Editor's Note:

Evansia Editor Transition

We would like to thank Scott LaGreca for eleven years of service as editor of Evansia! Scott always brought a positive energy to his work, and has been a very actively engaged member of ABLS. According to Scott, "it was a wonderful experience getting to know colleagues, amateurs and students alike, and to build many great relationships! I don't know how many articles came across my desk in total, but I can say that having "entire-issue articles" (articles that took up the entire issue) was always a joy to me! For example, Dale Vitt published a December issue which was entirely a Sphagnum key with descriptions and photos. Another such issue was John Atwood doing an index for a decade's worth of issues—that was the entire issue, just an index. Plus, the cover for that issue was a montage of all the covers from the past 10 years. I loved that!" Scott also discussed the joy of seeing what the active research was in the fields of lichenology and bryology, while also providing a space for fun articles such as field trip reports from the annual meetings. Those of us who have worked closely with Scott have always appreciated his kindness, patience, and thorough attention to the Evansia articles. Below are some selected quotes:

"He had a great vision for making Evansia more attractive to all members of the community. And he was really fun to work with." -Daphne Stone, ABLS President

"I enjoyed our time working together and wish him the best on whatever future endeavors he has. He was always very kind to me and the rest of the team." -Chris McConnell, KGL Association Management

"He was open to using bryophyte portal data and encouraged authors to make their checklist available through the portal with the cited voucher specimens, which makes state and regional species diversity publications easier to develop. He always made sure that the issues were out to print on time, showing our membership that we value their subscriptions." -Scott Schuette, former ABLS President and Treasurer

We now welcome Klara Scharnagl as the new editor of Evansia. Klara is excited to be in this role, and looks forward to working with many of you! Please send submissions to evansiaeditor[at]gmail[dot]com.

Moss sporophytes with a higher proportion of leptoids have higher water transport rates

Amy N. Okafor & Jessica M. Budke*

Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN 37996, U.S.A.
*Corresponding author's email: jbudke@utk.edu

Abstract: **The sporophytes of moss plants are dependent on the gametophytes for both photosynthesis and water, which makes conducting cells (hydroids and leptoids) an important part of the sporophyte anatomy. A previous study found that *Physcomitrium pyriforme,* which has shorter sporophytes, had higher rates of water transport than *Funaria hygrometrica,* which has taller sporophytes. The aim of this study is to test for differences in the conducting cell anatomy between these two moss species, which could be responsible for differences in water transport rates. We used histology methods to fix, embed, and section sporophyte seta and then quantified the numbers and sizes of the conducting cells. The results revealed that leptoids comprise a higher proportion of the conducting cell area in *P. pyriforme,* while hydroids comprise more of the conducting cell area in *F. hygrometrica.* These results point toward the leptoids playing a role in water transport in the moss sporophyte.**

***Keywords*:** anatomy, bryophytes, conducting cells, hydroids, seta

INTRODUCTION

Mosses are small, photosynthetic green plants that include over 13,000 morphologically diverse species (Patel et al. 2021). Mosses have sporophytes that are physically attached to the leafy gametophyte throughout their life and are dependent on the gametophyte for both photosynthates and water. Despite being classified as non-vascular, moss plants contain cells that are specialized for internal conduction and transport (i.e., hydroids and leptoids; Ligrone et al. 2000). Hydroids are water conducting cells that are dead at maturity and elongated, whereas leptoids are food conducting cells that are elongated and alive at maturity (Hébant 1977). Together these cells transport water and nutrients from the gametophyte to the sporophyte, which facilitates spore development (Scheirer 1980). This makes conducting cells an important part of the functional anatomy of the sporophyte and spore production.

Funariaceae is a family of mosses that have diverse sporophyte morphologies and can be easily grown in the laboratory. Across the approximately 255 species in the family, sporophytes have diverse capsule shapes and sizes as well as a variety of seta lengths, whereas the gametophyte morphology is quite uniform (Medina et al. 2018). The geographical distributions of Funariaceae are also diverse with many species occurring on more than one continent (Fife 1985). Sporophytes across the family range from only a few millimeters tall in *Physcomitrium patens* (Hedw.) Mitt. to over several centimeters tall in *Funaria hygrometrica* Hedw. This diversity makes species in the Funariaceae useful for comparative studies (Budke & Goffinet 2016).

In a previous study using two Funariaceae species (Whitaker & Budke 2021), we found that *Physcomitrium pyriforme* (Hedw.) Brid. sporophytes had higher rates of water transport (2.78 mm per min) than the taller sporophytes of *Funaria hygrometrica* (2.30 mm per min). This contradicted our hypothesis that taller sporophytes, extending beyond the still air of the boundary layer, would have higher rates of water transport compared to shorter sporophytes. Based on these findings, in this study we are testing for differences in the conducting cell anatomy between *P. pyriforme* and *F. hygrometrica*, which could be responsible for differences in the water transport rates. We predict that *P. pyriforme* sporophytes will have a larger number of and larger transverse area devoted to conducting cells compared to *F. hygrometrica.* Examining these cells might give us insights into the structure-function relationship for water transport rates in moss sporophytes.

MATERIALS AND METHODS

Study Taxa

Specimens of *Funaria hygrometrica* (Budke 145, CONN) and *Physcomitrium pyriforme* (Goffinet 9276, CONN) were used to generate laboratory cultures of gametophytes and sporophytes as outlined in Budke & Goffinet (2016). Both species were grown in the laboratory under the same temperature, light, water, and soil conditions. These species have diverse sporophyte morphologies with *F. hygrometrica* having an average height of 35 mm (Fig. 1A), while *P. pyriforme* is smaller with an average height of 15 mm (Fig. 1B; Whitaker & Budke 2021). *Funaria hygrometrica* also has larger capsules that have an average length of 2-3.5 mm (Budke et al. 2011), whereas *P. pyriforme* has smaller capsules with an average length of 1-3 mm (McIntosh 2007).

Histology

Laboratory grown sporophytes of *F. hygrometrica* and *P. pyriforme* with fully expanded capsules were collected and the middle 50% of the sporophyte stalk was fixed in formalin-aceto-alcohol (FAA). The tissues were fixed at room temperature for 4-8 hrs, and then overnight under a vacuum for a total of 24 hrs of fixation. Post-fixation specimens were placed into 70% ethanol for initial dehydration and then processed through a graded series of cold ethanol: 95% for 30 min and then 100% for 15 minutes, which was repeated twice. After dehydration, tissues were embedded in Spurrs resin (5g ERL 4221, 4g DER 736, 12.50g NSA, 0.14g DMAE) using a graded series. They were first placed in a 1:1 solution of resin to ethanol (100%) for 1.5 hrs, then 3:1 resin to ethanol for 3 hrs, and finally 100% freshly made resin overnight. These steps were carried out on a rolling mixer with perforated lids. Some of the 100% Spurrs resin was used to make shims that served as a platform to center the specimens in the resin and anchor the specimen labels. Specimens were placed in silicone molds on top of the shims and fresh 100% resin was added. The resin was polymerized overnight at 60-65°C.

Sectioning and Mounting

Once polymerized, the blocks containing the tissues were sectioned 700 to 1000 nm thick using a microtome (EM UC7; Leica Microsystems, Wetzlar, Germany) and glass knives. Sections were placed onto a drop of water on a glass slide coated with 2% formvar (Ruzin 1999), which helped the sections stick. The slides were then placed on a slide warmer at 45°C overnight. Next, the sections were stained with 1% toluidine blue and coverslips were mounted on the slides using permount and xylene. The slides were placed onto a slide warmer at 50°C overnight with one small fishing weight (3.013 g) placed on top of the coverslips to prevent bubbles from forming as the mounting media dried. After 12-24 hrs the slide warmer was turned off, but the weights were left in place for another 12-24 hrs.

Microscopy

A compound light microscope (BX60; Olympus, Tokyo, Japan) with an attached digital camera (EOS Rebel T6i; Canon, Tokyo, Japan) was used to take images of the stained tissue on the slides at 40X magnification.

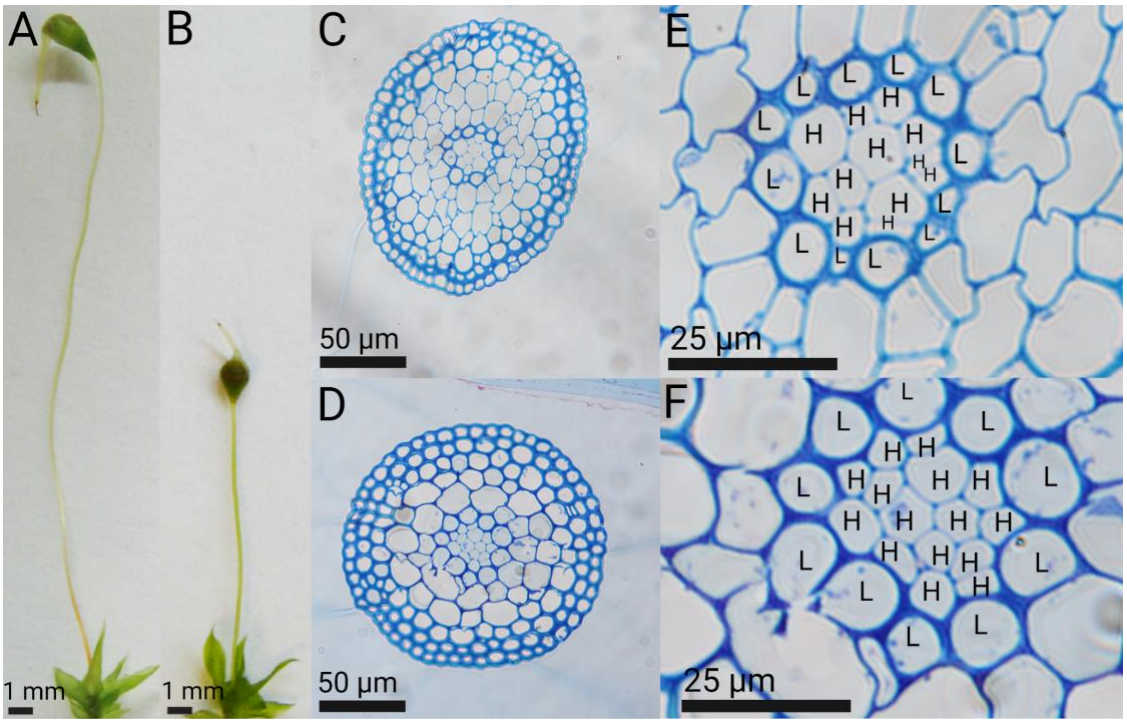

Figure 1. Sporophyte morphology and anatomy of Funariaceae species. A,C,E. *Funaria hygrometrica.* B,D,F. *Physcomitrium pyriforme.* A,B. External sporophyte morphology with expanded capsules topped by calyptra and attached leafy gametophytes at the base. Images used with permission under CC-BY, version 4.0 from Figure 1J,L in Budke & Goffinet (2016). C-F. Transverse sections through the middle of the sporophyte seta. E,F. Close-up of the conducting cells from C and D. L = leptoid cells; H = hydroid cells. This figure was created with BioRender.com.

Data Collection & Analysis

ImageJ was used to count cells and measure areas from the digital images (Schneider et al. 2012). Specifically, measurements and counts were made from the transverse sections of the seta, including the total seta area; the area devoted to conducting cells; hydroid cell area; leptoid cell area; as well as the number of conducting cells, hydroids, leptoids, and non-conducting

cells. Ratios between these area measurements and cell counts were also calculated, such as conducting cell area divided by seta area and conducting cell area divided by number of conducting cells. These data were recorded in an Excel spreadsheet (Microsoft, Washington, USA). RStudio (RStudio Team 2020) was used to perform statistical analysis and generate figures.

