How to dry a bryophyte: A review and experimental test of four methods to induce desiccation tolerance

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Abstract. A review of ∼290 research articles on bryophyte desiccation tolerance (DT) over the last century reveals four prominent methods that incorporate equilibrium drying. We compare these methods (called *Conventional*, *Wetted substrate*, *Step-down* and *Partial drying*) in terms of inducing the trait DT in four species of mosses occupying distinctly different evolutionary clades and known to exhibit an inducible strategy of DT (*Phascum cuspidatum*, *Funaria hygrometrica*, *Bryum argenteum* and *Syntrichia obtusissima*). *Conventional*=plants placed directly at 33% RH; *Wetted substrate*=plants dried at different rates (times) to 33% RH by wetting the substrate; *Step-down*=plants dried to equilibration in sequence from 100, 75, 54, then 33% RH; *Partial drying*=plants exposed to 100% RH prior to placement at 33% RH. Efficacy of each method was evaluated using postrehydration damage and recovery as assessed from chlorophyll fluorescence and leaf or tissue damage 7 days postrehydration. For each chlorophyll fluorescence measure, there was a significant three-way interaction between species, drying method and time. Three of the four methods produced good recovery after 24 h of rehydration, with the plants subjected to the *Conventional* method not recovering from desiccation. Photosynthetic damage immediately upon rehydration was reduced for the *Partial dry* method and similar for the *Step-down* and *Wetted substrate* methods. Tissue damage 7 d postrehydration was equivalent for the *Wetted substrate*, *Step-down* and *Partial dry* methods, and most plants died following the *Conventional* method of drying. Recovery following 24 h of rehydration was near control levels for all methods but the *Conventional*. Species differences were considerable among drying methods. The *Subturgor Hypothesis* is advanced to explain degree of induction of desiccation tolerance, and was generally supported, with the caveat that, among the three most successful methods tested, the *Step-down* method performed better than expected based upon time at subturgor.

Keywords. Equilibrating relative humidity, constitutive, inducible, suprasaturation, chlorophyll fluorescence.

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What is desiccation tolerance? The trait desiccation tolerance (DT, also desiccation tolerant) in plant biology is generally conceived as the ability of a plant to tolerate air-dryness (Alpert 2005), i.e., where the water content (WC) of the individual plant equilibrates with atmospheric conditions (lab or field). If the plant can revive and resume metabolic functions after such an event, it is considered as DT (if not it is desiccation-sensitive). Depending on how restrictive the view, DT can be quantitatively defined in two ways based upon the dry weight WC of the organism upon equilibration with the desiccating atmosphere: (1) ability to tolerate $\leq\sim$10% WC (more restrictive), or (2) ability to tolerate $\sim\leq$30% WC (less restrictive) (Wood 2007). Each definition has merits. A 10% WC (or slightly higher) based on dry weight corresponds to the interconversion point of cytoplasm from amorphous gel to a biological glass (Oliver et al. 2020, –80 to –100 MPa), and correlates well to an equilibrating relative humidity (*RHeq*) of

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$\sim$50% at 20°C (Fernandez-Marin et al. 2013; Hatanaka & Sugawara 2010). A 20–30% WC corresponds with an absence of bulk cytoplasmic water that shuts down most metabolic processes. The latter WC correlates to an *RHeq* of $\sim$75–85% (Alpert & Oechel 1987; Hoekstra et al. 2001), at which point leaf curling occurs in several species of mosses (Schonbeck & Bewley 1981; Stark 2017). Dehydration tolerance can be distinguished from desiccation tolerance by restricting the former to non-equilibrated exposure to RHs $>\sim$85% (Marks et al. 2019).

Factors, timeline and strategies of desiccation tolerance. What are the chief conditions attending the dry/wet/dry cycle that carry the most influence on plant fitness (as survival, growth and reproduction)? These conditions, termed "factors" of DT, may be numerous, were derived over decades (e.g., Glime 2017; Green et al. 2011; Norris 1990; Schonbeck & Bewley 1981), and were recently codified (Stark 2017) into four principal factors. Briefly, the factors of DT consist, in a timeline, of the rate of drying (*RoD*), the equilibrating relative humidity (*RHeq*, also known as the WC at equilibration, or *WCeq*), the duration of the dry period (*DD*), and the rate of rehydration (*RoR*). How a plant copes with the repetitive stress of drying and rehydration describes the evolutionary strategy of the species. Although two strategies are recognized (constitutive and inducible; CDT and IDT), most bryophytes incorporate responses that may vary with life phase (Hájek & Vicherova 2014; Stark et al. 2016) and often blend the two strategies into points that vary along a gradient of inducibility (Bu et al. 2017; Coe et al. 2021; Hatanaka & Sugawara 2007; Hellwege et al. 1994; Liu et al. 2019; McLetchie & Stark 2019; Stark & Brinda 2015).

Need for a common method of drying. Studies incorporating DT in bryophytes number in the hundreds, with at least 175 bryophyte species assessed for DT (Marks et al. 2021). Potential applications deriving from this body of research are also numerous, including among others, evaluating the genetic induction of the trait for eventual use in crop science and drought tolerance; deriving the strategy of DT among species and life phases; elaborating the physiology of inductive and constitutive protection; evaluating potential tradeoffs between the trait of DT and fitness; and analysis in functional trait-based phylogenetic studies. At present there is no consensus among researchers regarding a particular method of drying that is most likely to detect the ecophysiological signal of desiccation; this signal will doubtless be manifested in any genetic program conferring protection from desiccation (Marks et al. 2021). Our goal here is to review current methods in the field of DT, distill the literature into four testable methods, and subject these methods to experimental test in four DT species that are culturable, are fairly widely studied, and which represent at least three distinct bryophyte clades. The preferred method would be one that most efficiently captures the ecophysiological signal of desiccation, as assessed through postrehydration assays.

Literature review of methods employed. A total of 289 published studies (most of recent origin) that incorporated a bryophyte desiccation component was sorted based on how desiccating RH was treated. Six roughly defined methods emerged, separated chiefly by the use of single vs. multiple desiccating RHs in field or laboratory situations (**Table 1**). A more detailed table with references, species of study, brief notes, and the DT factors assessed is found in **Supplementary Table S1**. This initial look revealed six qualitatively different methods: (1) air-dry with *RHeq* not specified; (2) air-dry with *RHeq* specified or controlled; (3) drying to a single *RHeq*; (4) drying to multiple *RHeqs* to approximate different *RoDs*; (5) drying using single or multiple *RHeqs* and explicitly varying the *RoD*; and (6) incorporating a series of declining *RHeqs* within a Step-down approach. These descriptive groupings, when restricted to methods that can be experimentally tested and compared in a laboratory setting, reduce to the last four methods above. After combining areas of overlap from the latter 4 methods, and adding a method commonly used as a pretreatment in various methods, the literature on bryophyte DT reduces to the use of four predominant methods of drying (**Table 2**).

The logic employed in deriving four predominant methods of drying begins with a recognition that equilibrium drying is the basic method of experimentally desiccating bryophytes. Because of their poikilohydric condition, bryophytes naturally equilibrate their tissue water content to that of the surrounding atmosphere. Thus, the choice the researcher makes is how to get from plants at full

Table 1. Classification of bryophyte drying methods based on 289 studies incorporating desiccation, based on field and lab reports, for ~860 species, based on the type of equilibrium drying approach used. A few (<10) publications are listed for multiple methods. A more detailed table including references, species of study, relative humidities (RHs) tested, and additional details is presented in **Supplementary Table 1**; the total of 282 publications represents some cross-listed studies.

Method	Lab or Field	Number of Publications (Number of species)
Air-dry, RH not specified	Lab, Field	66 (156)
Air-dry, RH specified	Lab, Field	45 (140)
Single desiccating RH	Lab	69 (152)
Multiple desiccating RHs	Lab	75 (362)
Wetted substrate with 1+ desiccating RHs	Lab	14 (20)
Declining series of desiccating RHs (Step-down)	Lab	20 (33)

turgor to the target equilibrating RH. Direct equilibrium drying encompasses methods (3) and (4) above, and is the most prevalent method in use (*Conventional* method). Extending the drying time before equilibrium is reached by supplying a moist, quantifiable substrate encompasses method (5) above (*Wetted substrate* method). Incorporating a "stepped" series of declining equilibrium drying RHs includes elements of methods (3), (4), and (6) above (*Step-down* method). Finally, equilibrating plants to a saturated atmosphere prior to drying to a lower *RHeq* also encompasses elements of methods (3), (4), and (6) above but without a "stepped" decline in *RHeq* (*Partial drying* method). The methods above may be combined in various structures of methodology; here we focus on a comparison of individual methods.

The *Conventional* method is easily the most common methodology used in studies of bryophyte DT, and dates to its widespread adoption in the 1970s and 1980s (e.g., Bewley & Pacey 1978; Bewley et al. 1978), and is in wide use today (e.g., Yuquing et al. 2020). It has been called "equilibrium drying" (e.g., Xiao et al. 2018) for the direct placement of plants in desiccators targeting a specific *RHeq*. Because (1) all four methods addressed here reach a plant equilibrium WC and (2) the plant trait DT is evaluated by plant responses upon rehydration from drying to equilibrium with surrounding air, we consider "equilibrium drying" as an important element in all four methods tested here. The *Conventional* method purports to compare (usually) two rates of drying, with a rapid-dry (RD) event occurring at a lower *RHeq* (e.g., 0% using silica gel) and a slow-dry (SD) event occurring at a higher *RHeq* (e.g., 65% over a saturated solution of NH_4NO_3). This method assesses the "intensity" of DT because it combines *RoD* and *RHeq*, and is unable to achieve *RoDs* beyond about 12 h from full turgor to leaf curling (Bewley 1995); this latter weakness means that inductive processes may not have sufficient time to develop, even with what was described as a "very slow-dry" (Bewley 1995; the exceptions being at very high *RHeqs*, Koster et al. 2010). Because using multiple RHs tends to couple equilibrium plant WC with *RoD*, we refer to the *Conventional* method as targeting a single RH (with a single plant WC). Because the *RoD* is based on the *RHeq* and because *RHeq* effects are not controlled, the *Conventional* approach unintentionally conflates *RoD* and *RHeq* resulting in lower plant WC at the lower equilibrating RH, and so the data from such experiments cannot be construed as reflecting differences in *RoD* alone (reviewed in Stark 2017). Nevertheless, the *Conventional* method of drying is an integral part of two of the next three methods of drying.

