Moss sporophyte transpiration rates are higher when calyptrae are removed

Katherine R. Whitaker and Jessica M. Budke*
Department of Ecology and Evolutionary Biology, University of Tennessee, Knoxville, TN 37996
*E-mail address: **jbudke@utk.edu**

Abstract. **Moss sporophytes are physically attached to and dependent on the leafy gametophyte through their entire life. During early development, the sporophyte apex is covered by the calyptra, a cap of gametophytic tissue which protects the developing capsule. The aim of this study is to examine the influence of the calyptra on sporophyte transpiration rates in mosses. We used two laboratory-grown species with different sporophyte and calyptra sizes for this study: *Funaria hygrometrica* Hedw. and *Physcomitrium pyriforme* (Hedw.) Hampe. When the calyptra was removed from the sporophyte apex, there were significantly higher rates of sporophyte transpiration compared to individuals with the calyptra present. These results provide evidence supporting the importance and influence of the gametophyte calyptra on the movement of water in moss sporophytes. Changes in transpiration, and thus the pull of water and nutrients from the gametophyte into the sporophyte, may also have downstream effects on sporophyte reproductive output.**

Key words. **Bryophytes, calyptra, Funariaceae, maternal effects, transpiration rate.**

INTRODUCTION

In photosynthetic organisms, water is critical for transforming atmospheric carbon into complex carbohydrates. For plants that have moved onto land, water is a limiting resource and these plants have evolved morphological features to both transport and retain water, including conducting cells (Ligrone et al. 2000), stomata (Kenrick and Crane 1997), and the cuticle (Graham 1993). Transpiration is the process by which water moves through the plant body and ultimately evaporates into the atmosphere. In mosses, sporophytes lack roots and do not take up water directly from the surrounding environment; sporophyte internal water is derived exclusively from the attached maternal gametophyte (Browning and Gunning 1979). The acquisition and maintenance of hydration is especially critical for sporophytes at early developmental stages (Budke et al. 2013).

Moss sporophytes are physically attached to and dependent on the leafy gametophyte throughout their entire lifespan. This dependence includes photosynthates, inorganic nutrients, and water that are supplied by the maternal gametophyte and transported through the foot to the offspring sporophyte (Browning and Gunning 1979). Maternal gametophytes have limited resources to allocate between their own survival and future reproduction, and the survival and maturation of their offspring sporophytes (Haig and Wilczek 2006). This results in a lifelong conflict for resources between the maternal gametophyte and offspring sporophyte in mosses (Haig 2013). Changes in sporophyte transpiration may thus influence the pull of resources from the gametophyte into the attached sporophyte and ultimately play a significant role in moss parent-offspring conflict.

An additional interaction between the gametophyte and sporophyte phases occurs at the distal end of the sporophyte where the immature sporophyte apex is covered by the gametophyte

calyptra (Budke 2019). The calyptra has a relatively thick cuticle (Budke et al. 2011) that is formed early in development and protects the sporophyte apex until capsule maturation (Budke et al. 2013). In contrast, the cuticle of the young moss sporophyte is relatively thin (Budke et al. 2012) and exposure to low humidity at this stage, without the presence of the calyptra, results in wilting of the apex and often sporophyte death (pers. obs.). Thus moss calyptrae are important for protecting the immature sporophyte from desiccation and positively influence its survival, development, and fitness (Budke et al. 2013). The calyptra has also been found to play a role in sporophyte transpiration. Bopp and Stehle (1957) demonstrated that water moved through the sporophytes of *Funaria hygrometrica* 1.3× faster when the calyptra was removed compared to sporophytes with calyptrae present. These transpiration rates were measured at an early sporophyte developmental stage (spear-shaped), prior to both stomata development and capsule expansion, thus the transpiration in these plants was most likely due to water lost from cells of the sporophyte epidermis. Unfortunately, the laboratory conditions (light, temperature, humidity) were not reported in the study of Bopp and Stehle (1957), which hinder our ability to make direct comparisons to their results.

Funariaceae is a diverse family of mosses that is ideal for comparative studies. Members of this family are easily grown in a laboratory setting and have diverse sporophyte and calyptra morphologies (Budke and Goffinet 2016). They range from the minute *Physcomitrium patens* (Hedw.) Mitt. that has a small sporophyte (~1 mm tall) with a tiny calyptra (0.2 mm) to the relatively large *Funaria hygrometrica* Hedw. with a sporophyte that can range from 20-50 mm tall and a calyptra from 3-5 mm tall (McIntosh 2007; Budke and Goffinet 2016). Differences in sporophyte height and calyptra size may also influence sporophyte transpiration. Species with short sporophytes are protected by the still air present in the laminar boundary layer, which results in lower levels of evaporation of water from the plant to the atmosphere (Proctor 1982; Rice and Schneider 2004). Thus species with shorter sporophytes are expected to experience lower levels of transpiration compared to those with taller sporophytes that extend beyond the protective zone of the boundary layer.

