria in early daughter cells of the apical meristematic cell in *Marchantia polymorpha* before the formation of visible oil bodies. Light microscopy, 100×.

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APPENDIX 1

List of Marchantiophyta material studied. Index Herbariorum (Thiers, 2014) abbreviations are in parentheses. All vouchers were newly obtained in this study.

Taxon, Author, Family, Locality, Coordinates, Date, Voucher (Herbarium), Accession Number.

Marchantia polymorpha **subsp.** ***polymorpha*** L., Marchantiaceae, USA, NJ, Middlesex Co., New Brunswick, Raritan River, 40.48701, –74.42195, 31 July 2023, *Young* 566 (CHRB), CHRB-B-0007268.

Radula complanata (L.) Dumort., Radulaceae, USA, NJ, Middlesex Co., New Brunswick, Rutgers Ecological Preserve, 40.51670, –74.43726, 10 September 2023, *Young* 568 (CHRB), CHRB-B-0007271.