The *Wetted substrate* technique also evolved as a way to incorporate multiple rates of drying (Cui et al. 2011; Lienard et al. 2008; Pressel & Duckett 2010; Stark et al. 2013; Werner et al. 1991) but at the same *RHeq*, allowing *RoD* to be unlinked to *RHeq* in experiments. The first method to accomplish this separation involved a wind tunnel (Penny & Bay-

Table 2. Four prevalent methods in the literature of drying bryophytes for study of desiccation tolerance in the laboratory. *RHeq* = equilibrating relative humidity, *RoD* = rate of drying.

Method	Prevalence	Summary
Conventional	common	plants subjected directly to one or more *RHeqs*
Wetted Substrate	infrequent	plants dried upon a wetted substrate at >1 *RoD* to one or more *RHeqs*
Step-Down	infrequent	plants dried at higher *RHeqs* and then moved to lower *RHeqs*
Partial Drying	rare	plants partially dried in near-saturated atmosphere then moved to lower *RHeq*

field 1982), which then yielded to a dry or wetted substrate. Thus, it is possible to dry rapidly or slowly at very low or very high RHs (e.g., McLetchie & Stark 2019). This method capitalizes on the more simulative (to nature) drying design of placing hydrated plants on a moist substrate that gradually (or rapidly) loses its water to the environment. Such a method creates a RH near the plants of ~100% while plants are inside a desiccator targeting much lower RHs, a condition that probably has relevance during induction (Cui et al. 2011). Even during tissue dehydration, the subturgor state is lengthened in direct proportion to the amount of moisture in the substrate (and the plants do not have access to free water in the substrate). Therefore, the experimental moisture level in the substrate determines the rate of water loss from the plants (Lienard et al. 2008). This method is similar to the *Partial drying* method described below, with the difference being that in the *Wetted substrate* method WC declines during a prolonged period at 100% RH, whereas during *Partial drying* an equilibrated WC is reached quickly.

The *Step-down* technique originated in an abbreviated form for bryophytes near the same time and from the same lab as the *Conventional* method (Bewley 1974; Gwóźdź et al. 1974; Penny & Bayfield 1982), and slows down water loss by first placing the plants at a higher RH and then moving them, after equilibration, to a lower RH. As with the two previous methods above, this method allows study of different rates of drying (which may or may not conflate *RoD* with *RHeq* depending upon the design). Rather than taking shoots directly from full turgor to 33% RH, the shoots are taken from full turgor to, e.g., 95% RH, allowed to equilibrate, and then moved to a lower humidity, allowed to equilibrate, and so forth until the target of 33% RH is reached and equilibrated. In an expanded design, the *Step-down* approach can include a range of *RHeqs* (e.g., Schonbeck & Bewley 1981), although early techniques such as the latter did not intend to equilibrate WC at each stepped-down RH, but rather intended to slow water loss from plants (this may be an important distinction). As plants lose water from tissues during the *Step-down* process, at some point metabolic processes become impossible (crossing 85% *RHeq*, or at ~30% WC, Hoekstra et al. 2001); theoretically the time when shoots are metabolically active is longer than in the *Conventional* approach, but likely shorter than allowed in the *Wetted substrate* approach. Interestingly, Schonbeck & Bewley (1981) found that the rate of water loss is proportional to postrehydration injury up until ~30% WC (*RHeq*=~85%). At this WC leaves of *Syntrichia ruralis* begin to curl inward (desiccation morphology), and the rate of water loss from this WC to lower target WCs does not have biological effects on recovery; clearly this should be confirmed.

The *Partial drying* technique perhaps arose from the recognition that the physiological signal for DT occurs when plants are in a subturgor state (Abel 1956; Brown & Buck 1979; Lee & Stewart 1971). Beckett and his associates (Beckett 1999, 2001; Beckett et al. 2005; Marschall & Beckett 2005) held plants at subturgor for different times by exposing plants blotted to full turgor to an atmosphere of 100% RH, achieved by suspending plants in the head space over distilled water in a sealed environment. In a matter of hours, the plants initiate a "desiccation morphology" such as slight (but not full) leaf curling. This method, which resulted in a suspended condition of partial desiccation (subturgor and without subsequent water loss), found good evidence of hardening to DT in *Atrichum*. Specifically, protein synthesis critical to hardening to DT occurred during the partial dry event (Beckett 1999). This hardening was enhanced under lighted conditions during ABA tests (Beckett 2001). While a 3-day exposure to 100% RH maximized hardening to DT in *Atrichum* (Beckett 1999), with species of *Sphagnum* a longer period of partial drying (7 days) served this function (Hájek & Vicherová 2014). Notably, this method yielded mixed results in producing increases in sucrose synthesis among six species of mosses tested using a 24 h period at 100% RH (Smirnoff 1992). This method differs from the *Wetted substrate* method by achieving an equilibrium WC at subturgor; i.e., the plant weight does not change and there is no water loss once subturgor is reached (at *RHeq*=100%). Slight variations in WC using this method should be tested over various durations at or near 100% RH. *Partial drying* produces the longest time at subturgor, and if time at subturgor is crucial to hardening and subsequent survival (as appears to be the case), then this technique is predicted to correlate best to ecophysiological signals of DT. Recently this method was employed in *Physcomitrium patens*, after ~one wk at

Table 3. Four species of mosses used in assessments of methods of drying. Single clones were derived from each collection and cultured to adult shoots and/or protonema.

Species	Family	Origin of genotype
Bryum argenteum Hedw.	Bryaceae	Fayette County, Kentucky, USA, Nicholas McLetchie 2009
Funaria hygrometrica Hedw.	Funariaceae	Tulare County, California, USA, Llo Stark 2017
Phascum cuspidatum Schreb. ex Hedw.	Pottiaceae	Lake County, California, USA, John Brinda 3486
Syntrichia obtusissima (Müll. Hal.) R.H. Zander	Pottiaceae	Culberson County, Texas, USA, John Brinda 8306

89% RH, to induce ABA production and good recovery from desiccation, with some initial damage, following exposure to equilibration with 33% RH (Rathnayake et al. 2019).

Evaluating the methods. The criteria used to compare these four methods of drying in bryophytes center on an efficient detection of the ecophysiological signal of inducible desiccation tolerance (IDT). That is, how strong is this signal as judged by the ability to recover from the current desiccating event (acclimation). Signal strength will depend on the effectiveness of the method to confer protective changes in the plants during exposure to four days of partial or complete desiccation to equilibration with a relative humidity (RH) of 33%. These will be evaluated through postrehydration chlorophyll fluorescence and visual estimates of tissue damage. Ideally, evaluating these methods should also employ a priming response: tolerating a future desiccating event. However, preliminary data from our pilot experimental trials varied widely in this regard and await further methodological refinements.

Hypotheses. Our null hypothesis is that the four methods of drying should produce similar responses to acclimation, i.e., result in similar signals of hardening to desiccation tolerance. Because the physiological signals to hardening are likely to occur while plants are in a subturgor state, our secondary hypothesis is the method which extends subturgor time the furthest (*Partial drying* technique) will result in the strongest signals of inducibility to DT.

MATERIALS AND METHODS

Species and culturing protocol. Shoots of four species (collection localities given in **Table 3**) were placed into culture in plastic Petri dishes (inner diam. 35 mm) on pH-neutral, dry-autoclaved (121°C for 60 min), locally (NV) collected, sieved (500 μm) fine sand and allowed to regenerate in a growth chamber in a 12-h photoperiod (20°C lighted, 8°C darkened) at ~90 μmol m^{-2} s^{-1} PAR (photosynthetically active radiation). After several weeks, the plants were subcultured in order to produce a single clonal line free of visible microorganism contaminants (**Fig. 1A**). Several such subcultures were necessary. Plants used for the experiments had been through at least three regeneration cycles in culture prior to use (shoot to regenerated shoot), and were 3–6 mos. old (shoots) or 1 mo. old (protonema). Cultures were watered every few days, alternating sterile distilled water with a 30% inorganic nutrient solution (Hoagland & Arnon 1938) as needed in order to maintain a suprasaturated condition among the shoots, i.e., visible water present on the shoot apices or protonema at all times. Pre-experimental trials were conducted to ensure that the species behaved in an inducible ecological manner when dried to equilibration with 33% RH, i.e., exhibiting at least partial tolerance to desiccation when dried slowly and considerable damage when dried rapidly.

Experimental design. The four methods of drying bryophytes (*Conventional*, *Wetted substrate*, *Step-down*, and *Partial drying*; **Table 2**) were compared by drying shoots or protonemata from full turgor to a common target *RHeq*=33% over a common timeline of 4 days, with the last day spent equilibrating at 33% RH. All treatments therefore experienced the same time in a desiccated or partially desiccated state.