The aim of this study was to examine the influence of the calyptra on moss sporophyte transpiration rates under controlled and constant laboratory conditions. 1) We predicted that sporophytes with their calyptra removed will have a higher rate of transpiration compared to sporophytes with their calyptra present due to the removal of the dehydration protection provided by the calyptra; 2) we also predicted that moss species with taller sporophytes will have higher transpiration rates compared to species with shorter sporophytes because they lack the protective influence of the laminar boundary layer.

MATERIALS AND METHODS

Study Taxa

Funaria hygrometrica, the cord moss, is a cosmopolitan species that has relatively tall sporophytes that average 35 mm in height with calyptrae that are 3-5 mm tall, whereas *Physcomitrium pyriforme*, the goblet moss, has smaller sporophytes that average 15 mm tall with calyptrae that are 2-3 mm (McIntosh 2007; Budke and Goffinet 2016). These species are both in the Funariaceae and share a similar calyptra morphology with an inflated base topped by a narrow rostrum (Fig. 1A). Unlike the minute sporophytes and calyptra of *P. patens*, these two species are large enough for easy experimental manipulations, such as calyptra removal, during early

developmental stages. Spores collected from *F. hygrometrica* (*Budke 144*, CONN) and *P. pyriforme* (*Goffinet 9276*, CONN) were cultured in the laboratory for this study.

Growth Conditions

Leafy gametophytes of these two species were grown from spores on culture media in petri dishes. Protonema were ground using a mortar and pestle and then added to a rich, sandy-loam soil mix in PlantCon plant tissue culture containers (Fisher Scientific, Pittsburgh, Pennsylvania) with a single 5×5 mm opening cut in the top of each container to increase airflow. These cultures were then placed on a light cart at room temperature for a minimum of four months under ~60 μmol m^{-2} s^{-1} of light for 16 h light : 8 h dark. The leafy gametophytes were cold treated at 10°C under similar light levels with 8 h light : 16 h dark for two months (Percival Scientific GR-36VL, Perry, Iowa). This cold treatment simulated fall growing conditions and initiated gametangia formation with antheridia produced after approximately one month and archegonia after two months. Once gametangia developed, each population was flooded with deionized water for 24 h and then drained in order to stimulate fertilization. The population was then placed back into the original room temperature growing conditions to facilitate sporophyte development. The methods described here are modified from Budke et al. (2011).

Experimental Design

Immature, spear-shaped sporophytes of *Funaria hygrometrica* and *Physcomitrium pyriforme* sporophytes (developmental stages 3 and 4 as in Fig. 4C, D in Budke et al. 2012) were used for this study (Fig. 1A). When a majority of the sporophytes in a culture container reached these developmental stages, the population of mosses was moved to the laboratory from the light cart to begin the experiment. Forceps were used to remove an individual gametophyte with its attached sporophyte to avoid damaging the seta. The moss was then placed on a dry glass slide and a scalpel was used to cut the sporophyte directly above the vaginula, using a dissecting microscope to remove the sporophyte foot and gametophyte below. Sporophytes were then randomly assigned one of two treatments. The calyptra was left undisturbed on the apex (Fig. 1B) or was removed from the sporophyte apex (Fig. 1C). Forceps were then used to gently place the sporophyte into a 100 μL plastic tube with an internal depth of 15 mm containing 10 μL of a 10% methylene blue solution in water. The sporophyte leaned against the inside of the tube to maintain a vertical orientation. The tube containing the dye and sporophyte was then transferred into a growth chamber (Percival Scientific CU-22L) with internal conditions set to a constant temperature of 22°C, 55% relative humidity (RH), and 22.8 μmol m^{-2} s^{-1} of light and the sporophyte was allowed to transpire for a set period of time (as described below) based on preliminary trials. After the transpiration period, the growth chamber was opened, forceps were used to remove the sporophyte from the tube, and photos were taken immediately to record the dye movement. ImageJ (Schneider et al. 2012) was used to measure sporophyte height, sporophyte diameter, and the distance the dye moved up through the sporophyte from the images. The dye moved through the central strand of the sporophyte first (Fig. 1D, E) and then permeated the rest of the seta tissue (Fig. 1F).