T-tests were used to test for significant differences between the two species. Prior to these tests we carried out F-tests to compare variances between the data collected for the two species. If there was not a significant difference between the variances ($p > 0.05$), then the variance was set as equal in the following t-test. However, if there was a significant difference ($p < 0.05$), then the variance was set as unequal.

RESULTS

Based on our anatomical observations we tested for significant differences in conducting cell anatomy between *Physcomitrium pyriforme* (N = 7) and *Funaria hygrometrica* (N = 6; Fig. 1C-F). We did not find a significant difference in the number of conducting cells (leptoids + hydroids) and number of hydroids between the two species. In contrast, we found that *F. hygrometrica* had a larger number of leptoids compared to *P. pyriforme* (t = 2.2893; df = 11; $p = 0.04283$; Fig. 2A). In order to account for variation in seta area among individual sporophytes, the conducting cell areas were divided by the total seta area for each individual. The conducting cell area per seta was greater in *P. pyriforme* than in *F. hygrometrica* (t = -5.6457; df = 7.5267; $p = 0.0006019$; Fig. 2B).

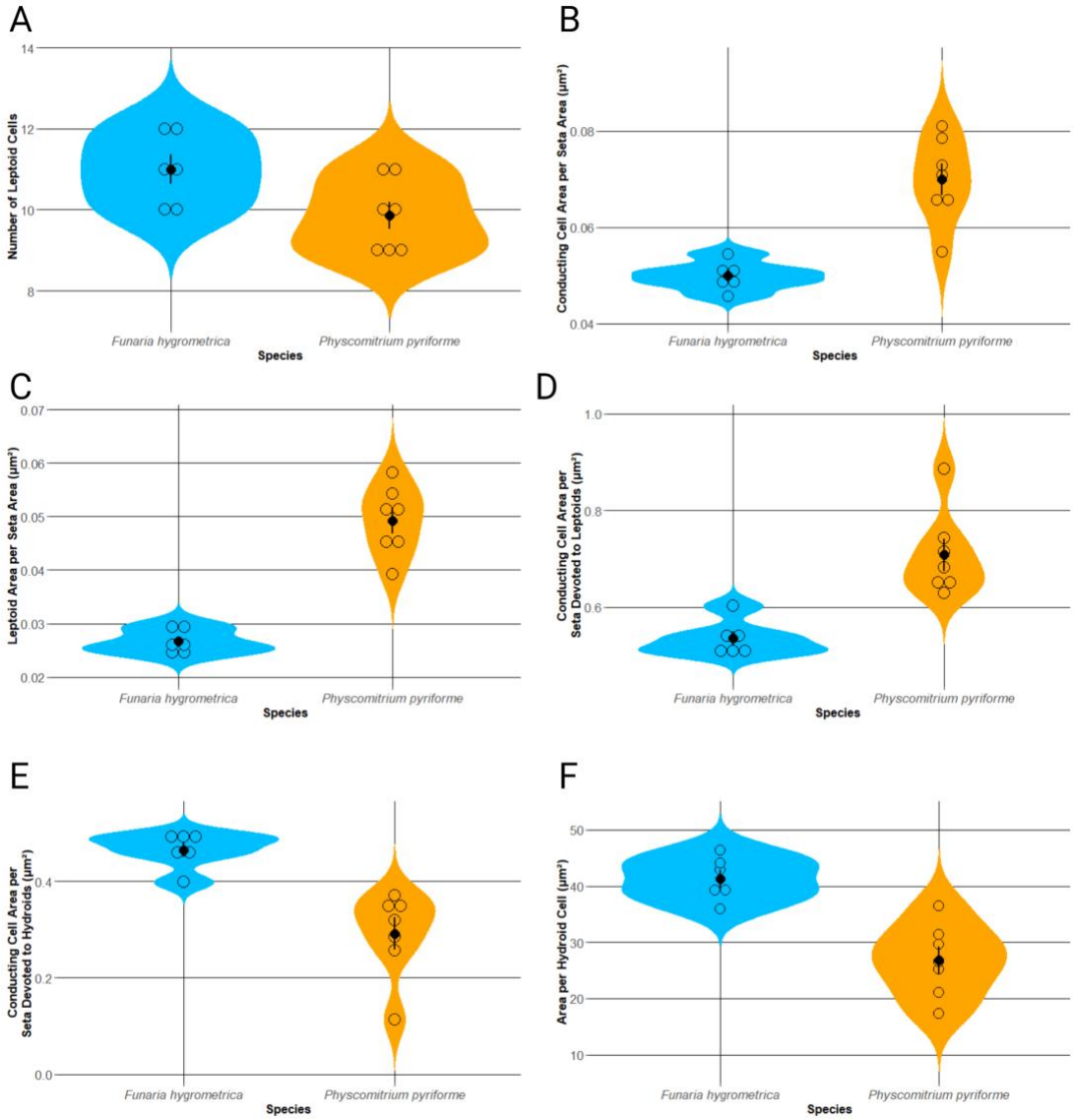

Figure 2. Violin plots with the data from each sample displayed as open circles, the mean as a solid circle, and standard error of the mean as bars. Data from *Funaria hygrometrica* (N = 6) is on the left of each panel in blue and *Physcomitrium pyriforme* (N = 7) is on the right in orange. (A) Number of leptoid cells. (B) Conducting cell area per seta area in µm^2. (C) Leptoid area per seta area in µm^2. (D) Conducting cell area per seta devoted to leptoids in µm^2. (E) Conducting cell area per seta devoted to hydroids in µm^2. (F) Area per hydroid cell in µm^2. This figure was created with RStudio and BioRender.com.

Examining the leptoids separately, *P. pyriforme* was found to have a larger leptoid area per seta than *F. hygrometrica* (t = -8.7787; df = 7.5939; p = 3.071e-05; Fig. 2C) and more of the conducting cell area per seta is devoted to leptoids (t = -4.4791; df = 11; p = 0.0009328; Fig. 2D). However, there was no significant difference in the size of the leptoid cells between the species. Examining the hydroids separately, *F. hygrometrica* had a more of the conducting cell area per seta devoted to hydroids (t = 4.4791; df = 11; p = 0.0009328; Fig. 2E) than *P. pyriforme*. Also, *F. hygrometrica* had a greater hydroid cell size (t = 4.8163; df = 11; p = 0.0005391; Fig. 2F).

DISCUSSION

We predicted that *Physcomitrium pyriforme* sporophytes would have a larger number of and larger transverse area devoted to conducting cells compared to *Funaria hygrometrica*. Our results support this prediction, showing not only that *P. pyriforme* sporophytes have a proportionally larger conducting cell area than *F. hygrometrica* sporophytes, but also that more of this area is dedicated to leptoids, rather than hydroids. In our previous study, *P. pyriforme* sporophytes were found to have higher rates of water transport than *F. hygrometrica* (Whitaker & Budke 2021). This points towards the leptoids playing an important and potentially larger role in water movement through the sporophyte in this moss species.

Hydroids and leptoids in bryophytes have anatomical similarities to xylem and phloem in tracheophytes. In both lineages, water conducting cells (hydroids and xylem) are located centrally with the food conducting cells (leptoids and phloem) surrounding them. Both leptoids and phloem sieve elements are elongated with thick walls and alive at maturity (Scheirer 1980). Leptoids retain a microtubular cytoskeleton in addition to mitochondria, which differs from phloem sieve elements that typically lack these organelles and are supported by companion cells (Woudenberg et al. 2022). Hydroids and xylem tracheids are also elongated cells that are both dead at maturity, lacking all cell contents, including organelles (Ligrone et al. 2000). In terms of their cell walls, the hydroids lack the secondary wall thickenings that are present in xylem (Scheirer 1980).

A significant difference between the hydroids of bryophytes and xylem of tracheophytes is the presence of lignin in the cell wall. Lignin are complex hydrophobic heteropolymers that are crosslinked to carbohydrate polymers (Ligrone et al. 2007). Hydroids lack lignin, but they do have lignin-like compounds in their cell walls. Both of these are aromatic compounds that contain cinnamyl, but lignin-like compounds lack methoxyl groups, which are present in lignin (Edelmann et al. 1998). While the lignin and lignin-like compounds both decrease lateral permeation of water through the cell walls, lignin is significantly more effective, resulting in xylem being better at retaining water compared to hydroids (Scheirer 1980). Additionally, hydroids have very thin cell walls, which not only makes them poor at mechanical support (Woudenberg et al. 2022), but also provides less volume for lignin-like compounds to be deposited into the cell walls. On the other hand, the thin, and potentially more flexible, cell walls of the hydroids may enable them to recover from cavitation events more easily, in comparison to xylem.

Since leptoids and phloem cells are anatomically similar, they may also have similar capacities for internal water conduction. Sugars are loaded into phloem sieve elements at the source (photosynthetic tissue) and are then unloaded at the sink (fruits, seeds, roots; Gould et al. 2005). The higher solute concentration at the source causes water to flow in the sieve elements via osmosis (De Schepper et al. 2013). This generates a pressure gradient resulting in the movement of both sugars and water from source to sink (Knoblauch et al. 2016). Moss gametophytes have lower rates of photosynthesis compared to vascular plants (Martin & Adamson 2001). Thus, mosses may have a higher proportion of water per unit sugar transported through the leptoids in comparison to the phloem of vascular plants, which could explain their role in water movement in moss sporophytes. Another way that moss leptoids could play a role is by facilitating water movement laterally into the hydroids, which have more permeable cell walls due to their lack of lignin. Additional research could help to determine the role leptoids play in water transport and whether it is facilitated in similar ways to phloem.

CONCLUSION

This study found that *P. pyriforme* has a higher proportion of conductive tissue, larger transverse area of the seta devoted to leptoids and that a larger proportion of the conducting cell area is composed of leptoids in comparison to *F. hygrometrica*. These results have the potential to explain why, despite having shorter sporophytes, *P. pyriforme* has higher rates of water transport compared to *F. hygrometrica* (Whitaker & Budke 2021). In combination, these findings point toward leptoids, when present, playing a role in sporophyte water movement, in combination with hydroids.

ACKNOWLEDGEMENTS

We appreciate the assistance of Jaydeep Kolape and the Advanced Microscopy and Imaging Center at the University of Tennessee, Knoxville. This research is supported by a grant to J.M.B. from the United States National Science Foundation (DEB-2046467).

LITERATURE CITED

Budke, J. M. & B. Goffinet. 2016. Comparative cuticle development reveals taller sporophytes are covered by thicker calyptra cuticles in mosses. Frontiers in Plant Science 7: 832.

Budke, J. M., B. Goffinet & C. S. Jones. 2011. A hundred-year-old question: is the moss calyptra covered by a cuticle? A case study of *Funaria hygrometrica*. Annals of Botany 107: 1279–1286.

De Schepper, V., T. De Swaef, I. Bauweraerts & K. Steppe. 2013. Phloem transport: a review of mechanisms and controls. Journal of Experimental Botany 64: 4839–4850.

Edelmann, H. G., C. Neinhuis, M. Jarvis, B. Evans, E. Fisher & W. Barthlott. 1998. Ultrastructure and chemistry of the cell wall of the moss Rhacocarpus purpurascens (Rhacocarpaceae): a puzzling architecture among plants. Planta 206: 315–332.

Fife, A. J. 1985. A Generic Revision of the Funariaceae (Bryophyta: Musci) Part I. The Journal of the Hattori Botanical Laboratory 58: 149–196.

Gould, N., M. R. Thorpe, O. Koroleva & P. E. H. Minchin. 2005. Phloem hydrostatic pressure relates to solute loading rate: a direct test of the Munch hypothesis. Functional Plant Biology 32: 1019–1026.

Hébant, C., 1977. The conducting tissue of bryophytes. Bryophytorum Bibliotheca 10.

Knoblauch, M., J. Knoblauch, D. Mullendore, J. Savage, B. Babst, S. Beecher, A. Dodgen, K. Jensen & N. Holbrook. 2016. Testing the Münch hypothesis of long distance phloem transport in plants. eLife 5: e15341.