Water content. Shoot or protonemal mat water content (WC) on a dry weight basis (N=3) was determined for each *RHeq* by removing a group of 5–20 vegetative (nonsporophytic) shoots, each ~3–5 mm in length cut near the substrate surface, or with protonemal mats a section of ~3 × 6 mm, blotting

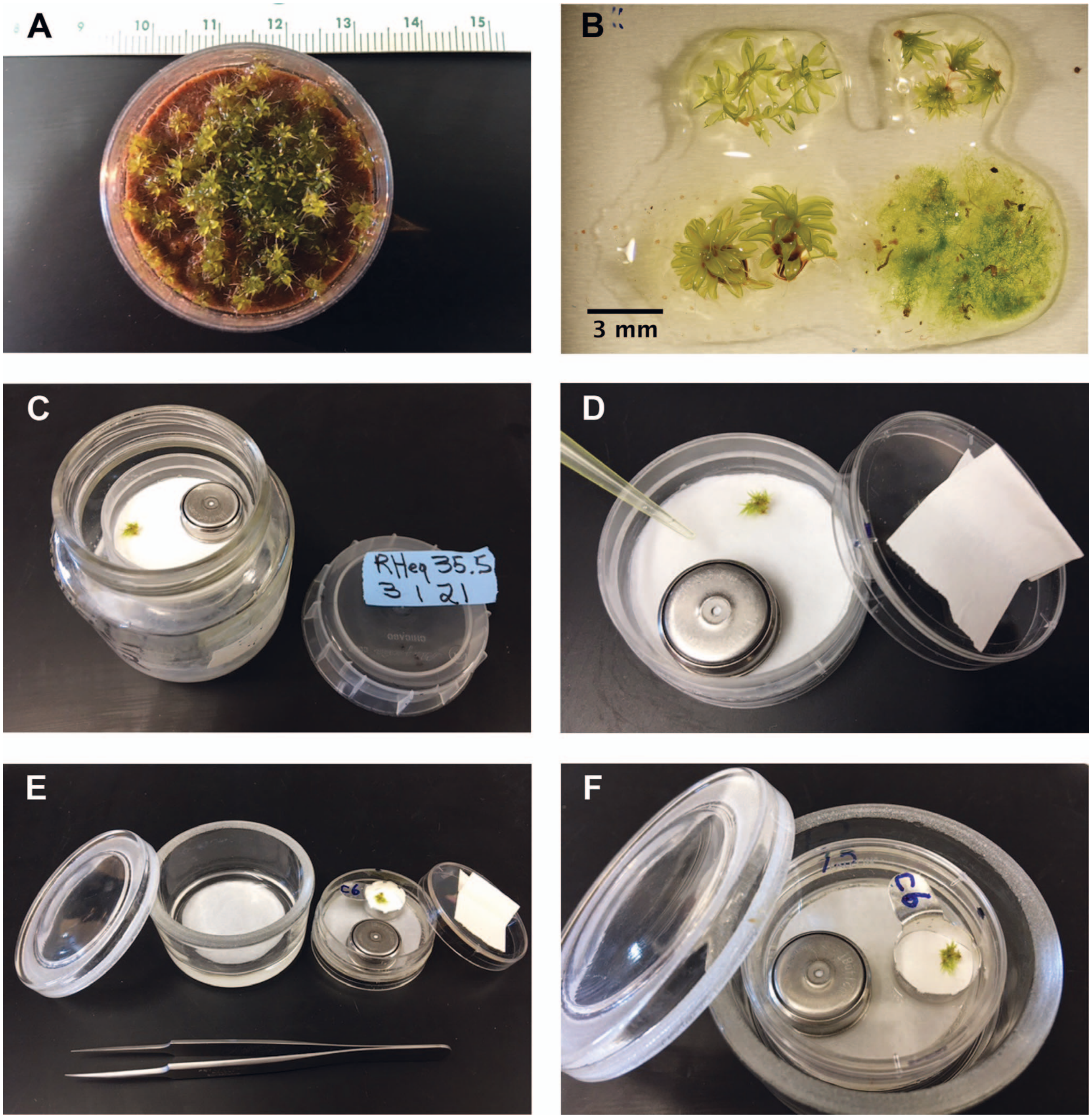

Figure 1. Experimental setup to compare methods used to assess bryophyte desiccation tolerance. **A.** Culture that is ~2 months in age, *Syntrichia obtusissima*. **B.** Shoots or protonema after removing from culture, cutting away lower portions, and rinsing in water, clockwise from top left, *Funaria hygrometrica*, *Phascum cuspidatum*, *Bryum argenteum* protonema, *Syntrichia obtusissima*. **C.** Setup for *Conventional* method and the *Step-down* method (lower equilibrating relative humidities). **D.** Setup for *Wetted substrate* method. **E–F.** Setup and closeup for *Partial dry* method and the first exposure for the *Step-down* method. *iButtons* present in **C–F**, Petri dishes used have inner diameter of 35 mm, pipet used in **D**, aluminum weigh boats used in **E** and **F**, 1-ply (**C**, **E**, **F**), or 3-ply (**D**) *Whatman* #1 filter paper used.

free water from each shoot group or protonemal mat for up to 30 s on a chemical wipe, followed by placing the shoot group or protonemal mat in a desiccator targeting either 100, 75, 54 or 33% RH. After equilibrating the plants for 3 d in desiccators, plants were weighed and then oven-dried for 2 d at 80°C followed immediately by reweighing. WC was calculated as follows: [(Equilibrated Wt – Oven-dry

Wt)/(Oven-dry Wt)] ×100, yielding a percentage dry weight (DW).

Desiccating technique. All manipulations and desiccation trials were conducted in an environmental room set to 20°C and constant dim light (~3 PAR). The approach for shoots (gametophores) was identical for the three species except that accommodations were made in the fluorescence clip for plant size differences (fewer shoots for the larger *Syntrichia*, and more shoots for the smaller *Funaria*). Protonemal mats used were ~3–6 mm × ~3–6 mm (length × width) and otherwise handled similarly to shoots. Two to five adult vegetative (nonsporophytic, although very small embryos may be present in *Phascum*) shoots were removed from a culture, cut at the substrate surface, rinsed several times of any particulates in sterile distilled water on a microscope slide (**Fig. 1B**), and placed in a chlorophyll fluorescence clip on a chemical wipe dipped in water to maintain superincumbent water (suprasaturation) on the plants. Shoots in the clip were dark adapted for 10 min and then assessed for chlorophyll fluorescence described below under *Chlorophyll fluorescence*. These data served as control levels of fluorescence, to which the desiccated and rehydrated shoots and protonemata were compared (as a percentage of control readings from the same plants). These shoot clusters or protonemal mats remained contiguous throughout the experiment, i.e., with shoots in physical contact with one another. Shoots or protonemata were transferred from the clip to a drop of sterile water, and then blotted to full turgor by placing on a chemical wipe and observing at 60× under a dissecting microscope until most visible external water disappeared from the shoot apices; the blotting took ~5–15 sec for shoot tips of *Phascum*, *Funaria*, and *Syntrichia*, and ~30 sec for *Bryum* protonemal mats. Blotting the mosses in this way and the use of very few shoots in each assay eliminate the potential to extend rates of dehydration by species-specific colony water storage locations in the bryophyte canopy (Cruz de Carvalho et al. 2019), which was outside the scope of the current study, and thereby created equivalent rates of drying across the four taxa used.

For the *Conventional* method, shoots or protonemal mats at full turgor were placed on a single sheet of filter paper (Whatman #1) in an empty unlidded 35 mm wide plastic Petri dish in the head space of a desiccator containing a saturated salt solution of $MgCl_2$, equating to ~33% RH at 20°C (~ –150 MPa; **Fig. 1C**). Shoots remained in the desiccator for 4 days. For the *Wetted substrate* method, shoots or protonemal mats at full turgor were placed on 3-ply filter paper prewetted with 400 μL sterile water (no visibly free water) in a lidded Petri dish inside a 33% desiccator as above, for 4 days (**Fig. 1D**, further details of the *Wetted substrate* method are found in Stark 2017). For the *Step-down* method *Day 1*, shoots or protonemal mats at full turgor were placed on a 1-ply filter paper in an aluminum weigh boat (13 mm diameter) in a lidded Petri dish inside a lidded watch glass (**Figure 1E-F**), with both the Petri dish and the watch glass (but not the weigh boat, which has unwetted 1-ply filter paper) having 1-ply filter paper saturated with sterile water and including visibly free water, and allowed to equilibrate for 24 h (~100% RH, ~0 MPa). On *Day 2*, the weigh boat with shoots or a protonemal mat and *iButton* were lifted from the Petri dish/watch glass chamber and transferred to a 75% desiccator on a single sheet of filter paper (above a saturated solution of NaCl (~ –39 MPa) and allowed to equilibrate for 24 h in an unlidded Petri dish. On *Day 3*, the Petri dish was transferred to a 54% desiccator [above a saturated solution of $Mg(NO_3)_2$ (~ –83 MPa)] and allowed to equilibrate for 24 h. On *Day 4*, the Petri dish was transferred to a 33% desiccator and allowed to equilibrate for 24 h (~ –150 MPa). For the *Partial dry* method, shoots or protonemal mats at full turgor were handled as in the *Step-down* method, i.e., placed on a single sheet of filter paper in a weigh boat in a lidded Petri dish with a saturated 1-ply filter paper inside a lidded watch glass also having a single sheet of saturated filter paper including visibly free water, but remained at 100% RH for 3 days, then transferred to a 33% desiccator and allowed to equilibrate for 24 h. In all exposures, an *iButton* (Embedded Data Systems, Lawrenceburg, KY, U.S.A.) was positioned adjacent to the shoot group or protonemal mat to allow RH measurements experienced by the shoots and to document the RH over experimental trials. Different setups producing 100% or 98% RH were piloted to reduce condensation while still achieving 100% RH, and we eventually settled on the double chamber (lidded Petri dish inside of a lidded watch glass).

Chlorophyll fluorescence. Groups of 2–5 shoots of each species or protonemal mats of *Bryum* were rehydrated (or used directly from cultures if controls) in a fluorescence clip on a chemical wipe dipped in water allowing the plants to remain hydrated (suprasaturated) during the procedure, dark-adapted for 10 m, and assessed using a modulated chlorophyll fluorometer (FMS2, Hansatech, King's Lynn, UK) at 10 m and 24 h postrehydration. Measurement parameters were set to a saturation intensity of 40 (1600 μmol m^{-2} s^{-1}) and a pulse duration of 0.4 s. Gain and modulation were adjusted to achieve an adequate signal per manufacturer directions. Desiccated shoots and protonemal mats fully rehydrate (visibly) in <10 m. In between fluorescence readings, plants were allowed to remain hydrated in an open fluorescence clip kept in an environmental room maintained at a constant dim light ($\sim$3 μmol m^{-2} s^{-1} PAR) and 20°C. The saturation pulse method (Bilger et al. 1995) was employed to determine the minimal fluorescence (F_0), the fluorescence when all reaction centers of photosystem II (PSII) are open; maximal fluorescence (F_m), a rough comparative measure of the amount of light absorbed by chlorophyll and thus of the total potential photosynthetic activity of the plant material especially if the same shoots are used in a time series (Logan et al. 2007) and shoot biomass is kept constant (Proctor 2003); maximum photochemical efficiency of dark-adapted PSII ($F_\mathrm{v}/F_\mathrm{m}$) followed by an assessment of the effective quantum yield of PSII photochemistry (Φ_PSII) and *qP*, the latter two measurements determined as described in Genty et al. (1989). $F_\mathrm{v}/F_\mathrm{m}$ is a measure of general physiological condition; Φ_PSII represents the fraction of excitation energy flowing through PSII and hence an indicator of active photosynthesis (Green & Proctor 2016); and *qP* is a "measure of the oxidation state of the first electron acceptor Q_A" (Proctor et al. 2007a), and represents the proportion of PSII reaction centers that are open (Maxwell & Johnson 2000). Because we first assessed control shoots and then dried these same shoots, we compared fluorescence values as a percentage of control levels, as in Hájek & Vicherová (2014).