Based on preliminary tests of crystal violet, methylene blue, and sudan II dye solutions, methylene blue was found to be the clearest to visualize the movement of the dye through the sporophyte. Preliminary trials were used to determine the average time for the methylene blue solution to reach the middle of the sporophyte of each species, both with the calyptra present (Fig.

1B) and removed from the apex (Fig. 1C). Thus the following times were used for each experimental treatment: 5 min for *Funaria hygrometrica* with calyptra present, 2 min for *F. hygrometrica* calyptra removed, 3 min *Physcomitrium pyriforme* with calyptra present, 1 min *P. pyriforme* calyptra removed. Height, diameter, and rate of dye movement up through the central strand (transpiration rate) of the sporophyte were measured for both species.

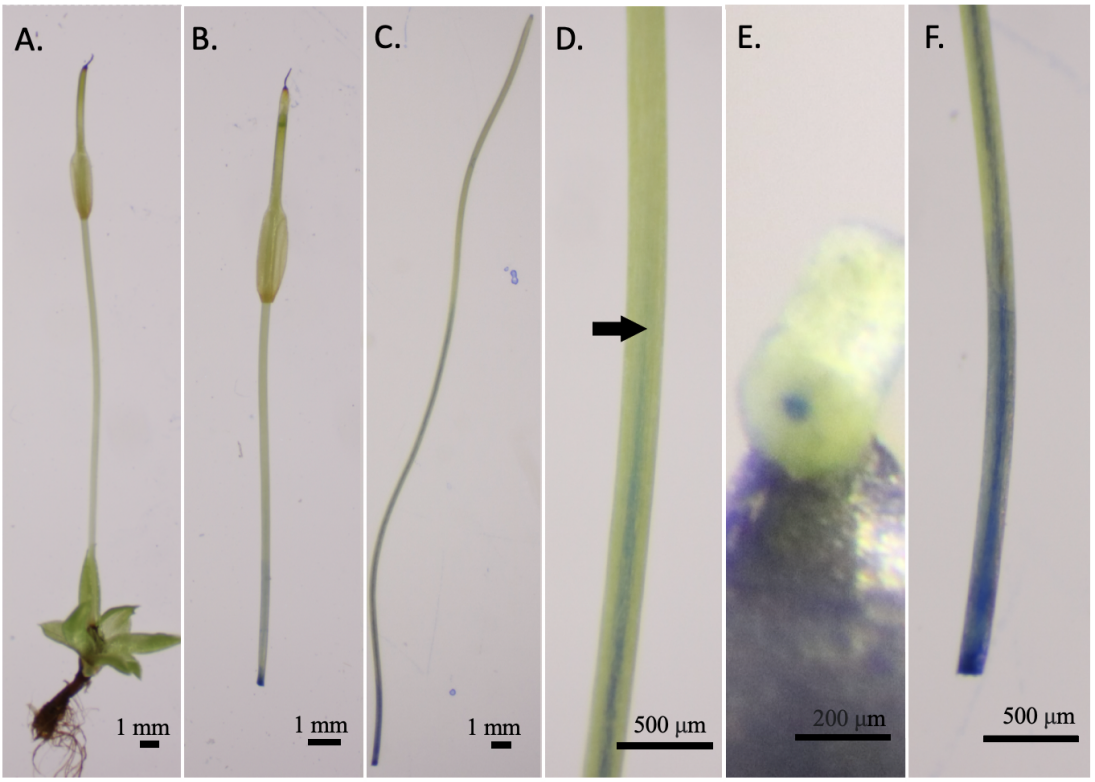

Figure 1. Sporophytes of *Funaria hygrometrica* at the spear-shaped stage of development that were used for this experiment (stages 3-4 as in Budke et al. 2012). **(A-C)** Each sporophyte is a unique individual. **(A)** Sporophyte with the calyptra on the apex and the leafy gametophyte attached. **(B)** Sporophyte with the leafy gametophyte removed. **(C)** Sporophyte with both the leafy gametophyte and calyptra removed. **(D-F)** Sporophyte images are all of the same individual. **(D)** Magnification of the sporophyte in C with an arrow indicating the height of dye movement through the water conducting cells of the central strand. **(E)** Transverse section of the sporophyte seta on the tip of a metal forcep showing the dye movement through the central strand. **(F)** Magnification of the sporophyte base of C showing the dye moving strongly through the central strand of the sporophyte and then spreading laterally into the nearby cells of the sporophyte seta.