Ligrone, R., A. Carafa, J. G. Duckett, K. S. Renzaglia & K. Ruel. 2007. Immunocytochemical detection of lignin-related epitopes in cell walls in bryophytes and charlean alga *Nitella*. Plant Systematics and Evolution 270: 257–272.

Ligrone, R., J. G. Duckett & K. S. Renzaglia. 2000. Conducting tissues and phyletic relationships of bryophytes. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 355: 795–813.

Martin, C. E., & V. J. Adamson. 2001. Photosynthetic capacity of mosses relative to vascular plants. Journal of Bryology 23: 319-323.

McIntosh, T. T. 2007. 12. Funariaceae Schwägrichen, pp. 180–199. In: Flora of North America Editorial Committee (eds.). Flora of North America North of Mexico, Vol. 27, Bryophytes: Mosses, part 1. Oxford University Press, New York, NY.

Medina, R., M. Johnson, Y. Liu, N. Wilding, T. Hedderson, N. Wickett & B. Goffinet. 2018. Evolutionary dynamism in bryophytes: phylogenomic inferences confirm rapid radiation in the moss family Funariaceae. Molecular Phylogenetics and Evolution 120: 240–247.

Patel, N., R. Medina, M. Johnson & B. Goffinet. 2021. Karyotypic diversity and cryptic speciation: have we vastly underestimated moss species diversity? Bryophyte Diversity and Evolution 43: 150–163.

RStudio Team. 2020. RStudio: Integrated Development for R. RStudio, PBC, Boston, MA. URL http://www.rstudio.com/.

Ruzin, S. E., 1999. Plant microtechnique and microscopy. Oxford University Press, New York, NY.

Scheirer, D. C. 1980. Differentiation of bryophyte conducting tissues: structure and histochemistry. Bulletin of the Torrey Botanical Club 107: 298–307.

Schneider, C. A., W. S. Rasband & K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9: 671–675.

Whitaker, K. R. & J. M. Budke. 2021. Moss sporophyte transpiration rates are higher when calyptrae are removed. Evansia 38: 100–108.

Woudenberg, S., J. Renema, A. M. F. Tomescu, B. De Rybel & D. Weijers. 2022. Deep origin and gradual evolution of transporting tissues: perspectives from across the land plants. Plant Physiology 190: 85–99.

Speed Spotting TLC Plates with Micropipette Tips

Bruce McCune

Department of Botany and Plant Pathology, Oregon State University, Corvallis, Oregon 97331, U.S.A.
Author email: **mccuneb@oregonstate.edu**

***Abstract*. Spotting thin-layer chromatography (TLC) plates can be sped up dramatically by using micropipette tips for simultaneous extraction and delivery of lichen substances. High quality results can be obtained in half the time or less for the most time-consuming step in TLC of lichens, spotting the plates.**

***Keywords*.** Lichenized fungi, methods, secondary metabolites, specialized metabolites

Introduction

Thin-layer chromatography (TLC) remains a valuable tool for lichenologists, both for research on systematic questions and for identification of species. Its mainstream use for over 50 years has resulted in a large body of knowledge on the evolution, ecology, functions, and applications of secondary metabolites in lichens. I have routinely used TLC since 1979, after first being taught Chicita Culberson's basic method in a lichenology class taught by Mason Hale in 1977, on a picnic table outside the University of Montana Biological Station on Flathead Lake.

The method remains fundamentally the same as popularized by Culberson (1972): acetone extracts are spotted on a starting line on silica-gel plates using a capillary tube. Plates are developed in air-tight glass tanks containing a solvent system that creeps up the plate separating compounds by their polarity. When the solvent front reaches a finish line or top of the plate, the plates are removed from the tank, evaporated dry, then the spots visualized with UV light, treated with dilute sulfuric acid and heated in an oven.

The slowest part of the TLC process is spotting the plates with the lichen extracts, which typically takes 2 hr or more. In my lab our standard has been 20–30 touches per spot with a loaded capillary, with 18 starting spots (3 control lanes, 15 test specimen lanes) on a 20 × 10 cm plate (details and illustrations in McCune 2017, vol. 1). The capillary is loaded by touching the puddle of lichen-infused acetone on a glass slide or in a plastic microtube. We almost always do this for two plates simultaneously, destined for two different solvent systems, which help to resolve overlapping spots or compounds hidden by another compound.

In this article I report on trials using disposable plastic micropipette tips, searching for a method to make the spotting process both faster and more efficient. Pipette tips are consumed in huge numbers by molecular biology labs, including all labs performing DNA extractions and sequencing. These are used only once, then discarded. Reusing them for spotting TLC plates eliminates the costs and decreases waste of capillary tubes, glass slides, and plastic microtubes.

Methods

For lichen substance extraction and delivery we used the smallest size pipette tip consumed by our lab, for 0.1-20 µl of fluid, using just the tip; the pipettor itself not being needed. The tips serve as miniature funnels to make acetone extractions for TLC. A lichen fragment and a drop of acetone are placed into the wide end of the tip, using forceps for the fragment and a standard dropper bottle for the acetone. Capillarity draws the acetone over the lichen fragment and into the fine point of the tip. This is then manually spotted on the plate, as would be done with a cylindrical capillary tube.

Supplies for the speed spotting process are listed below:

- Acetone dropper bottle
- Forceps
- Numbered rack for pipette tips in order of appearance on the plate
- Silica gel TLC plates with starting spots and labels marked in pencil
- 0.1-20 µl pipette tips (larger tips will deliver the acetone too rapidly)
- Two paper towels (one to cover the parts of the plates not yet spotted and one to the side for dealing with flow rate problems as described below)

Step-by-step use of the micropipette tip method is given in Table 1. Figure 1 shows the tip rack (homemade from wood) and setup for use.

Table 1. Steps in setting up and using a micropipette tip to apply acetone extracts of lichens to TLC plates.

Step	Comments
1. Label and arrange plates with your usual method. Starting spots should be 1 cm apart.	Reduce spotting errors by labeling the tip rack 1 through 15 and control and a piece of paper or cardboard adjacent to the plates with the same labels.
2. Place small fragments in wide end of the tip.	Fragments with total volume of several mm^3 of lichen
3. Add two drops of acetone to wide end of tip	Depending on the amount of lichen, much of the first drop is absorbed by the lichen and the second drop will fill the lower part of the capillary. If the lichen fragment is small, use only one drop. If you added too much acetone, drops will fall out of the tip.
4. Briefly touch loaded tip to starting spot on plate, trying to keep area of absorbance to 1 cm or less. Repeat for second plate	Watch the tip carefully for drops appearing that can fall unexpectedly. If that is about to happen, hold over paper towel.
5. Repeat until flow stops	Usually, 2 or 3 touches at each of two starting points. Wait for the previous touch to evaporate before applying more.
6. Add one drop acetone to wide end of the tip.	Never add more than one drop at this point to avoid fallout from tip.
7. Repeat touches to starting spots until flow stops.	Usually, 3 more touches at each of two starting points for a total of 5–6 touches per starting spot.

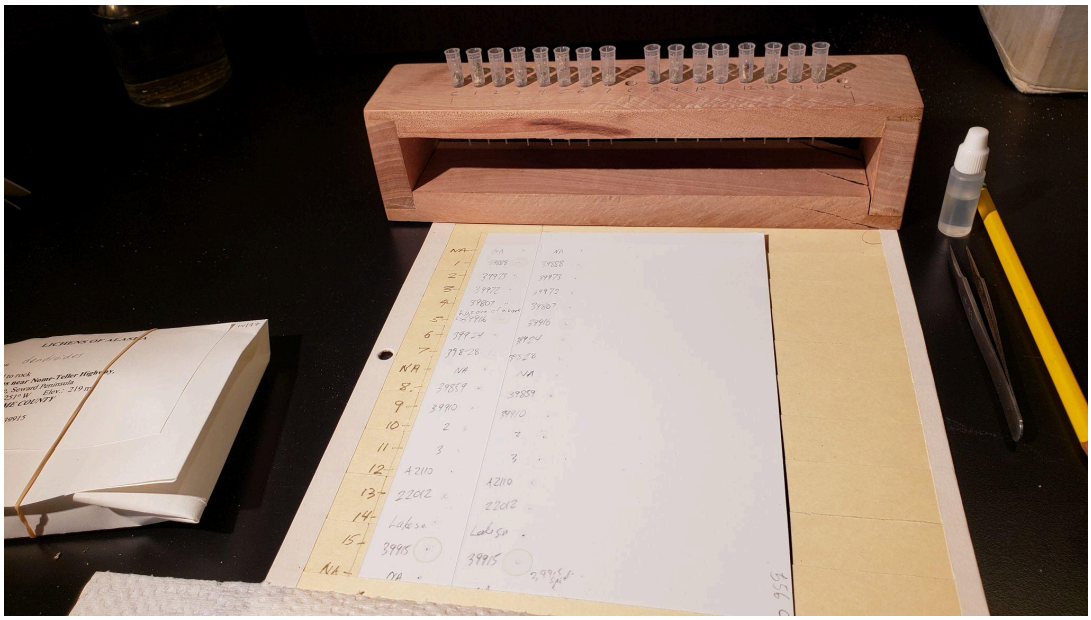

Figure 1. Setup for speed-spotting TLC plates. A numbered wooden rack holds the pipette tips loaded with lichen fragments; two overlapping labeled silica gel plates are ready for spotting. The two identical plates will be run in different solvent systems. The forceps and acetone dropper bottle are used for plucking lichen fragments and loading the tips.

Results and Discussion

The advantages in efficiency of substance delivery and speed from using micropipette tips as opposed to traditional application with a capillary were immediately apparent, cutting the time to spot two plates by more than half, from over 2 hours to about 1 hour. With 15 test specimens per plate, that is 4 min per specimen. The faster application comes from not needing to reload the capillary between spots and from more efficient extraction and delivery of lichen substances. In the traditional method, much extract remains as a dried residue on the glass slide or in the microtube used for extraction. With

pipette tips, all of the acetone extract is funneled towards the delivery point. Five or six touches with the tip is sufficient as opposed to about 20–30 touches with a capillary from a glass slide.

Compared to our previous method of applying 20–30 touches with a capillary tube, 5–6 touches with the pipette tip method results in larger diameter spots, so that the sides of the spots often touch each other. We have not found that this affects our ability to interpret the plates, nor does it appear to change the Rf values. In fact, more lichen substance is applied to the plate, so even though spots are larger, they are also more deeply colored and easier to interpret after development (Figure 2).