Leaf and protonemal damage. Following the assessment of chlorophyll fluorescence, shoots were placed on 2-ply saturated filter paper inside a Petri dish and allowed to remain in the growth chamber at culture settings (described above) for 7 d in a suprasaturated condition, watered with sterile water as needed. Visual leaf damage was assessed on day 7 by examining the shoots at 60× and assigning each leaf along a shoot to one of 3 categories: entirely chlorophyllose (green, value=1), partially chlorophyllose (value=0.5), or entirely chlorotic (brown, value=0). For assessing protonemal mat damage, chlorophyllose tissue area was estimated visually to the nearest 10%.

Statistics. Due to the inherent censoring in the leaf damage data, tobit regression models (Tobin 1958) were used to test for the effects of drying method and species. Multiple comparisons with Tukey contrasts were used to compare across these groups. A three-way mixed ANOVA was used to test for the effects of species, drying method, and time on photosynthetic performance. Bonferroni adjustments were applied as needed when making subsequent pairwise comparisons using t-tests. All analyses were performed using R version 4.0.3 (R Core Team 2020).

RESULTS

Fluorescence controls, water content and equilibrating relative humidities. Control values for four chlorophyll fluorescence measures reflect differences among species, and on balance indicate healthy study cultures, with F_v/F_m values >0.7 (**Table 4**). Water content on a dry weight basis was highest and quite variable by species at full turgor. WC at equilibration with 100% RH at 20°C was about half the WC at full turgor, with higher values observed in *Funaria* shoots. WC then declined sharply at an *RHeq* of 75% (to a WC $\sim$20%) and eventually to $\sim$9.5% a *RHeq*=33% (**Table 4**), with fairly similar WCs across species for equilibrating RHs of 75%, 54%, and 33%. Assessing WC of *Bryum* protonemal mats proved challenging, owing to the sand grains that are difficult to rinse out, even when we ashed the protonema and subtracted out the weight of the sand grains; protonemal WC data are not given here. During the drying treatments, RH experienced by the plants reflected the differences in each method of drying (**Fig. 2**). The *Wetted substrate* method resulted in initial (first 48 h) RHs of slightly less than 100%, whereas in both the *Step-down* and *Partial dry* methods the initial RH was $\geq$100%; this difference is likely the result of (1) free water in the *Step-down* and *Partial dry* methods, whereas in the *Wetted substrate* method the free water was absorbed

Table 4. Control values for selected chlorophyll fluorescence parameters and water contents (based on dry weight at equilibration with four relative humidities at 20°C) of the four species of study. Plants from cultures maintained in a continuous state of suprasaturation were dark-adapted for 10 min, values represent means ± one SE, N=32 (fluorescence), N=3 (WCs at 100, 75, 54, 33% equilibrating relative humidity), N=12 (WCs at full turgor). WC = [(Equilibrated Wt − Oven-dry Wt)/(Oven-dry Wt)] ×100. See Methods for tissue preparation details. WC = water content, Wt = weight. Water contents for protonema of *Bryum argenteum* proved to be difficult to determine (ND=not determined), see text in Methods.

Chlorophyll fluorescence

	F_m	F_v/F_m	Φ_{PSII}	qP
Bryum argenteum	572.22 ± 30.82	0.753 ± 0.005	0.706 ± 0.006	0.935 ± 0.003
Funaria hygrometrica	487.25 ± 25.51	0.789 ± 0.008	0.752 ± 0.009	0.935 ± 0.004
Phascum cuspidatum	481.09 ± 23.74	0.790 ± 0.010	0.772 ± 0.012	0.951 ± 0.003
Syntrichia obtusissima	646.63 ± 25.42	0.718 ± 0.007	0.672 ± 0.009	0.906 ± 0.004

Water content (%)

	Full turgor	**100%**	**75%**	**54%**	**33%**
Bryum argenteum	ND	ND	ND	ND	ND
Funaria hygrometrica	609.52 ± 47.19	328.95 ± 36.96	19.12 ± 0.56	11.26 ± 1.81	8.95 ± 0.58
Phascum cuspidatum	370.13 ± 18.92	189.38 ± 9.95	20.50 ± 1.60	12.58 ± 1.70	9.43 ± 0.76
Syntrichia obtusissima	456.86 ± 10.25	182.83 ± 6.18	22.17 ± 2.07	11.98 ± 1.20	10.25 ± 1.88

by the filter paper, and (2) small iButton errors present at very high RHs.

Chlorophyll fluorescence. For F_m (**Fig. 3**), there was a statistically significant three-way interaction between species, drying method, and time ($F=7.307$, $p<0.0001$). There were also two statistically significant simple two-way interactions between species and drying method at time points T0 ($F=4.25$, $p<0.0001$) and T24 ($F=7.42$, $p<0.0001$), but not for the control measurement ($F=0.541$, $p=0.842$). There were statistically significant simple main effects of drying method on F_m for all species at both T0 ($F>5.32$, $p<0.005$) and T24 ($F>17.2$, $p<0.0001$) except for *Funaria* at T24 ($F=1.92$, $p=0.149$). Subsequent pairwise comparisons at T0 indicated the mean F_m following the *Partial dry* treatment was different for all species ($p<0.00802$) except *Bryum* as compared with the *Step-down* or *Wetted substrate* methods ($p>0.139$) and *Phascum* as compared to only the *Wetted substrate* method. In addition, the *Wetted substrate* method differed from both the *Conventional* and *Step-down* methods in *Phascum* ($p<0.00003$), while the *Conventional* method differed from *Wetted substrate* and *Step-down* methods in *Syntrichia* ($p<0.0281$). Subsequent pairwise comparisons showed a clear pattern at T24, with all species (*Funaria* excluded) having their mean F_m following the *Conventional* drying treatment significantly different than the other three methods ($p<0.00208$) with no differences among any of the other methods ($p>0.231$).

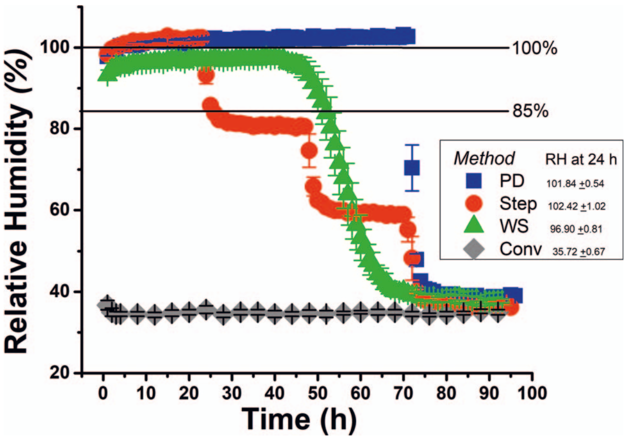

Figure 2. Typical variation in relative humidity (RH) experienced by shoots or protonema of bryophytes during the four methods of drying to equilibration with 33% RH at 20°C, as assessed hourly by *iButtons* (data presented every 3 h for *Conventional* method; Conv = *Conventional* method, WS = *Wetted substrate* method, Step = *Step-down* method, PD = *Partially dry* method; N=3, means ±one SE, error bars not visible are within the symbol). Data at the 24, 48, and 72 h timepoints are offset slightly for synchrony; the first point in each case is at hour 1.

Similarly, there were statistically significant simple main effects of species on F_m for all drying methods at both T0 ($F>4.16$, $p<0.015$) and T24 ($F>3.09$, $p<0.043$). Subsequent pairwise comparisons showed that the mean F_m of *Syntrichia* significantly differed from all other species following