Statistical Analyses

Generalized linear mixed models were used to assess the influence of the following parameters on the response variable, transpiration rate. The full model included *treatment* (whether the calyptra was present or removed) and *species* as the primary fixed effects, the covariates of *sporophyte height* and *sporophyte diameter* as additional fixed effects, and experiment date as a

random effect. Starting with a full model, which included all of the variables outlined above, we then ran a stepwise reduced regression model to determine which effects significantly influenced

the response variable of transpiration rate. The analysis was performed using R 3.0.2 (R Core Team 2020) and RStudio (RStudio Team 2020).

RESULTS

Sample sizes were $N = 34$ for *Funaria hygrometrica* with calyptra present, $N = 36$ for *F. hygrometrica* with calyptra removed, $N = 25$ for *Physcomitrium pyriforme* with calyptra present, and $N = 25$ for *P. pyriforme* with calyptra removed. All sporophytes included in the experiment were at an immature, spear-shaped stage of development as outlined above and shown in Fig. 1A, but varied in height at the time of the experiment. The *F. hygrometrica* sporophytes were 10 to 33 mm tall and *P. pyriforme* sporophytes were 10 to 22 mm tall (Fig. 2).

The stepwise reduced regression model determined that both experiment date and sporophyte diameter ($P = 0.15$) did not have a significant effect on the response variable transpiration rate and thus were removed from the final reduced model. Thus the final reduced model included treatment, species, and sporophyte height as fixed effects. A significant effect of treatment was found; when the calyptra was removed, there were higher rates of sporophyte transpiration compared to individuals with the calyptra present (ANOVA: $F_{1, 109} = 414.84$, $P < 0.0001$; Fig. 2). On average, the transpiration rate was 2.65× higher for sporophytes with their calyptra removed (Table 1). Species had a nearly significant effect on the transpiration rate with sporophytes of *Physcomitrium pyriforme* having a slightly higher rate compared to *Funaria hygrometrica* (ANOVA: $F_{1, 3} = 8.31$, $P = 0.057$; Fig. 2; Table 1). Additionally, a significant interaction was found between treatment and species on the transpiration rates (ANOVA: $F_{1, 109} = 13.21$, $P < 0.0001$). The covariate sporophyte height also had a significant effect on the rate of transpiration (ANOVA: $F_{1, 111} = 97.55$, $P < 0.0001$; Fig. 2) and thus the transpiration rates were standardized by sporophyte height for comparison (Table 1). Even after standardizing for sporophyte height, sporophytes with their calyptra removed had a higher transpiration rate compared to those with their calyptra present, and the rates were higher for *P. pyriforme* compared to *F. hygrometrica* (Table 1).

In order to further compare the transpiration rates of *Funaria hygrometrica* and *Physcomitrium pyriforme*, the slope of the regression lines was determined (Fig. 2). In *F. hygrometrica* with calyptra present on the apex, each centimeter increase in sporophyte height corresponded to a 0.14 increase in the transpiration rate (mm per min), whereas with calyptra removed the transpiration rate increased by 0.21 mm/min for each cm increase in sporophyte height. In *P. pyriforme* with calyptra present, a one cm increase in sporophyte height corresponded to a 0.19 mm/min increase in the rate of transpiration, whereas with the calyptra removed, the transpiration rate increased to 0.45 mm/min for each cm increase in sporophyte height.