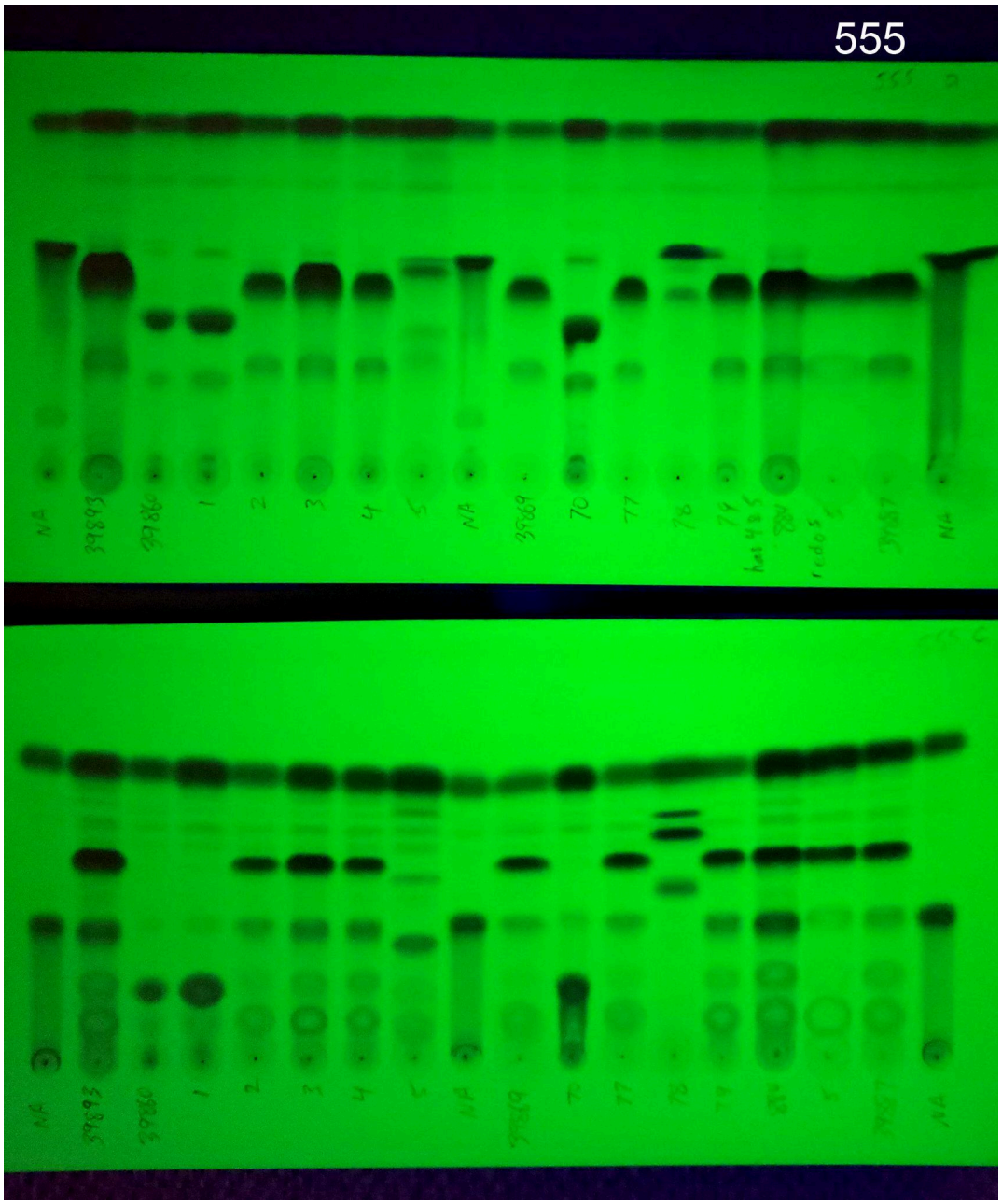

Figure 2. Speed spotting results viewed under short-wave UV light, using specimens from the genus *Stereocaulon*. TLC plates were developed in solvent systems A and C of Culberson (1972). The fully developed plates and labeled substances are shown in Figure 3. The background is bright greenish because the silica gel contains a UV 254 nm indicator dye. Note that spots for major substances are distinct and dark, even though only 5-6 touches were used per spot. The top row of spots is atranorin. Norstictic controls are in the first, last, and center lanes. A spotting error is visible in the far-right lane of the upper plate. Note the prong on the norstictic acid spot extending to the plate margin. This was caused by a single drop of an overfilled tip falling from the tip before it was positioned correctly.

The main drawback to using pipette tips is that the delivery rate can be quite variable. It can be too fast if too much acetone is applied and drops spontaneously fall from the end of the tip.

The delivery rate can be too slow if minute fragments of lichen clog the lower part of the tip. This was problematic with *Stereocaulon*, which often is so crumbly that breaking off a branch tip with forceps and dropping it in the wide end of the capillary also delivers minute fragments that make the delivery erratic. This can be much reduced by setting the fragment down after breaking it off, then picking it up again with forceps. This allows the tiny fragments to fall off before placing the main fragment in the tube. We tried to control this by using tips with built-in filter barriers, but these effectively blocked the capillary action.

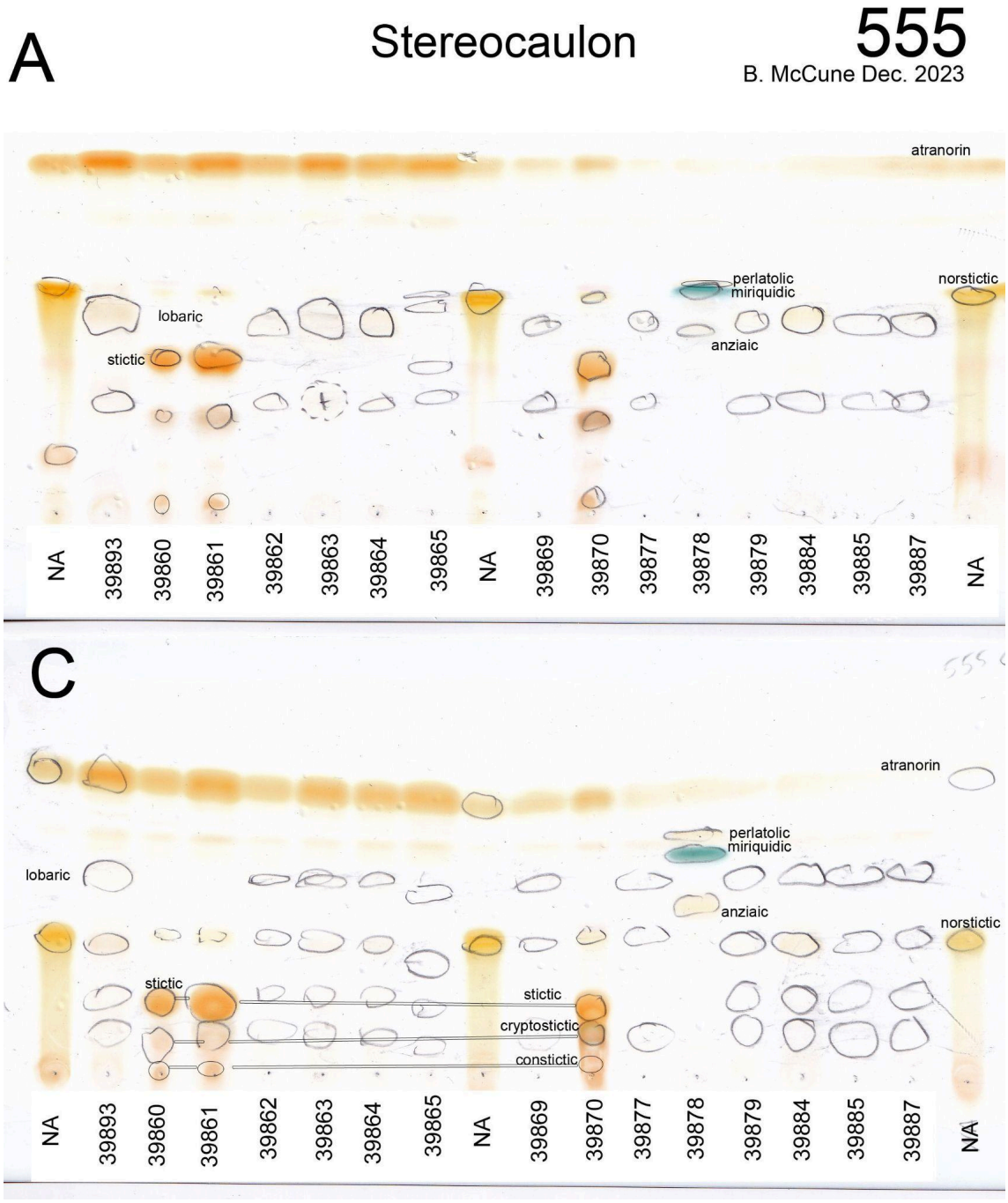

Figure 3. Speed spotting results after acid + heat treatment, same TLC plates as in Figure 2.

If the flow is blocked by fragments, you can often get it going by gently tapping the point of the tip on a paper towel on your work surface. This can result in a sudden gush, so do this away from your plates. With a little practice, these spotting errors were minimal.

TLC practitioners can minimize expense and waste by the single re-use of pipette tips that would otherwise go directly into the waste stream from a molecular biology lab. Even if you have no connection to such a lab, you can currently buy a bag of 1000, 5 µl tips for US$10 (penny apiece) online. Eppendorf (microcentrifuge tubes with attached

caps) cost about twice that, plus one needs to rinse the capillary with acetone between specimens. You might, however, prefer microtubes over pipette tips if dealing with pulverized material or if you wish to save the extracted fragments.

Deciding how much extract to load on the starting spots is one of the persistent problems of TLC. My earlier reference to our usual 20–30 touches per spot when using a capillary tube is lab lore, rather than my prescription. Individual people settle on their own target number, depending on their results. The problem defies a standard solution because concentrations of lichen substances vary so much among species or even among specimens of the same species. Also, from my experience, having trained dozens of lab helpers over the years, different people deliver different amounts of extract, depending on the diameter of their chosen capillary (usually drawn to a fine tip), the size of the lichen fragment, how much acetone is used, and the target diameter of the starting spot. Some of these problems also apply to the pipette tip method, so one should take my prescription of five touches with the pipette tip per starting spot as a general suggestion, rather than a rigid protocol.

My only regret with this method is that I did not try it sooner in my career. The time savings of over one person-hour per pair of plates would have quickly added up to months of lab time and a lot less wasted glass and plastic.

Acknowledgements

I thank Patricia Muir for helpful suggestions on the manuscript and Dale Ruzicka for testing the method. Jason Hollinger went above and beyond the call of duty by performing and communicating his own tests, along with insightful comments and discussion on the manuscript.

Literature Cited

Culberson, C.F. 1972. Improved conditions and new data for the identification of lichen products by a standardized thin-layer chromatographic method. Journal of Chromatography 72: 113–125.

McCune, B. 2017. Microlichens of the Pacific Northwest. Volumes 1 & 2. Wild Blueberry Media, Corvallis, Oregon.

Germination trials in newly reported tubers of *Timmiella crassinervis*

Alexander Russell [a], Theresa A. Clark [b], Joshua L. Greenwood [a], Wagner Luiz dos Santos [c], Nicole Pietrasiak [a], and Lloyd R. Stark [a,d]

[a] School of Life Sciences, University of Nevada, 4505 Maryland Parkway, Las Vegas, Nevada 89154-4004, USA
[b] Department of Evolution, Ecology, & Behavior, University of Minnesota Twin Cities, St. Paul, Minnesota 55455, USA
[c] Department of Plant Biology, Universidade Estadual de Campinas, Monteiro Lobato - 250, Barão Geraldo, Campinas, São Paulo, Brazil
[d] Email: LRS@UNLV.nevada.edu

***Abstract*. Tubers are described for the first time in the moss genus *Timmiella* (Pottiaceae). Using uniclonal cultures, tubers were produced after at least 3 months, were spherical, often occurred in groups ("nests"), ranged in size from 10–600 µm in diameter, and had a water content of ~4% (dry wt basis) while yet retaining an oil-like residue. On a per individual shoot basis, belowground biomass in cultures equaled aboveground biomass. Tubers germinated in ~3 days from planting by producing multiple protonemal filaments, similar to detached shoot tips and rhizoids, but much more rapidly than detached leaves. Germinated tubers eventually formed a colony (producing 50 protonemal shoots and extending horizontally 11 mm) in ~30 days, measures very similar to detached shoot tips, and faster than rhizoids and detached leaves. The presence of *Timmiella crassinervis* in frequently burned regions of chaparral suggests fire frequency in California may have selected for greater belowground allocation.**

***Keywords*:** asexual reproduction, propagules, biomass allocation, uniclonal cultures, southern California, regeneration

INTRODUCTION

During culturing of *Timmiella* plants from California (USA) in 2015 as part of an undergraduate research project on desiccation tolerance, large spherical tubers were noticed by the first author. This is noteworthy because tubers and other types of specialized asexual reproductive structures are unknown for the genus (Zander 2007). Here we describe the tubers, estimate tuber biomass in relation to aboveground and belowground biomass of individual plants, determine the water content of shoots and tubers, and compare the germination times of tubers to the regeneration times of detached leaves, whole shoots, and rhizoid strands. In addition, we compare the ability of tubers to generate a colony of ~50 shoots with that of detached leaves, whole shoots, and rhizoid strands. In field populations of *Timmiella crassinervis* (Hampe) L. F. Kock, sporophytes were rare or absent, and sex expression infrequent. In cultures, sex expression was not seen; as a result spores could not be included in our germination analyses.