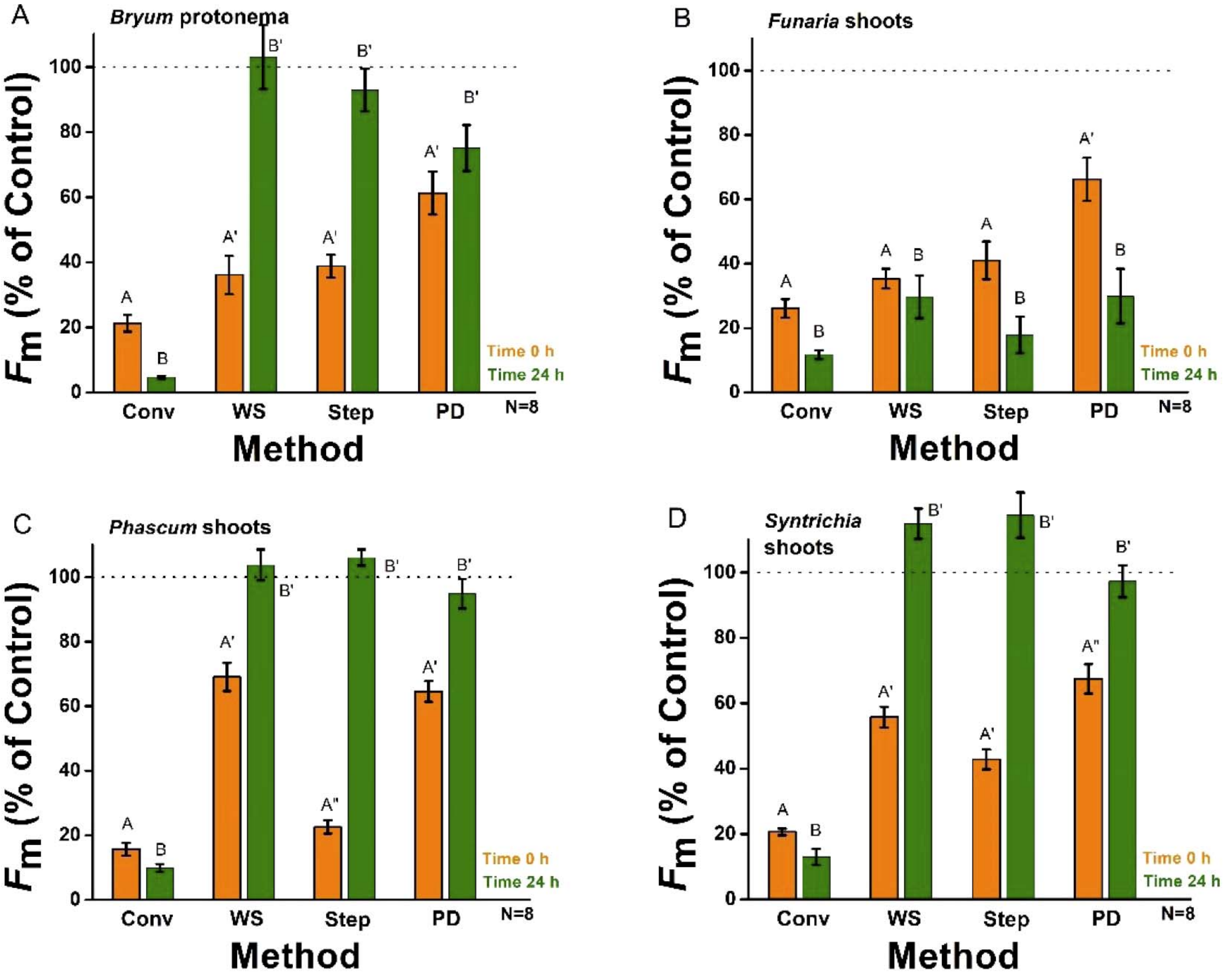

Figure 3. Maximal chlorophyll fluorescence (F_m) responses 10 min (0 h) and 24 h postrehydration in shoots or protonema of four species exposed to the four methods of drying for *Bryum argenteum* (*Bryum*) (**A**), *Funaria hygrometrica* (*Funaria*) (**B**), *Phascum cuspidatum* (*Phascum*) (**C**), and *Syntrichia obtusissima* (*Syntrichia*) (**D**). Conv = *Conventional* method, WS = *Wetted substrate* method, Step = *Step-down* method, PD = *Partially dry* method; means ±one SE, pairwise comparisons are within each postrehydration time (T0 and T24), e.g., A and A′ differ significantly at $p<0.05$.

the *Partial dry* treatments at both time points ($p<0.00273$). Another pattern was the significant difference between *Funaria* and all other species at T24 ($p<0.00375$) except for the *Conventional* treatment ($p>0.866$). Other significant differences were between *Bryum* and *Syntrichia* at T24 following the *Conventional* treatment ($p=0.0363$) and at T0 following the *Wetted substrate* treatment ($p=0.0448$). Also, *Funaria* differed from both *Phascum* and *Syntrichia* at T0 following the *Wetted substrate* treatment ($p<0.0227$), and finally *Phascum* and *Syntrichia* were significantly different at T0 following both the *Step-down* and *Conventional* treatments ($p<0.016$).

For F_v/F_m (**Fig. 4**), there was a statistically significant three-way interaction between species, drying method, and time ($F=3.01$, $p=0.00031$). There were also two statistically significant simple two-way interactions between species and drying method at time points T0 ($F=197$, $p<0.0001$) and T24 ($F=175$, $p<0.0001$), but not for the control measurement ($F=0.918$, $p=0.512$). There were statistically significant simple main effects of drying method on F_v/F_m for all species at both T0 ($F>26.6$, $p<0.0001$) and T24 ($F>9.1$, $p<0.000229$). Furthermore, subsequent pairwise comparisons showed the mean F_v/F_m for the *Conventional* drying method to be significantly different than the other three methods for all species at both T0 and T24

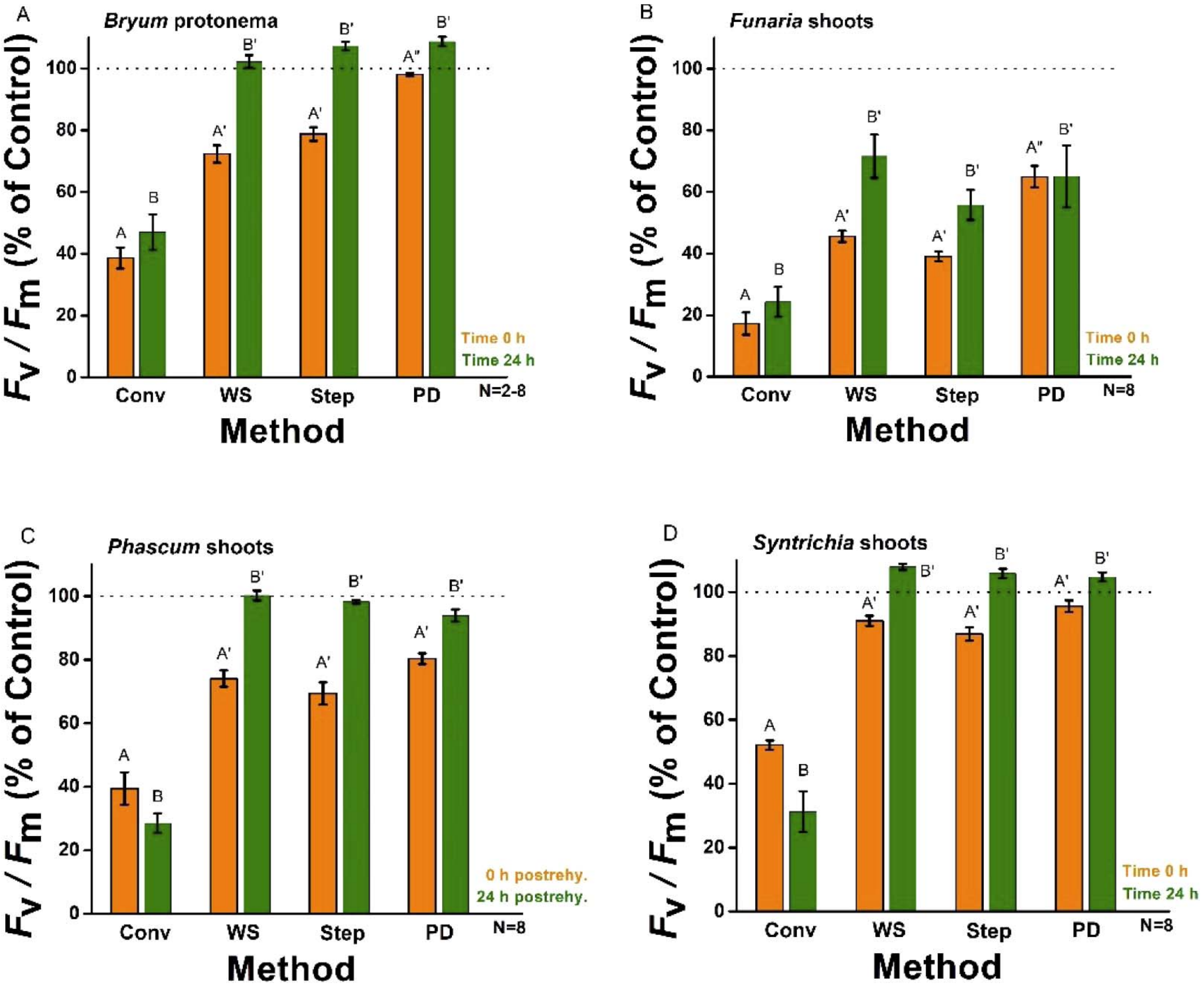

Figure 4. Maximum photochemical efficiency of dark-adapted PSII (F_v/F_m) responses 10 min (0 h) and 24 h postrehydration in shoots or protonema of four species exposed to the four methods of drying for *Bryum argenteum* (*Bryum*) (**A**), *Funaria hygrometrica* (*Funaria*) (**B**), *Phascum cuspidatum* (*Phascum*) (**C**), and *Syntrichia obtusissima* (*Syntrichia*) (**D**). Conv = *Conventional* method, WS = *Wetted substrate* method, Step = *Step-down* method, PD = *Partially dry* method; means ±one SE, pairwise comparisons are within each postrehydration time (T0 and T24), e.g., A and A′ differ significantly at $p<0.05$.

($p<0.0384$). In addition, the mean F_v/F_m for the *Partial dry* method was significantly different from the *Step-down* and *Wetted substrate* methods but only for *Bryum* and *Funaria* and only at T0 ($p<0.000372$). Similarly, there were statistically significant simple main effects of species on F_v/F_m for all drying methods at both T0 ($F>12.7$, $p<0.00002$) and T24 ($F>3.37$, $p<0.032$). Subsequent pairwise comparisons showed the mean F_v/F_m for *Funaria* to be significantly different from all the other species ($p<0.0421$) except as compared to *Phascum* and *Syntrichia* following recovery from the *Conventional* drying method ($p=1$). The only other significant differences were between *Bryum* and *Phascum* at T0 following a *Partial dry* ($p=0.0029$) as well as between *Syntrichia* and both *Bryum* and *Phascum* following the *Wetted substrate* treatment ($p<0.0456$).