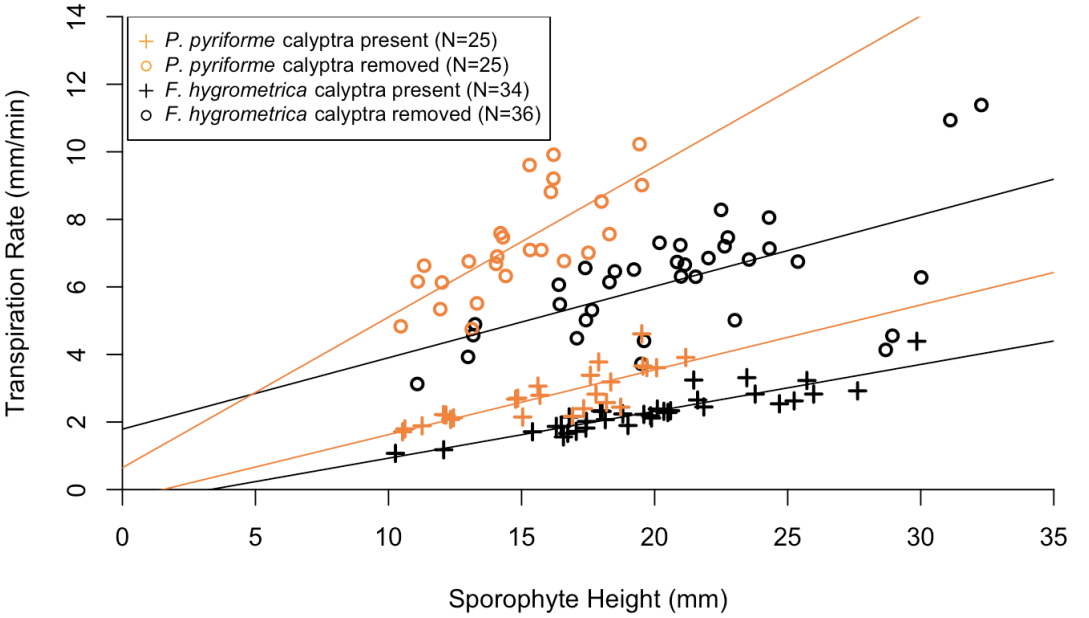

Figure 2. Transpiration rate (mm/min) plotted by sporophyte height (mm) for *Funaria hygrometrica* and *Physcomitrium pyriforme* sporophytes with either the calyptra present or removed. Transpiration rate was determined by measuring the distance a dye moved up through the central strand of the seta during a set period of time. Lines are from a basic linear regression.

Table 1. Average transpiration rate (mm/min) and average rate standardized for sporophyte height [(mm/min)/mm] for *Funaria hygrometrica* and *Physcomitrium pyriforme* sporophytes with either the calyptra present or removed.

		Transpiration Rate (mm/min)	Transpiration Rate Standardized for Sporophyte Height (mm/min)/mm
Funaria hygrometrica	Calyptra Present ($N = 34$)	2.30	0.12
	Calyptra Removed ($N = 36$)	6.18	0.29
Physcomitrium pyriforme	Calyptra Present ($N = 25$)	2.78	0.17
	Calyptra Removed ($N = 25$)	7.28	0.49

DISCUSSION

These results provide additional evidence supporting the influence of the calyptra on sporophyte transpiration in mosses. This influence may play an important role in the parent-offspring conflict by limiting the resources the offspring sporophyte is able to take from the maternal gametophyte during its development. We predicted that 1) sporophytes with their calyptra removed would have a higher rate of transpiration compared to sporophytes with their calyptra present due to the lack of dehydration protection provided by the calyptra and 2) that moss species with taller sporophytes would have higher transpiration rates compared to species with shorter sporophytes since they lack the protective influence of the laminar boundary layer.

Our first prediction was supported by the data presented above. The average transpiration rate (mm/min) with the calyptra removed for both species combined was 2.65× faster compared to those with the calyptra present (Table 1). This increase in transpiration rate was higher than the earlier study where Bopp and Stehle (1957) found a 1.3× faster transpiration rate in sporophytes of *Funaria hygrometrica* with the calyptra removed compared to those with the calyptra present on the apex. Unfortunately, that study did not report the experimental conditions and thus differences in light, temperature, and/or humidity during the two experiments may be driving the differences between their results and ours. However, both studies demonstrate that the calyptra decreases sporophyte transpiration rates, which can be attributed to a decrease in water loss from the sporophyte apex (Budke et al. 2013). This decrease in transpiration has the potential to also decrease the ability of the offspring sporophyte to pull resources from the maternal gametophyte (Haig 2013). Therefore, evolutionary changes in calyptra morphology that increase cuticle thickness, number/length of calyptra hairs, or calyptra size could be predicted to have a negative influence on the offspring sporophyte's ability to acquire resources from the maternal plant, thus enabling the maternal plant to retain more resources for its own survival and/or future reproduction. In this way the gametophyte calyptra may play a critical role in parent-offspring conflict over resources in mosses.