Taxonomic comments. *Timmiella* is a widespread genus of 13 species distributed in North, Central, and South America, Europe, Asia, Africa, and the Pacific Islands. It is characterized by plane leaf margins, wide costae, and leaf cells medially bistratose, the ventral layer bulging (Zander 2007). Typical field habitat is on shaded soil, clay, along road sides or ravine bottoms, and boulder crevices (Zander 2007 and field observations LS). Two species occur in California (USA), with Norris and Shevock (2004) noting that where the two species co-occur, *T. crassinervis* tends to occur in the more humid NW California, with *T. anomala* (Bruch & Schimper) Limpricht more common in the drier southern portion of California. However, both species are known from southern California, and using the more reliable sporophyte traits (seta length and theca dimensions), with peristomes and sex-expressing plants mostly unavailable, the main collection used in the study is identified as *T. crassinervis*. Although a distinguishing feature is the leaf base either flared (*T. crassinervis*) or not abruptly wider than the limb (*T. anomala*), we found this trait to vary widely in culture. In addition, plant sexual condition is variable, and a taxonomic revision may place these two species into synonomy (Zander 2007). *Timmiella crassinervis* is native to western North America, from British Columbia to Arizona, and Hawaii (Zander 2007).

METHODS

Material placed into culture and field tuber notes. Collections from five locations in southern California (USA) chaparral habitat were placed into culture. These collections were originally made for other purposes (phenology, desiccation tolerance), and much of the rhizoid layer was often not collected. Nevertheless, tubers were found in most field collections, as given below. A single shoot from each field collection was subcultured away from any visual contamination and propagated in single clone lab cultures over several years. Attempts to remove a putative rhizosphere parasite were unsuccessful; regenerative protonemal filaments absent of the fungus eventually formed a colony of plants with infected tubers, thus we suggest the parasite is systemic in nature (i.e., intracellular or retained upon tuber germination). The primary

cultures (genotypes) used in our analyses are from near Julian, California (called *Banner Grade*), and from near Poway, California (called *Poway*). A set of tuber diameters was earlier made on the *Iron Mountain* genotype. Other genotypes are listed below and behaved similarly in culture. Collection locality and field tuber notes are given below.

Banner Grade: USA, California, San Diego County, along Hwy 78 just E of Julian, 33.0844 N, -116.57241 W, collected Rob Smith, 28 March 2013 (UNLV). Tubers unconnected to the rhizoid system and covered with what we assume, based on its behavior in culture, is a black rhizosphere fungus.

Poway: USA, California, San Diego County, city of Poway, roadside trail along Espola Rd (S5), ~33.01563 N, -117.02376 W, collected Llo Stark 15 Nov 2013. Tubers attached to plants and dangling from rhizoids (Figure 1A).

Iron Mountain: USA, California, San Diego County, trail to Iron Mountain, north of Lakeside, 32.97737 N, -116.96468 W, collected Llo Stark 20 June 2011. Tubers not seen in the field (very small) collection but commonly produced in culture.

Ojai: USA, California, Ventura County, Taft Gardens & Nature Preserve, loose wet soil after heavy rains, 34.45180 N, -119.33998 W, collected Llo Stark 1 Jan 2023. Tubers observed in the freshly collected hydrated material, although not observed in the desiccated material.

Tujunga: USA, California, Los Angeles County, roadside trail near Singing Springs, CA, along Big Tujunga Canyon Rd (since burned), 34.32344 N, -118.13947 W, collected Llo Stark 3 August 2002 as single shoots. Tubers rare in field collection, one tuber observed free among shoots.

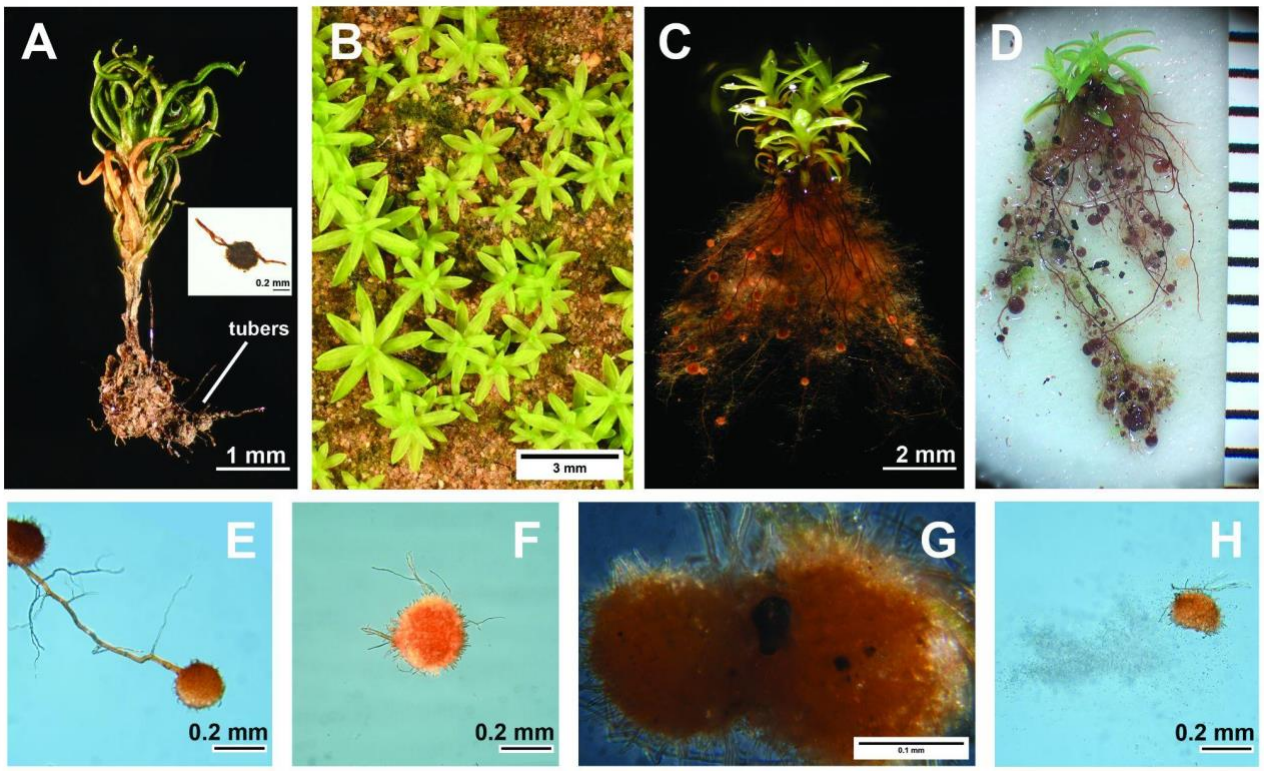

Figure 1. Tubers from *Timmiella crassinervis* field collections and cultures. (**A**) Shoot with tubers collected in the field from the *Poway* location, with inset of field tuber from the *Banner Grade* location. (**B**) Mature culture of *T. crassinervis*, *Banner Grade* genotype. (**C**) Tubers attached to a plant from a 10-month-old culture showing rhizoid interconnectons with tubers, *Iron Mountain* genotype. **(D**) Tubers attached to a plant from a 10-month-old culture, *Banner Grade* genotype (mm scale at right). (**E-G**) Examples of tubers from cultures of the *Iron Mountain* genotype, black regions are a putative fungal parasite. (**H**) Ruptured tuber.

Culturing protocol. Experiments were conducted using three of the genotypes above in which single clone cultures were derived. Shoot tips were cleaned (rinsed in sterile water) and placed into culture (plastic Petri dishes, inner diameter 35 mm, Percival E30B growth chamber, Boone, Iowa, USA) on locally collected pH-neutral sand sieved at 355 µm and dry-autoclaved 60 min at 121°C. Cultures were decontaminated of visible microorganisms through successive subculturing of shoot apices, watered weekly with sterile distilled water and monthly with a 30% Hoagland's inorganic nutrient solution

(Hoagland & Arnon 1938). Multiple cultures were grown under a 12 h photoperiod (20°C lighted, 8°C darkened), with photosynthetic active radiation (PAR) ~90 µmol m^{-2} s^{-1}. The *Banner Grade*, *Poway*, and *Iron Mountain* genotypes used here had been subcultured and grown to maturity (Figure 1B) through several (uncounted) asexual generations over the course of several years. In 2022 and 2023, these cultures were transferred from the campus to a home lab under similar conditions above but using grow lamps in lieu of a growth chamber, with somewhat lower light levels (24—90 PAR) and higher temperatures (18–24°C), rotating Petri dishes daily across a counter. Once a mature culture was established (~6 months to produce medium-sized tubers), a shoot was removed from the culture to include the associated sand and rhizoids, this shoot gently rinsed 2—3 times in sterile water on a microscope slide, and the tubers teased away from their rhizoids. A set of 192 tubers from the *Iron Mountain* genotype was measured in diameter, with all available tubers selected from a culture colony section 1 cm^2. Tubers were measured with a slide micrometer to the nearest 10 µm; those tubers used in experiments were medium to large sizes (~150—400 µm in diameter) in order to facilitate experimental manipulation and make detecting germination possible using a dissecting microscope of up to 50× magnification. All structures studied (tubers, detached leaves, shoot apices, rhizoids) were regenerated on sand media prepared as above.