For Φ_{PSII} (**Fig. 5**), there was a statistically significant three-way interaction between species, drying method, and time ($F=3.801$, $p<0.0001$). There were also two statistically significant simple two-way interactions between species and drying method at time points T0 ($F=8.23$, $p<0.0001$) and T24 ($F=5.2$, $p<0.0001$), but not for the control measurement ($F=0.848$, $p=0.574$). There were sta-

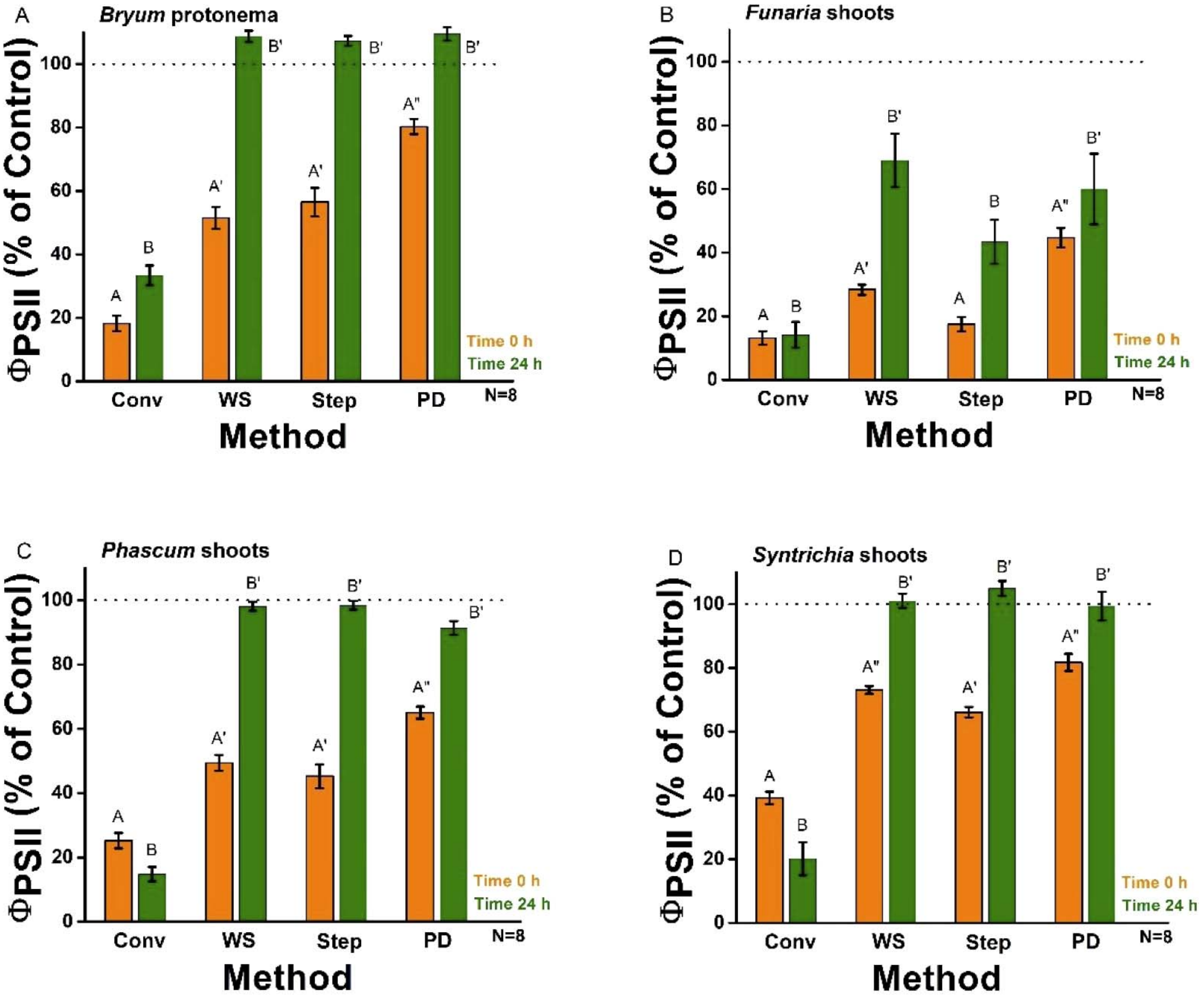

Figure 5. Effective quantum yield of PSII photochemistry (Φ_{PSII}) responses 10 min (0 h) and 24 h postrehydration in shoots or protonema of four species exposed to the four methods of drying for *Bryum argenteum* (*Bryum*) (**A**), *Funaria hygrometrica* (*Funaria*) (**B**), *Phascum cuspidatum* (*Phascum*) (**C**), and *Syntrichia obtusissima* (*Syntrichia*) (**D**). Conv = *Conventional* method, WS = *Wetted substrate* method, Step = *Step-down* method, PD = *Partially dry* method; means ±one SE, pairwise comparisons are within each postrehydration time (T0 and T24), e.g., A and A′ differ significantly at $p<0.05$.

tistically significant simple main effects of drying method on Φ_{PSII} for all species at both T0 ($F>40.8$, $p<0.0001$) and T24 ($F>8.64$, $p<0.000323$). Furthermore, subsequent pairwise comparisons showed the mean Φ_{PSII} for the *Conventional* drying method to be significantly different than the other three methods for all species at both T0 and T24 ($p<0.00199$) except as compared to the *Step-down* method in *Funaria* at either time point ($p>0.12$). In addition, the mean Φ_{PSII} for the *Partial dry* method was significantly different from the *Step-down* and *Wetted substrate* methods for all species at T0 ($p<0.000388$) except for the comparison between the *Partial dry* and *Wetted substrate* methods in *Syntrichia* ($p=0.451$). The *Step-down* and *Wetted substrate* methods were only significantly different in *Syntrichia* and *Funaria* at T0 ($p<0.0496$). Similarly, there were statistically significant simple main effects of species on Φ_{PSII} for all drying methods at both T0 ($F>19.3$, $p<0.0001$) and T24 ($F>4.38$, $p<0.012$). Subsequent pairwise comparisons showed the mean Φ_{PSII} for *Funaria* to be significantly different from all the other species ($p<0.0219$) except as compared to *Phascum* and *Syntrichia* following recovery from the *Conventional* drying method ($p=1$) or as compared to *Bryum* at T0 ($p=1$). Other significant differences were between *Bryum* and *Phascum* at T24 following the *Conventional* treatment ($p=0.0315$) and at T0 following the *Partial dry* treatment ($p=0.0239$), and finally between *Syntrichia* and both *Bryum* and *Phascum* following the *Wetted substrate* and *Conventional* treatments at T0 ($p<0.0296$).

For qP (**Supplementary Fig. S1**), there was a statistically significant three-way interaction between species, drying method, and time ($F=2.617$, $p=0.002$). There was also a statistically significant simple two-way interaction between species and drying method at T0 ($F=5.55$, $p<0.0001$), but not for T24 ($F=1.94$, $p=0.054$) or the control measurement ($F=0.246$, $p=0.987$). There were statistically significant simple main effects of drying method on *qP* for all species at T0 ($F>3.53$, $p<0.028$). Furthermore, subsequent pairwise comparisons showed the mean *qP* for the *Conventional* drying method to be significantly different than the other three methods but only for *Syntrichia* and *Bryum* ($p<0.0481$). In addition, the mean *qP* for the *Partial dry* method was significantly different from the *Step-down* method in *Phascum* and *Funaria* ($p<0.0411$) but not in the other species. Finally, the mean *qP* was significantly different between the *Partial dry* and *Wetted substrate* methods only for *Bryum* ($p=0.0109$). Similarly, there were statistically significant simple main effects of species on *qP* for all drying methods at T0 ($F>3.97$, $p<0.018$). Subsequent pairwise comparisons showed the mean *qP* for all species were significantly different following the *Partial dry* and *Step-down* methods ($p<0.0418$) except for the comparison between *Bryum* and *Syntrichia* ($p=1$). In addition, the mean *qP* for *Syntrichia* differed from all other species following the *Wetted substrate* treatment ($p<0.0208$), and finally the mean *qP* of *Syntrichia* and *Bryum* differed following the *Conventional* treatment ($p=0.0118$).

Tissue damage. Drying method had a significant effect on tissue (leaf or protonemal) damage, but that effect was limited to the *Conventional* method, which resulted in more damage than the other three methods ($p<0.0001$, $|z|>16.943$; **Fig. 6**). When compared to each other, the remaining three methods did not produce significantly different levels of tissue damage ($p>0.761$, $z<1.368$). All comparisons of tissue damage across species were significantly different ($p<0.0001$, $|z|>5.415$) except for the comparison between *Syntrichia* and *Phascum*, which showed no difference ($p=0.983$, $z=0.756$).

DISCUSSION

Methods in use and need for replicability among studies. A total of 289 studies (published papers) was reviewed on desiccation tolerance (DT) in bryophytes in which a drying method was described by the authors. These studies date from 1921 to 2021, do not represent an exhaustive list, and encompass ~860 species of bryophytes; some redundancy of species was inevitable and not teased apart here (**Supplementary Table S1**). Field and laboratory studies were combined and organized into six categories discussed in the *Introduction*, based on how the desiccating procedure was administered (air-dry RH unspecified; air-dry RH specified; using a single desiccator; using multiple desiccators; varying rates of drying; and the *Step-down* technique). Commonalities among the various methods of drying above were formulated with laboratory techniques in mind in order to derive four prevalent methods of drying bryophytes that are testable in the laboratory and in current use.

The four predominant methods distill fairly easily into methods currently in use: *Conventional*, *Wetted substrate*, *Step-down*, and *Partial drying* (**Table 2**). Each method necessarily begins with plants blotted to full turgor. Briefly, the *Conventional* method is in broad use, where plants are placed directly at one or more targeted *RHeqs* (equilibrating relative humidities). The other three methods are in more restrictive use. In the *Wetted substrate* method the rate of drying (*RoD*) is varied at a single or multiple *RHeqs*. In the *Step-down* method plants are "stepped down" from a high *RHeq* through a declining series of *RHeqs*, equilibrated at each step. Finally, the *Partial drying* method is the most infrequently used, and consists of leaving plants in humid air (*RHeq* ~100%) for a period of time prior to moving to the targeted *RHeq* of the experiment.