Our second prediction, that moss species with taller sporophytes (*Funaria hygrometrica*) would have higher transpiration rates compared to species with shorter sporophytes (*Physcomitrium pyriforme*), was not supported by our results. In our reduced model, the interaction between the fixed effects of species and treatment had a significant influence on the transpiration rate, however, the species were reversed relative to our prediction; sporophytes of *F. hygrometrica* (Fig. 2 in black) had lower transpiration rates compared to the sporophytes of *P. pyriforme* (Fig. 2 in orange) for individuals both with their calyptra present and removed. Our prediction was based on the idea that species with taller sporophytes extend higher above the still air of the surface boundary layer (Rice et al. 2001), resulting in higher transpiration rates. On average the sporophytes *F. hygrometrica* included in this study (20.4 mm, $N = 70$), were taller than those of *P. pyriforme* (15.4 mm, $N = 50$), however, there was broad overlap in sporophyte height between the two species (Fig. 2). Thus differences between these species are likely to be driven by factors other than sporophyte height. The difference in transpiration rates between the species also cannot be explained by sporophyte diameter, since this parameter was not significant and was removed from our reduced model. An alternative and untested explanation for the species-level differences in transpiration rates are differences in the number and/or diameter of water conducting cells located in the central strand of the seta (Hébant 1977). Ongoing research in our laboratory is exploring the water conducting cells

of these two species to see whether anatomical differences may explain the differences in transpiration rates observed in this study for sporophytes of similar heights (Fig. 2).

CONCLUSIONS

Moss calyptrae are an important and understudied plant structure (Budke 2019). They protect the immature sporophyte from desiccation and positively influence sporophyte survival, development, and fitness (Budke et al. 2013). This study adds support to the earlier results of Bopp and Stehle (1957) who demonstrated that moss sporophyte transpiration is faster when gametophyte calyptrae are removed from the immature sporophyte apex. Our comparative approach, examining both *Funaria hygrometrica* and *Physcomitrium pyriforme*, demonstrates species-level differences in transpiration rate that could be due to differences in water conducting cell anatomy. Exploring structure-function relationships of calyptra comparatively across phylogenetically diverse species, will enable us to continue to broaden our understanding of maternal effects and the parent-offspring conflict in mosses.

ACKNOWLEDGEMENTS

We would like to thank Mark Wienhold for help with preliminary trials of this research, Brian Stevens and Jim Fordyce for helpful discussions and assistance with statistical analyses, and members of the Budke laboratory group, and an anonymous reviewer, for feedback on an earlier version of this manuscript.

LITERATURE CITED

Bopp, M. and E. Stehle. 1957. Zur Frage der Wasserleitung im Gametophyten und Sporophyten der Laubmoose. Zeitschrift für Botanik 45: 161–174.

Budke, J.M. 2019. The moss calyptra: A maternal structure influencing offspring development. The Bryologist 122: 471–491.

Budke, J.M. and 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 and 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.

Budke, J.M., B. Goffinet and C.S. Jones. 2012. The cuticle on the gametophyte calyptra matures before the sporophyte cuticle in the moss *Funaria hygrometrica* (Funariaceae). American Journal of Botany 99: 14–22.

Budke, J.M., B. Goffinet and C.S. Jones. 2013. Dehydration protection provided by a maternal cuticle improves offspring fitness in the moss *Funaria hygrometrica*. Annals of Botany 111: 781–789.

Browning, A.J. and B.E.S. Gunning. 1979. Structure and function of transfer cells in the sporophyte haustorium of *Funaria hygrometrica* Hedw: III. Translocation of assimilate into the attached sporophyte and along the seta of attached and excised sporophytes. Journal of Experimental Botany 30: 1265–1273.

Graham, L. E. 1993. Origin of land plants. New York: John Wiley and Sons, Inc.

Haig, D. and A. Wilczek. 2006. Sexual conflict and the alternation of haploid and diploid generations. Philosophical Transactions of the Royal Society B 361: 335–343.

Haig, D. 2013. Filial mistletoes: the functional morphology of moss sporophytes. Annals of Botany 111: 337–345.

Hébant, C. 1977. The conducting tissues of bryophytes. Vaduz, Liechtenstein: J. Cramer.

Kenrick, P. and P. Crane. 1997. The origin and early evolution of plants on land. Nature 389: 33–39.

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

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, Bryophytes: Mosses, Part 1, Vol. 27. New York: Oxford University Press.

Proctor, M.C.F. 1982. Physiological ecology: water relations, light and temperature responses, carbon balance, pp. 333–381. *In*: A.J.E. Smith (ed.), Bryophyte Ecology. London: Chapman and Hall.

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

R Core Team. 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Rice, S.K., D. Collins and A.M. Anderson. 2001. Functional significance of variation in bryophyte canopy structure. American Journal of Botany 88: 1568–1576.

Rice, S.K. and N. Schneider. 2004. Cushion size, surface roughness, and the control of water balance and carbon flux in the cushion moss *Leucobryum glaucum* (Leucobryaceae). American Journal of Botany 91: 1164–1172.

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