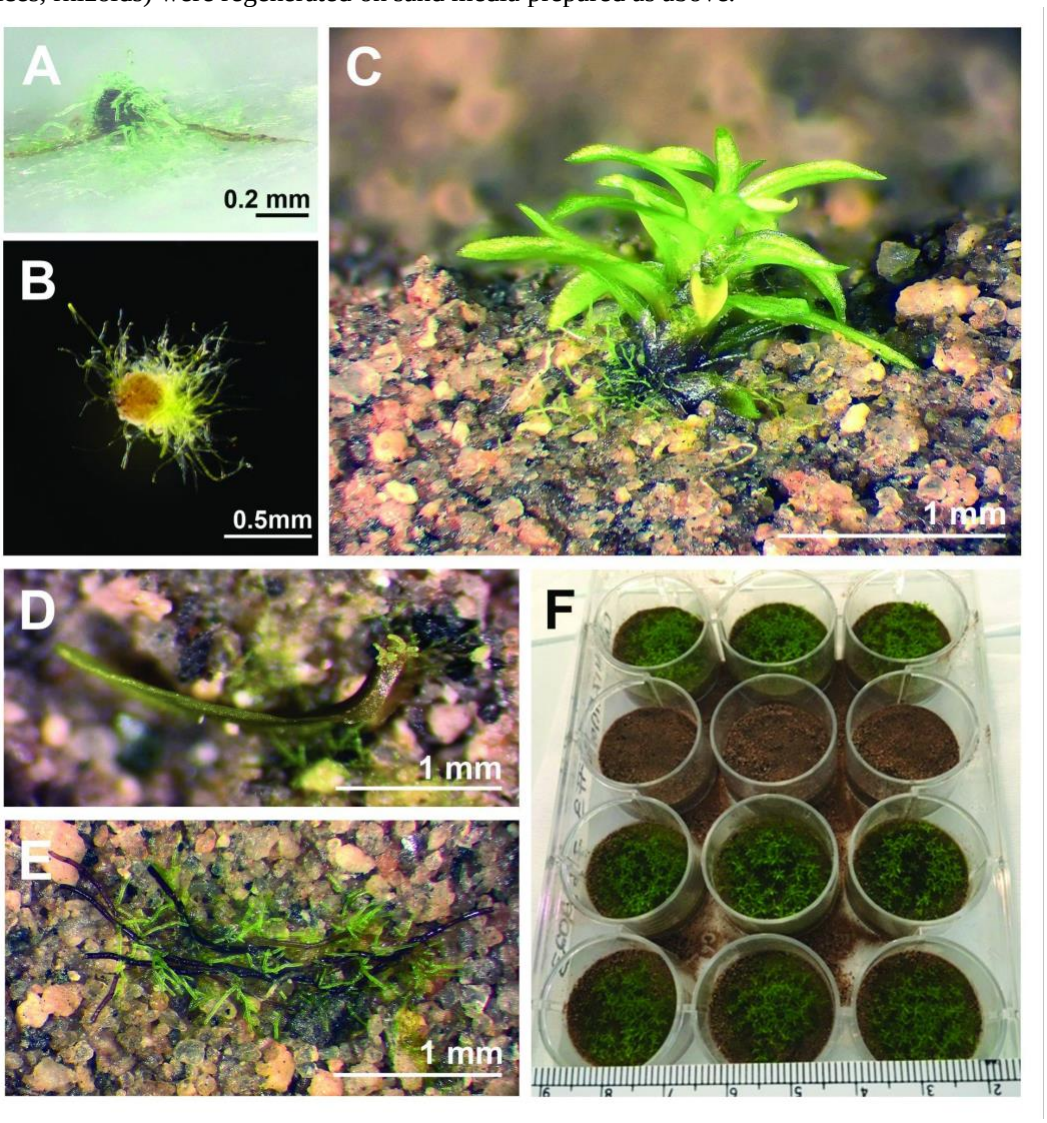

Figure 2. Germination and regeneration of tubers, shoots, detached leaves, and rhizoids of *Timmiella crassinervis*. (**A**) Tuber germination day 8 (*Banner Grade* genotype), on filter paper. (**B**) Tuber germination day 14 (*Banner Grade* genotype), originally germinated on saturated filter paper and shown here submerged in water. (**C**) Shoot tip regeneration day 6 (*Tujunga* genotype). (**D**) Detached leaf regenerating by day 37 (*Banner Grade* genotype). (**E**) Rhizoid regeneration day 6 (*Tujunga* genotype). **(F)** wellplate cultures used in regeneration experiment, showing colonies regenerated from single fragments.

Aboveground vs. Belowground biomass and Water content. Five individual mature plants from the *Poway* genotype were gently removed from culture to include both above- and belowground tissues, and rinsed of debris in water several times. The base of the shoot was excised with a straight edge, separating above- and belowground structures. Shoots were

normally 2—4 mm in length, from the shoot base to the tip of the longest leaf. Aboveground biomass included any rhizoids originating along the shoot, and belowground biomass included any subterranean shoot material. Fresh weight (FW) of above- and belowground material was taken to the nearest microgram after a 4 sec blot on a chemical wipe. The belowground portion was then rehydrated and the tubers and the tuber nest material (fine rhizoids interconnecting tubers) separated from other rhizoids. The number of tubers exceeding ~10 µm in diameter was counted, and using a slide micrometer the range of tuber sizes (diameter or the longest dimension) was estimated to the nearest 10 µm. The tuber with nest material was then gathered, blotted, and weighed (FW). All material was air-dried overnight (iButton readings for temperature 22.8 ±0.03°C, mean ± one standard error, range 20.6—25.6°C, relative humidity 28.5 ±0.02%, range 26.7—30.9%) and weighed again (AirDW). Water content (WC) required more biomass and was conducted on a separate set of plants also from the *Poway culture*, separated into aboveground (shoots, including adventitious rhizoids, 8 shoots per replicate, N=5) and tuber nests (including interconnecting rhizoids, ~50—100+ tubers per replicate, N=5) and rinsed of debris in sterile water. Rhizoids proved difficult to handle independent of tuber nests and a WC of free rhizoids was not taken. After taking fresh weights (FW) of shoots and tuber nests (plants taken from a water droplet and blotted 4 s on a chemical wipe, then weighed immediately), each was dried in desiccators of MgCl to equilibrate over 48 h at ~33% RH, re-weighed, and then oven dried (3 d at 80°C) and a final weighing. WC was calculated as [Experimental Wt – Oven DW / Oven DW] × 100, yielding a percentage dry mass (DW) for plants at full turgor and desiccated at 33% RH.

*Germination, regeneration, and colony formation***.** From a *Banner Grade* mature culture, 15 shoots including their rhizospheres were carefully pulled from the culture. The upper 2—3 mm shoot tip was excised (*Shoot tip*). From each shoot tip, a single leaf was removed toward the base of the cut shoot (*Detached leaf*). From each lower shoot, several rhizoid strands measuring ~3 mm in length were removed and cleaned of any protonema or debris (*Rhizoids*). From within the rhizoid mass, a single medium to large tuber (diameter ~ 200—300 µm) was isolated and cleaned of all but a single attached rhizoid (if present) to allow for handling (*Tuber*). Plantings occurred in five, 12-hole plastic wellplates (22 mm diameter × 22 mm depth) filled about halfway with locally collected pH-neutral sand that had been prepared as above, the sand hydrated with sterile distilled water (Figure 2F). Alternative trials on agar and filter paper resulted in delayed germination/regeneration for most fragments, and therefore sand was used. Each fragment was centrally positioned on the media, with shoot tips planted lying as flat as possible against the media. For each fragment type, N=15. The fragments were positioned with as much contact with the substrate as possible without burying the fragment. Watering was daily by pipet on an as needed basis to ensure the plants were continuously at full turgor over the course of the experiment (46 d).

On a daily basis, each fragment was examined for germination (tubers) or regeneration (detached leaves, shoot tips, rhizoids). In all cases, the first structure emerging was a protonemal filament. In some cases, especially with shoot tips, the lower portion of the shoot was obscured by the upper leaves; germination was noted as the day a protonema first became visible at 50× magnification. Response variables included the day of first germination/regeneration, days to 5 shoots, days to 10 shoots, days to 50 shoots, days to protonema reaching the edge of the wellhole, and days to shoot production at the edge of the wellhole, with "edge" meaning appearing or reaching a distance of ≤1mm from the edge of the wellhole. The edge of the wellhole is construed experimentally as reaching the edge of the colony, and at this point the colony consisted of >50 mature shoots (Figure 2F). For the duration of the colony formation experiment (46 d) the mean temperture was 23.8 ±0.01°C, range 20—28°C, and mean RH (outside of the wellplates) was 34 ±0.01%, range 23—44%, as measured by iButton adjacent to the wellplates.

Statistical comparisons. For biomass comparisons (aboveground vs. belowground), ANOVA was employed after confirming normality of the data. For both the water content level and germination/regeneration comparisons, we applied the Kruskal-Wallis test after finding the data not normally distributed after transformation. We then conducted pairwise comparisons with the Dunn test with Bonferroni adjustment. All analyses were performed using RStudio version 4.2.1. The Dunn test was executed using the Dunn.test package (Dinno & Dinno 2017).

RESULTS

Description of cultured tubers. Tubers of *T. crassinervis* were produced in culture from a single tuber inoculant in about 3 months (*Banner Grade* genotype). Shoots in culture for several months can produce copious numbers of tubers interconnected by rhizoids and often in "nests", where clusters of ~10—30+ tubers occur in close proximity interconnected with fine rhizoids (Figure 1C-D), whereas tuber nests were not observed on field-collected plants, just single (or few) tubers (Fig. 1A). This could be a product of the method of collection, where underground portions are often excluded by the collector, especially if collected for other purposes. Tubers of *T. crassinervis* are mostly spherical, sometimes irregularly spherical to oblong, and can be compound (connate), where 2-3(4) tubers grow attached to one another (Figure 1E-G). Tuber diameter (longest dimension) can vary from 10 µm in very young tubers to up to 600 µm in large spherical or compound tubers (Figure 1G) in older cultures, with two multiply compound tubers from the oldest *Poway* culture (several years) reaching 750 and 800 µm in the longest dimension. Superficial tuber cells are protuberant, irregularly shaped, from 7–42 µm in diameter (Figure 1F), and these cells are covered with short rhizoidal hairs, each ~5 µm in width and up to 30 µm long in

larger tubers. Internal tuber cells comprise a homogeneous cellular "pith" center. Sand grains are frequently adhered to the tuber surface, obscuring the cells. Lipid droplets (as in Zhang & Hong 2011) were not observed in tubers of *T. crassinervis*. When pressure is applied to hydrated tubers, a viscous whitish fluid is exuded that tests negative for starch (Figure 1H). When tubers air-dried to ~35% RH are crushed, a fluid does not exude but the tuber material is moist with perhaps an oil.

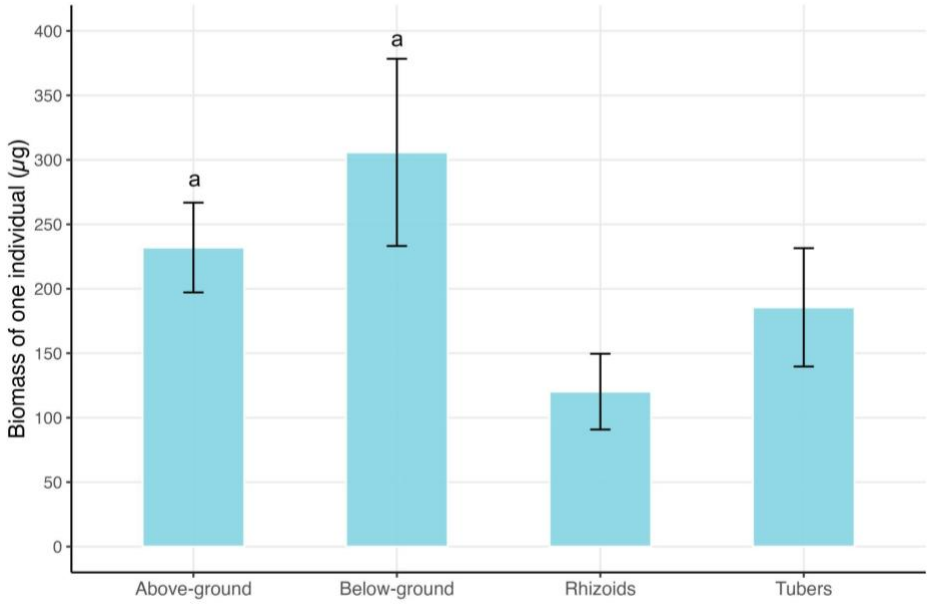

Figure 3. Air-dry (~23°C, RH ~28.5%) biomass of individual plants of *Timmiella crassinervis*, with belowground biomass separated into tubers and rhizoids. Bars with the same letter are not statistically different (P=0.09).

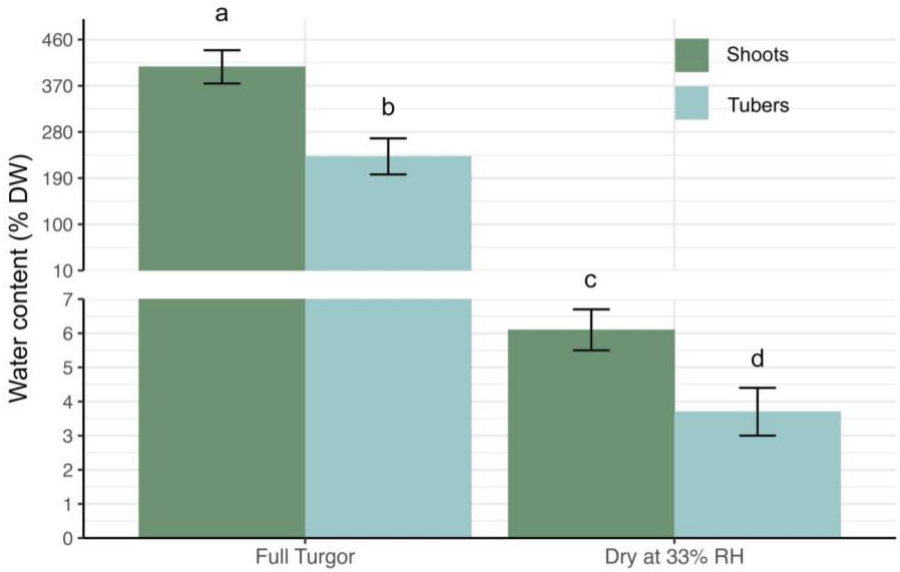

Figure 4. Water content (WC) of shoots and nests of tubers of *Timmiella crassinervis* at full-turgor (blotted for 4 s) and equilibrated at 33% RH, expressed as a percent of the oven-dry weight (3 d at 80°C). Bars with different letters are statistically different (P<0.001).