The need for such an empirical comparison of methods is apparent given lab-to-lab replicability concerns across a range of purposes from gene expression to determination of fitness outcomes and ecological strategies that may be critical. Adoption

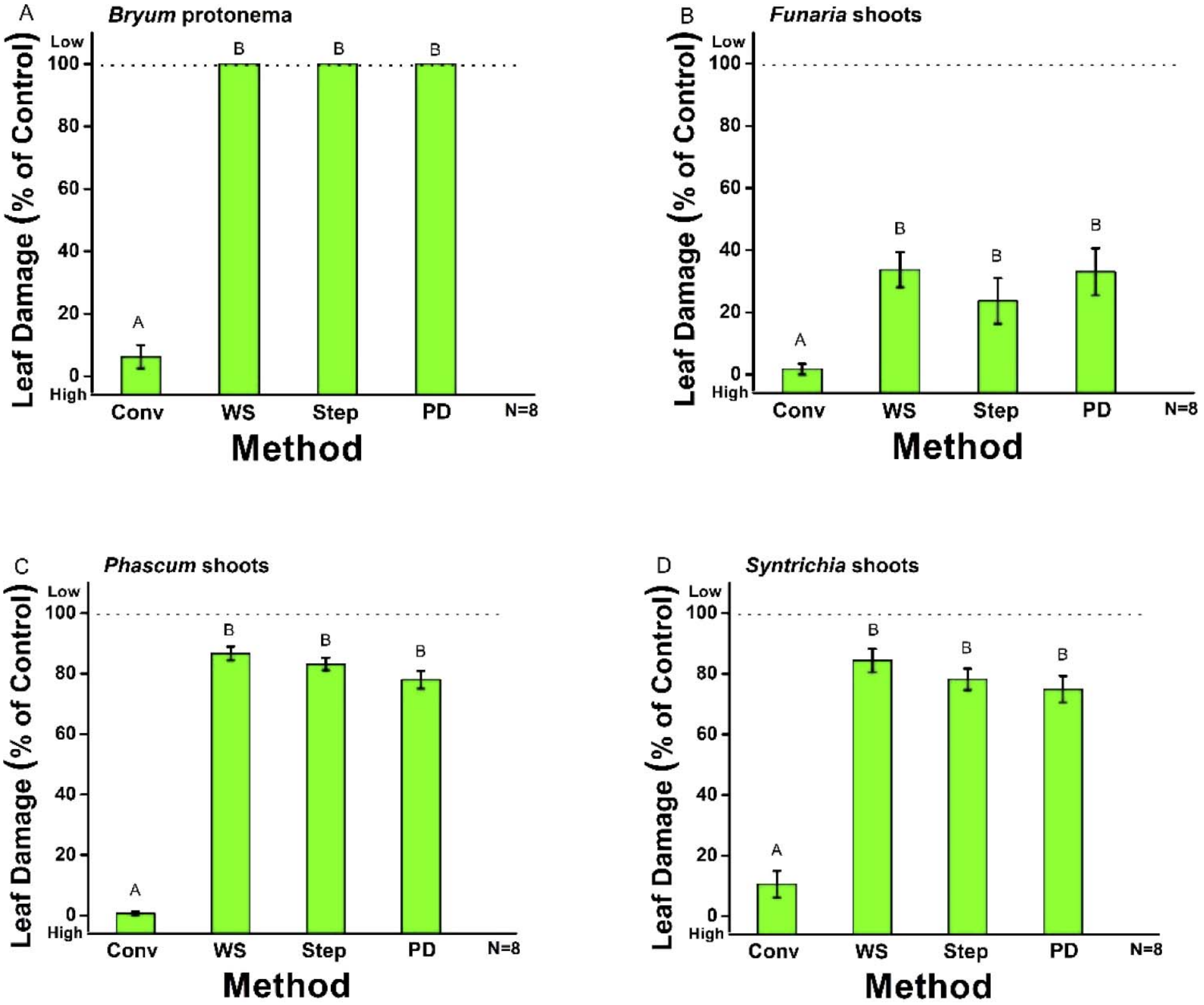

Figure 6. Visual leaf damage 7 days postrehydration in shoots or protonema of four species exposed to the four methods of drying for *Bryum argenteum* (*Bryum*) (**A**), *Funaria hygrometrica* (*Funaria*) (**B**), *Phascum cuspidatum* (*Phascum*) (**C**), and *Syntrichia obtusissima* (*Syntrichia*) (**D**). Conv = *Conventional* method, WS = *Wetted substrate* method, Step = *Step-down* method, PD = *Partially dry* method; means ±one SE, different letters differ significantly at $p < 0.05$.

of a common method, or at least utilizing one of the four methods compared herein, allows results to be comparable among species and labs, for the following purposes: (1) detecting the limits of DT in a species; (2) drying plants to elicit hormonal responses to stress; (3) detecting the ecological strategy of a species or life phase; and (4) generating a genomic response, e.g., a transcriptome produced during drying (as opposed to the transcriptome produced during the first 24 h of rehydration, as is commonly done). This inductive signal, to be maximized, is based on the best method to elicit a physiological response to desiccation, i.e., that method that optimizes a protective and/or hardening response and which incurs the least photosynthetic damage.

Selecting species for testing. The selection of species to serve as a test of these methods is critical and doubtless represents a potential weakness of this noncomprehensive study. The "ideal" species would be clonal, amenable to culture, fairly well studied, and importantly, exhibit indications of a moderately inducible DT strategy (IDT) that is neither extraordinarily sensitive to desiccation (e.g., *Physcomitrium patens*, also known as *Physcomitrella*) nor approaching the ecological strategy of constitutive DT (CDT, e.g., some species of *Syntrichia* and adult shoots of *Bryum argenteum*). Although life phases are important to survival strategies and may vary in their degree and strategy of DT, we focus here mainly on adult shoots (3 species) and protonema (1 species). Four species were selected for study that in lab preexperimental trials exhibited indications of incorporating a strategy of IDT: shoots of *Phascum cuspidatum*, *Syntrichia obtusissima*, *Funaria hygrometrica*, and protonema of *Bryum argenteum* (these species referred to henceforth by genus). Three of these species have been widely studied, are distributed nearly worldwide, represent relatively unrelated clades including 3 families, and all but one (*Syntrichia*) is regarded as incorporating a DT strategy of IDT (Werner et al. 1991 for *Funaria*; Greenwood et al. 2019 for protonema of *Bryum*; Coe et al. 2021 for *Syntrichia*; unpublished trials, LRS for *Phascum*). In preexperimental trials, these four species exhibited the desired response traits to a severe desiccating event (to equilibration with 33% RH): the shoots or protonemata were killed by a rapid-dry event (<10 min from full turgor to leaf curling) and yet survived with slight or no damage when dried more slowly (48 h from full turgor to leaf curling). A moderately inducible desiccation tolerance strategy (IDT) was therefore indicated.

Evaluating and ranking the methods. The most relevant criterion against which to compare the performance of these methods is the capacity of the method to capture the inductive signals of DT that produce hardening to DT (acclimation and priming). During subturgor the evidence for induction of DT in mosses is clear (Beckett 1999, 2001; Hájek & Vicherová 2014; Mayaba & Beckett 2003). To assess acclimation, we used chlorophyll fluorescence (immediately upon rehydration and 24 h postrehydration) and visual tissue damage (7 d postrehydration). Assessing priming, the ability to withstand a future severe drying event, proved, in our preliminary trials, to be too variable to test at this time. These assessments provide evidence of protective measures actualized during the drying period in order to withstand the initial drying event (acclimation). Once DT is induced and the plants are rehydrated, if maintained at full turgor the plants deharden to DT over a matter of days (Schonbeck & Bewley 1981) to a week (Brinda et al. 2016; Liu et al. 2019), and in rare cases hardening is not completely dissipated for 3 weeks (Hájek & Vicherová 2014). In the present study, methods of drying were deemed effective to the degree that, upon rehydration, they result in (1) the least photosystem damage immediately upon rehydration, (2) the most efficient recovery of photosystems at 24 h postrehydration, and (3) the least cellular necrosis in leaves and protonemata as viewed 7 days postrehydration.

Photosystem **damage** can be inferred from chlorophyll fluorescence readings taken immediately upon rehydration (termed here time zero, or T0 readings). The depression of T0 readings relative to controls should represent damage incurred from drying in these dehardened cultures. Photosystem **recovery** can be inferred from either assessing fluorescence readings reached 24-h postrehydration (T24, as done here), or assessing the difference in fluorescence between T0 and T24 h postrehydration; T24 readings (or T24 – T0) should represent the extent of postrehydration recovery/repair. Using these metrics, the most pronounced finding was the heavy damage incurred upon rehydration (equating to an inability to induce DT) coupled with the inability to recover from the *Conventional* method (**Figs. 3–5, Table 5**). The three alternative methods of drying (*Partial drying*, *Wetted substrate*, *Step-down*) resulted in far less photosystem damage (T0) and significantly higher photosystem recovery (T24) than the *Conventional* method (**Figs. 3–5**). Using the two perhaps most powerful (informative) response measures of chlorophyll fluorescence (F_v/F_m, Φ_{PSII}), the *Partial drying* method produced higher T0 readings for *Bryum* and *Funaria* (F_v/F_m) and in *Bryum*, *Funaria*, and *Phascum* (Φ_{PSII}). In *Syntrichia*, the *Partial dry* and *Wetted substrate* methods produced the highest T0 readings (Φ_{PSII}) and yet the *Wetted substrate*, *Step-down*, and *Partial dry* methods produced equivalent readings for both T0 and T24 (F_v/F_m). Excellent recovery in three species (*Syntrichia*, *Bryum*, *Phascum*), often exceeding even Control values (F_v/F_m, Φ_{PSII}) was observed in all drying methods excepting the *Conventional* method; recovery was incomplete in *Funaria*, indicating it was the most sensitive of the species tested and would benefit from extended drying times (*Wetted substrate*), extended time at high humidity (*Partial dry*), or interpolation of additional equilibrating RHs (*Step-down*). Given these results, it seems clear that the *Conventional* method is not appropriate for testing of DT in dehardened

Table 5. Ranking the methods of equilibrium drying (1=strongest response, 3=weakest response, with different rankings statistically significant from one another at p≤0.05) based on chlorophyll fluorescence (F_v/F_m, Φ_{PSII}, qP, F_m) immediately upon rehydration (T0 h) and 24 h postrehydration (T24 h) for the four species tested. *Bryum*=*B. argenteum*, *Funaria*=*F. hygrometrica*, *Phascum*=*P. cuspidatum*, *Syntrichia*=*S. obtusissima*.