Biomass and water content. As noted in Glime (2017a), data reporting belowground productivity are "woefully lacking", and rhizoids are often not considered when measuring biomass (Söderström & Gunnarsson 2003). Perhaps surprisingly, the AirDW of belowground plant biomass in cultures of *T. crassinervis* was equal to (with a higher nonsignificant mean, P=0.09) the aboveground biomass, with a majority of the belowground biomass consisting of tubers and the "nest" of rhizoids immediately surrounding the tubers (Figure 3). Shoot water content (WC) at full turgor was roughly twice that of tubers (P<0.01), and the WC at equilibration with 33% RH was lower (P<0.01) than expected for both shoots (6.1%) and especially tubers (3.7%) (Figure 4). After equilibrating at both 33% and 0% RH, the tubers, upon crushing, yet

appear moistened with a highly viscous fluid, and they tend to stick to dissecting tools. This could indicate the presence of oils in dried tubers. It will be interesting to identify the compounds in this exudate.

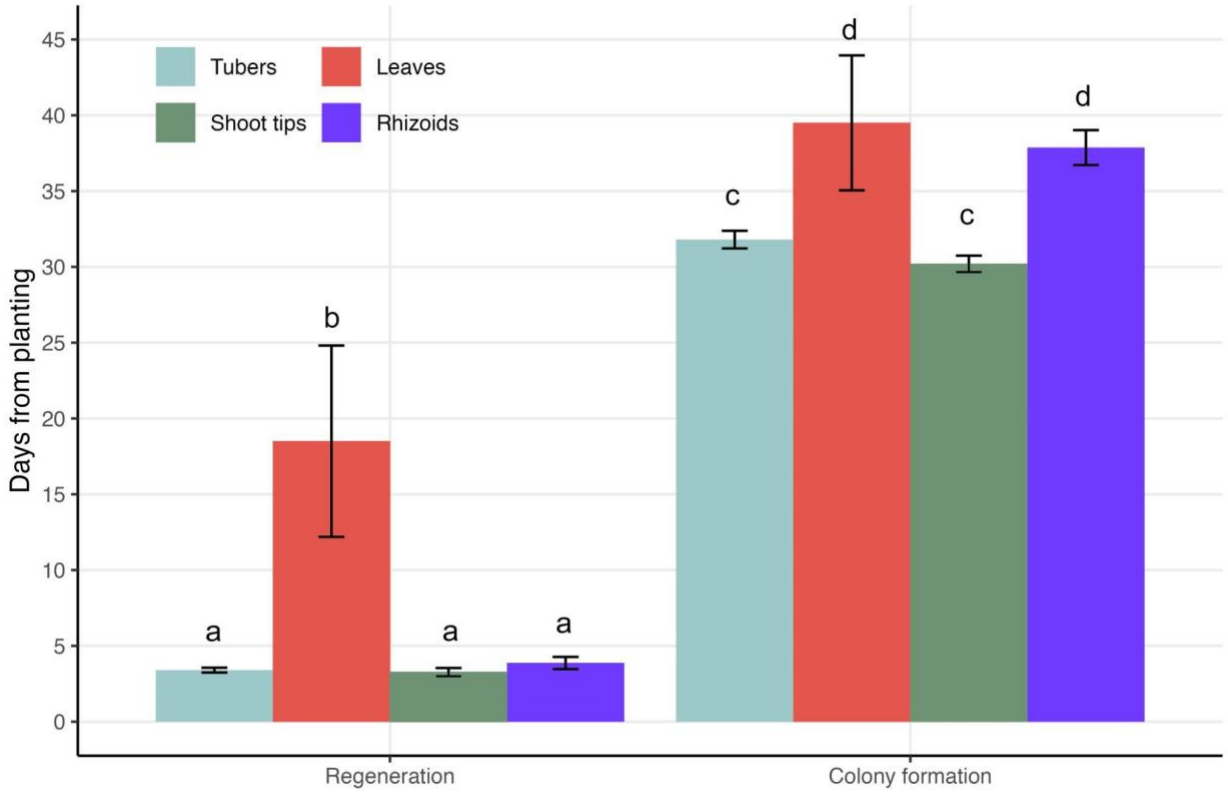

Figure 5. A comparison of four fragments of *Timmiella crassinervis* cultured plants for the day of first germination (tubers) or regeneration (leaves, shoot tips, rhizoids) and the time to colony formation (protonemal shoots reaching periphery of wellplate culture after planting in the center of the wellhole with a radius 11 mm). Bars with different letters are statistically different ($P<0.001$).

Germination, regeneration, and colony formation. All 75 fragments regenerated or germinated with the exception of 9 detached leaves (Figure 2A-E). Similarly, all fragments except leaves regenerated in 3—5 days, with detached leaves exhibiting a delayed regeneration or no regeneration at all (Table 1, Figure 5). Tubers, shoot tips, and rhizoids did not differ from one another in time to germination/regeneration ($P>0.05$), with leaves taking significantly longer to regenerate ($P<0.01$) (Table 1). Colony formation, assessed as having a regenerant shoot form at the edge of the wellhole, was fastest for shoot tips and tubers (~31 d), with rhizoids and detached leaves taking significantly longer ($P<0.05$) to form a colony (Figure 5).

DISCUSSION

Subterranean diaspores produced from rhizoids have been referred to as tubers or rhizoid gemmae (Risse 1987; Glime 2017b). Tubers have key features that distinguish them from other vegetative disapores: the absence of specialized abscission mechanisms, the presence of rhizoid side branches in the filament system producing tubers, and the presence of an apical cell (Duckett & Ligrone 1992; Frey & Kürschner 2011). Although gemmae produced on rhizoids technically lack an apical cell, subterranean tubers and gemmae are likely to have similar ecological survival strategies. In tubiferous species, tubers gradually enlarge and accumulate lipid or starch reserves while attached to perennial rhizoids (Duckett & Matcham 1995). Their age is inadequately known, but some species (e.g. *Ditrichum cylindricum*) may produce tubers still viable even 25–30 years from the date of collection (Duckett & Pressel 2003).While tubers of *Bryum bicolor* are produced early during the growth of the gametophore and function in proliferation (Glime 2017c), our *Timmiella* cultures did not produce tubers until a colony of mature shoots had formed, or after a period of about 3–6 months in a 35 mm diameter (substrate ~3 mm deep) Petri dish, with nests of tubers not forming for many months. Perennial rhizoids connect to each and every tuber and probably function in the conduction of photosynthate into the tuber array (Duckett & Matcham 1995). It is interesting to note that once tubers begin forming in good numbers, the growth of the shoots vertically slows or ceases while not becoming senescent (although not measured here).

Life strategy. *Timmiella* likely falls into the Colonist strategy for ephemerals, with "high asexual reproductive effort by subterranean tubers on rhizoids, sexual reproduction rare, and usually short turf" (Glime 2017c, based on During 1979, 1992). About 85% of known tubiferous species of mosses are dioicous (Whitehouse 1966; Risse 1987), and the majority of tubiferous species rarely produce spores (Longton & Schuster 1983). Southern California populations of *Timmiella crassinervis* sampled here are consistent with this trend, with sporophytes rare in two field collections (*Banner Grade* and *Tujunga*) and sex expression absent in most field collections and all lab cultures. Such a pattern suggests a tradeoff between sexual and asexual reproduction, with asexual reproduction given primacy in *T. crassinervis*. The pattern may also reflect a drain in resources not only in filling tubers, but also feeding the putative rhizosphere fungal associate.

Tuber size. Compared to the range of tubers presented in Whitehouse (1966) and Preston (2004), tubers of *T. crassinervis* are on the large size of the spectrum of European mosses, with only a few species exceeding the *Timmiella* size maximum. However, *Bryum billardierei* tubers pictured in Malcolm and Malcolm (2000), are 1—2 mm in diameter, perhaps the largest moss tuber of which we are aware, with *Phaeoceros laevis* tubers also reaching 2 mm. Compound (=connate) tubers are known in *Ditrichum cornubicum* (Arts 1994) and several species of *Bryum* and *Tortula* (Risse 1993).

Tuber biomass. The high biomass of tubers in *T. crassinervis* may be a result of culturing plants in relatively shallow media (~3 mm deep) where plants are unable to extend rhizoids deeper, and instead focus on filling tubers. Tubers are perennating organs in nature, and have been shown to tolerate drought or cold better than shoots in some species, capable of tolerating at least 10 years of desiccation (reviewed in Risse 1987). We have yet to observe nests of tubers in field collected material of *Timmiella*, but these observations are limited, and the closely packed "sward of … rhizoidal tubers" of *Discelium nudum* from the field (Duckett & Pressel 2003) may be similar to the nests of tubers in cultures of *T. crassinervis*. In addition, Reese (1988) may have observed these nests as tubers within "wefts of rhizoids" in *Weissia controversa*. Tuber nests of *T. crassinervis* tend to occur close to the substrate surface, near the shoot base and also under protonemata. Although tubers may be encouraged to form under low nutrient conditions (Duckett & Ligrone 1992), in recent *T. crassinervis* cultures few tubers were formed until a nutrient solution was added.

Table 1. A comparison of four gametophyte structures of cultured *Timmiella crassinervis* in their ability to germinate (tubers) or regenerate (detached leaves, shoot tips, rhizoids) and for protonemata to reach the edge of the wellhole. A single clone from Banner Grade, California was used. Emergence=germination or regeneration; "edge" = the periphery of the Petri dish; values are means ± one S.E., N=15. See Figure 4 for additional data. Rows with different letters are statistically different (P<0.001)

Structure	Percent emergence	First shoot (days)	10 Shoots (days)	50 Shoots (days)	Protonema reach periphery of dish (days)
Tuber	100	11.33 ±0.93 *a*	21.73 ±0.48 *a*	28.67 ±0.48 *a*	24.27 ±0.52 *a*
Leaf	40	26.33 ±3.28 *b*	32.50 ±3.50 *b*	38.50 ±6.50 *b*	31.50 ±6.50 *b*
Shoot tip	100	11.93 ±1.03 *a*	25.47 ±0.82 *b*	29.62 ±1.09 *ab*	23.20 ±0.55 *a*
Rhizoid	100	20.53 ±3.16 *b*	26.40 ±2.85 *b*	33.50 ±4.03 *ab*	29.86 ±2.92 *b*

Germination and regeneration. For mosses, the typical germination pattern is for a single gemma or tuber to produce a single shoot while a single spore can produce multiple protonemal shoots (Glime 2017d). In *T. crassinervis*, however, each of the 15 tubers tested germinated by multiple protonemal filaments, which eventually formed a lawn of protonemata followed by abundant shoot production. In ongoing pilot experiments with *T. crassinervis* tubers, >100 tuber germinations have been observed, with a large majority of tubers germinating by protonema, and a few germinating by a shoot. This pattern is similar to that described for *Gemmabryum tenuisetum, G. dichotomum* (=*Bryum bicolor*), *Gemmabryum violaceum*, and *Ephemerum recurvifolium*, with tubers producing a spread of protonemata and often a shoot, or just a spread of protonemata (Ashton & Raju 2001; Duckett & Ligrone 1992; El-Saadawi & Zanaty 1990; Pressel et al. 2005). In *Haplodontium notarisii* tubers simultaneously germinate with a gametophore and a caulonemal filament (Arts 1988). In previous pilots in our lab, 192 tubers of the *Iron Mountain* clone of *T. crassinervis* were germinated under water for 21 d, and these produced both shoots directly

(177 tubers) and protonemata directly (184 tubers). In many cases (80 tubers), multiple shoot production occurred from a single tuber, with a maximum of 4 shoots produced by single tubers (9 tubers).

Tubers and shoot tip fragments germinated/regenerated quickly, in about 3.3 days, with rhizoid strands taking about a half day longer, and detached leaves much later if at all. It took tubers and shoot tips 11—12 days to produce the first shoot. This compares to protonemal cultures of the related *T. anomala* producing a first shoot in 20 days (Chopra & Rekhi 1980) or 28 days (Kapur & Chopra 1989), with the light and temperature conditions similar to the present experiment, but the media used (agar vs. sand) differed. Low regeneration from detached leaves was also noted in snowbed samples (Miller & Ambrose 1976) and in slower leaf fragment regeneration compared to gemmae in *Dicranum flagellare* (Glime 2017e). The relatively rapid germination rates of tubers and an absence of lipid droplets (as photographed in Zhang & Hong 2011 for *Fissidens macaoensis*) suggest the presence of starch rather than lipids in tubers of *T. crassinervis* (Duckett & Pressel 2003; Pressel et al. 2005), although our preliminary starch test was negative and starch grains were not observed in tuber cells or in the viscous fluid inside the tuber. Based on several previous studies summarized in Newton and Mishler (1994), we expected that colony establishment (repopulation) should be faster from tubers than fragments. Surprisingly, shoot tip fragments formed a colony of shoots in culture just as rapidly as tubers. Nevertheless, tubers formed a colony 7—10 days faster than rhizoids and detached leaves. Although we could not directly compare colony formation rate between fragments and spores, it appears that *T. anomala* spores form protonemal shoots more slowly (5 shoots after 35 days) but produce a similarly sized protonemal mat (21 mm diameter in 27 days (Chopra and Rekhi 1980) than corresponding numbers for tubers and detached shoots of *T. crassinervis* (>50 shoots after 35 days, 21 (22) mm diameter protonemata in 24 days, respectively). Shoot proliferation may be slower on agar for this species, while protonemal growth on the media surface was similar on agar vs. sand. In a rare comparison of regeneration rates (in terms of the shoot length regenerated after two weeks) of shoot tips, tubers, and shoot fragments, Egunyomi (1980) found that (deciduous) shoot tips produced the longest shoots, followed by tubers and then shoot fragments.

Wildfire hypothesis. The rich reserve materials (e.g., lipids, starch) of perennating tubers likely serve the species in (i) adapting to disturbed habitats, and (ii) surviving unfavorable conditions such as drought and cold (Risse 1987). The production of tubers in *T. crassinervis* may be related to its occurrence in the California chaparral, where tubers may have evolved as a means to survive the frequent fires in this ecosystem. Although we did not collect *Timmiella* in obviously disturbed areas, disturbance by fire (and flood) is a relatively common occurrence in the chaparral portions of the world. In fact, one of our source populations (*Tujunga*) had burned in the course of this study. It will be interesting to see the components of the fluid within these interior tuber cells, with a function perhaps to assist and supply the germinative surface cells through wetting, drying, and fire cycles. In addition, given the sympatry of the two closely related species in California (*T. crassinervis* and *T. anomala*), it will be interesting to see if *T. anomala* also has tubers, or if tubiferous populations distinguish *T. crassinervis* from *T. anomala*. To this end, *T. anomala* from Arizona (USA) is currently in culture to determine if tubers can be produced.

ACKNOWLEDGEMENTS

We thank the Angeles National Forest (William J. Brown, Jr., Rare Plant Coordinator) for a collecting permit to sample the Tujunga site, Mr. John Taft for permission to gather *Timmiella* and other mosses at the Taft Gardens & Nature Preserve (Ojai, CA, USA), Chris Harrell and Brent Mishler for arranging a permit to collect bryophytes as part of the *So Be Free* moss foray near Julian, CA, USA, Marcus Hooker for receiving voucher specimens, and Robin Riker and Nicholas McLetchie for meaningful input on propagule biology. Original funding for this research was provided by a National Science Foundation Research Infrastructure Improvement Award IIA-1301726 to AR and LS. We thank Janice Glime for presenting her book online on the Ecology of Bryophytes; it has served as an ongoing wealth of information on all aspects of bryophyte ecology, Robin Riker for rendering the photoplates, and Claudio Delgadillo for reviewing the manuscript.

LITERATURE CITED

Arts, T. 1988. The occurrence of drought-resistant rhizoidal tubers in *Haplodontium notarisii*. Lindbergia 14: 131–132.

Arts, T. 1994. Rhizoidal tubers and protonemal gemmae in European *Ditrichum* species. Journal of Bryology 18: 43–61.

Ashton, N. W. and M. V. S. Raju. 2001. Development and germination of rhizoidal gemmae of *Bryum violaceum*. Cryptogamie, Bryologie 2001: 3–11.

Chopra, R. N. and A. Rekhi. 1980. Studies on protonemal differentiation and bud formation in *Timmiella anomala*. Phytomorphology 29: 179–184.

Dinno, A. and M. A. Dinno. 2017. Package 'dunn.test'. *CRAN Repos*, 10, 1–7.

Duckett, J. G. and R. Ligrone. 1992. A survey of diaspore liberation mechanisms and germination patterns in mosses. Journal of Bryology 17: 335–354.

Duckett, J. G. and H. W. Matcham. 1995. Studies of protonemal morphogenesis in mosses VII. The perennial rhizoids and gemmiferous protonema of *Dicranella heteromalla* (Hedw.) Schimp. Journal of Bryology 18: 407–424.

Duckett, J. G. and S. Pressel. 2003. Studies of protonemal morphogenesis in mosses. IX. *Discelium nudum*: exquisite pioneer of unstable clay banks. Journal of Bryology 25: 241–245.

During, H. J. 1979. Life strategies of bryophytes; a preliminary review. Lindbergia 5: 2–18.

During, H. J. 1992. Ecological classification of bryophytes and lichens. p. 1–31 *in* J. W. Bates and A. M. Farmer (eds.), Bryophytes and lichens in a changing environment. Clarendon Press, Oxford.

Egunyomi, A. 1980. Observations on the taxonomy and propagules of *Bryum nitens* Hook. (*B. cruegeri* Hampe). Journal of Bryology 11: 129–131.

El-Saadawi, W. E. and M. S. Zanaty. 1990. *Bryum bicolor* Dicks. and *Funaria hygrometrica* Hedw. develop remarkable persisting structures in extreme environment. Journal of the Hattori Botanical Lab 68: 285–291.

Frey, W. and H. Kürschner. 2011. Asexual reproduction, habitat colonization and habitat maintenance in bryophytes. Flora 206: 173–184.

Glime, J. M. 2017a. Productivity. Chapt. 12. In: Glime, J. M. Bryophyte Ecology. Volume 1. Physiological Ecology. Ebook sponsored by Michigan Technological University and the International Association of Bryologists. Last updated 18 July 2020 and available at http://digitalcommons.mtu.edu/bryophyte-ecology/>, accessed 22 September 2023.

Glime, J. M. 2017b. Adaptive Strategies: Vegetative Propagules. Chapt. 4-10. In: Glime, J. M. Bryophyte Ecology. Volume 1. Physiological Ecology. Ebook sponsored by Michigan Technological University and the International Association of Bryologists. Last updated 24 April 2021and available at http://digitalcommons.mtu.edu/bryophyte-ecology/>, accessed 18 March 2023.

Glime, J. M. 2017c. Adaptive Strategies: Life Cycles. Chapt. 4-6. In: Glime, J. M. Bryophyte Ecology. Volume 1. Physiological Ecology. Ebook sponsored by Michigan Technological University and the International Association of Bryologists. Last updated 5 June 2020 and available at http://digitalcommons.mtu.edu/bryophyte-ecology/>, accessed 6 March 2023.

Glime, J. M. 2017d. Adaptive Strategies: Vegetative vs Sexual Diaspores. Chapt. 4-7. In: Glime, J. M. Bryophyte Ecology. Volume 1. Physiological Ecology. Ebook sponsored by Michigan Technological University and the International Association of Bryologists. Last updated 24 December 2021 and available at http://digitalcommons.mtu.edu/bryophyte-ecology/>, accessed 10 March 2023.

Glime, J. M. 2017e. Ecophysiology of Development: Brood Bodies. Chapt. 5-7. In: Glime, J. M. Bryophyte Ecology. Volume 1. Physiological Ecology. Ebook sponsored by Michigan Technological University and the International Association of Bryologists. Last updated 17 July 2020 and available at http://digitalcommons.mtu.edu/bryophyte-ecology/>, accessed 2 April 2023.

Hoagland, D. R. and D. I. Arnon. 1938. The water-culture method for growing plants without soil. California Agricultural Experiment Station Circular 347: 1–39.

Kapur, A. and R. N. Chopra. 1989. Effects of some metal ions on protonemal growth and bud formation in the moss *Timmiella anomala* grown in aseptic cultures. Journal of the Hattori Botanical Laboratory 66: 283–298.

Longton, R. E. and R. M. Schuster. 1983. Reproductive biology. p. 386–462 *in* R. M. Schuster (ed.), New Manual of Bryology, Vol. 1. Hattori Botanical Laboratory, Nichinan.

Malcolm, B. and N. Malcolm. 2000. Mosses and other Bryophytes. An Illustrated Glossary. Micro-optics Press, Nelson, New Zealand.

Miller, N. G. and L. J. H. Ambrose. 1976. Growth in culture of wind-blown bryophyte gametophyte fragments from arctic Canada. Bryologist 79: 55–63.

Newton, A. E. and B. D. Mishler. 1994. The evolutionary significance of asexual reproduction in mosses. Journal of the Hattori Botanical Laboratory 76: 127–145.

Norris, D. H. and J. R. Shevock. 2004. Contributions toward a bryoflora of California: II. A key to the mosses. Madroño 51: 133–269.

Pressel, S., H. W. Matcham, and J. G. Duckett. 2005. Studies of protonemal morphogenesis in mosses. X. Ephemeraceae revisited; new dimensions underground. Journal of Bryology 27: 311–318.

Preston, C. D. 2004. An updated list of British and Irish bryophytes from which tubers have been reported. Field Bryology 83: 2–13.

Reese, W. D. 1988. Rhizoid gemmae in *Weissia controversa* Hedw. Bryologist 91: 184–185.

Risse, S. 1987. Rhizoid gemmae in mosses. Lindbergia 13: 111–126.

Risse, S. 1993. Very large tubers in *Bryum bicolor* Dicks. Journal of Bryology 17: 505–509.

Söderström, L. and U. Gunnarsson. 2003. Life History Strategies. A catalogue of population biology parameters for bryophytes occurring in North-Western Europe. Manual v. 1.0. BryoPlanet, Trondheim. 68 pp.

Whitehouse, H. L. K. 1966. The occurrence of tubers in European mosses. Transactions of the British Bryological Society 5: 103–116.

Zander, R. H. 2007. *Timmiella*. p. 481–482 in Flora North America Editorial Committee (eds.), Flora North America, Vol. 27, Bryophytes: Mosses, part 1. Oxford University Press, New York.

Zhang, L. and P. L. Hong. 2011. A new species of *Fissidens* with remarkable rhizoidal tubers and gemmae from Macao, China. Journal of Bryology 33: 50–53.