	METHOD							
RESPONSE	***Conventional***		***Wetted Substrate***		***Step-down***		***Partial Dry***	
at Time 0 h	F_v/F_m	Φ_{PSII}	F_v/F_m	Φ_{PSII}	F_v/F_m	Φ_{PSII}	F_v/F_m	Φ_{PSII}
Bryum	3	3	2	2	2	2	1	1
Funaria	3	3	2	2	2	3	1	1
Phascum	2	3	1	2	1	2	1	1
Syntrichia	2	3	1	1	1	2	1	1
at Time 24 h	F_v/F_m	Φ_{PSII}	F_v/F_m	Φ_{PSII}	F_v/F_m	Φ_{PSII}	F_v/F_m	Φ_{PSII}
Bryum	2	2	1	1	1	1	1	1
Funaria	2	2	1	1	1	2	1	1
Phascum	2	2	1	1	1	1	1	1
Syntrichia	2	2	1	1	1	1	1	1
	Conventional		***Wetted Substrate***		***Step-down***		***Partial Dry***	
at Time 0 h	qP	F_m	qP	F_m	qP	F_m	qP	F_m
Bryum	1	2	2	1	1	1	1	1
Funaria	1	2	1	2	1	2	1	1
Phascum	1	3	1	1	1	2	1	1
Syntrichia	1	3	1	2	1	2	1	1
at Time 24 h	qP	F_m	qP	F_m	qP	F_m	qP	F_m
Bryum	1	2	1	1	1	1	1	1
Funaria	1	1	1	1	1	1	1	1
Phascum	1	2	1	1	1	1	1	1
Syntrichia	1	2	1	1	1	1	1	1

or sensitive species. Furthermore, the *Partial drying* method may offer some advantages when attempting to induce DT in particularly sensitive species (Rathnayake et al. 2019). For these species, there is a narrow window of stress that invokes the DT response and beyond this damage is too severe for recovery to occur.

Visual leaf and protonemal damage observed 7 d postrehydration did not necessarily reflect T24 fluorescence readings across species. Only protonemal mats of *Bryum* showed no visually discernable damage, with the other species exhibiting some (*Phascum*, *Syntrichia*) or severe (*Funaria*) tissue necrosis. Among the latter three species, the three methods (*Wetted substrate*, *Step-down*, *Partial drying*) resulted in similarly high recovery (∼80% of controls) for two species, and similarly severe damage (∼30% of controls) in *Funaria*. We acknowledge that (1) tissue death is correlated but not necessarily coupled with measures of photosynthetic function, and (2) visual assessments of tissue damage are less exact than more quantitative measures such as fluorescence, and could be improved through photographic analyses of color differences.

Acclimation vs. priming. As adopted here, acclimation refers to the ability to recover from a desiccating event; priming refers to the ability to recover from a future desiccating event. We postulate that the responsible mechanisms of each are in common, and one (priming) represents an extension of the other (acclimation). That is, during a gradual drying event, a period of subturgor allows the bryophyte to detect drying pressure and then (if time allows) induce protective measures that reduce damage during both drying and subsequent rehydration. These measures would include synthesis/mobilization of sucrose, antioxidants, osmoregulative compounds, and specific proteins such as heat shock and LEA (late embryogenesis abundant) known to improve desiccation tolerance. Once a plant is acclimated via gradual drying, maintaining that metabolic condition of readiness while hydrated is time dependent and known as priming. At some point (days) the proteins, high sucrose levels, antioxidants, and osmoregulatory compounds will begin to degrade/dissipate into alternative cell metabolism pathways, decreasing the ability of the plant to tolerate a rapid drying event for which it has reduced protection; this course of losing the hardening signal is known as dehardening. It is measured by comparing chlorophyll fluorescence readings of control (never dried) plants with readings following a desiccating event. Species are expected to differ in their ability to be primed for a RD event, i.e., in their dehardening time, similar to our findings on acclimation here.

The subturgor hypothesis. A natural outgrowth of Richard P. Beckett's work on *Atrichum* (Beckett 1999, 2001; Beckett et al. 2005) with perhaps forerunners in Gupta (1978) and Schonbeck & Bewley (1981), the *Subturgor hypothesis* predicts that the method exposing plants to longer subturgor periods should exhibit the greatest inductive (acclimation and priming) signals. An extremely long uninterrupted subturgor period is probably unsustainable in nature; neither the length of the subturgor period nor the effects of light intensity during subturgor were explored here. Although bryophytes have been viewed as spending most of their time either desiccated or hydrated with little time spent in transition (Proctor et al. 2007b), even in a desert, patches of *Crossidium* spent more time in a partially dried state (10%) than in a hydrated state (8%), casting some doubt on this assumption (Stark 2005). While exposure to an atmosphere over distilled water (100% RH) was suitable to harden *Atrichum* (Beckett 1999), hardening through the *Partial drying* method can also be achieved at *RHeqs* $<$100%, as demonstrated by the amazing hardening of *Sphagnum* species to DT by exposure to 7 days at 98.5–99.0% RH (Hájek & Vicherová 2014). When RH falls below 98.5%, desiccation can be lethal or very stressful in *Sphagnum* (Abel 1956; Clymo 1973), but efficacious (89% RH) in inducing DT in the sensitive *Physcomitrium patens* (Rathnayake et al. 2019). The *Partial drying* and *Step-down* methods produced a slightly elevated RH than the *Wetted substrate* method ($\sim$100% vs. $\sim$97% RH during the first 24 h, **Fig. 2**), and this difference may help in explaining the better performance of the *Partial drying* method for two of the more sensitive species tested (*Funaria* shoots and *Bryum* protonemata) for T0 F_v/F_m and Φ_{PSII} as well as the surprisingly good performance of the *Step-down* method. However, at least 7 genera of mosses undergo hardening to DT after a 24-h exposure to lower RHs (96%; from Proctor & Pence 2002, derived from Höfler 1945 and Abel 1956). Similarly, the subturgor exposures occur with the *Wetted substrate* method of drying, where shoots are maintained near 100% RH as the plants lose WC (e.g., Pressel & Duckett 2010; Stark et al. 2013; Xiao et al. 2018). Such drying events are also known to protect bryophytes from a future RD event (e.g., Brinda et al. 2016; Werner et al. 1991), although this phenomenon was (oddly) not found in *Syntrichia ruralis* (Schonbeck & Bewley 1981).

Subturgor can be construed, for poikilohydric organisms like bryophytes, to be a condition where WC is at or above $\sim$30% on a dry-weight basis, equating to an *RHeq* $\geq\sim$85% (Coe et al. 2021; Lakatos 2011) at 20°C. Above this threshold of WC, metabolic activity is possible because bulk water is present (Hoekstra et al. 2001). Thus, time at subturgor should predict the efficacy of inductive mechanisms creating DT (acclimation, priming). However, in *Syntrichia caninervis*, exposure to an equilibrating RH of 85% (28–29% WC) did not result in protection from desiccation, but rather caused photosynthetic damage (Coe et al. 2021). Nevertheless, using this criterion of time at or above 85% RH, the greatest time at subturgor is experienced in the *Partial drying* method, followed in order by the *Wetted substrate* method, the *Step-down* method, and the *Conventional* method (**Fig. 2**). The *Subturgor hypothesis* (using F_v/F_m and Φ_{PSII} at T0) was generally sustained across species. However, at T24 recovery was robust (*Funaria* excepted) with no advantages accrued by partial drying over the other methods, where mostly excellent recovery was shown. Therefore, if recovery 24-h postrehydration is targeted, then no substantial differences were observed among *Partial drying*, *Step-down*, and *Wetted substrate* methods of drying, at least within the parameters of the present study. The advantage of partial drying may lie in mitigating damage from an ensuing desiccation event, rather than improving repair/recovery. Such evidence is in support of the *Subturgor hypothesis*, which predicts that longer periods of subturgor (experienced during partial hydration, also known as partial drying) should induce DT by mitigating the effects of drying. One caution: while depressed T0 F_v/F_m levels may indicate damage, such "damage" may reflect not only cellular/membrane damage but also downregulation of photosynthesis in response to stress through deactivation of the D1 protein (a key subunit of PSII) that shuts down the PSII reaction centers (Aro et al. 1993). Upon appropriate rehydration conditions, D1 damage is rapidly reversible, while other types of cellular damage are slower to recover or are irreversible and may lead to cell death (Vasilikiotis & Melis 1994).

Time at subturgor was 3 d for *Partial drying*, 2 d for *Wetted substrate*, 1 d for *Step-down*, and 0 d for *Conventional* methods of drying. The *Partial drying* method generally produced the highest T0 fluorescence readings, indicative of incurring the least photosystem damage. This advantage largely disappeared once recovery occurred over the next 24 h. Consistent with the *Subturgor hypothesis*, the *Conventional* method of drying resulted in heavy damage and reduced/no recovery across all species tested. The most sensitive species tested (*Funaria*) exhibited elevated fluorescence of F_v/F_m and Φ_{PSII} at T0 in the *Partial drying* treatment, indicating that *Partial drying* provides the optimal method for protection from damage incurred during drying. Recovery fluorescence readings of F_v/F_m and Φ_{PSII} at T24 exceeded 100% of control values for *Bryum* and *Syntrichia*, and was near 100% in *Phascum*. Notable also was the better than expected performance of the *Step-down* method, as predicted from the *Subturgor hypothesis*, vis-a-vis the *Wetted substrate* and *Partial drying* methods. Despite a subturgor exposure of only 24 h, the *Step-down* method produced roughly equivalent F_v/F_m and Φ_{PSII} levels to the *Wetted substrate* method. This indicates that an environment of high humidity has high inductive value to toleration of desiccation. Yet to be tested is the optimal WC for induction of DT. Plants can be subjected to, for example, the *Partial drying* method at *RHeqs* of 100, 98, 93, 85, and 75% for equivalent durations, then dried to a target RH, rehydrated and tested. It is probable that the optimal partial drying *RHeq* varies among species in a narrow band from 95–100% (cf. Hájek & Vicherová 2014 for *Sphagnum* species).

Partial-drying vs. prehydration. Both partial drying and prehydration enlist the same exposure to 100% RH for a given time. Whereas partial-drying occurs when taking plants from full turgor to equilibration with 100% RH (or at least 85%), prehydration occurs when taking plants from a desiccated state to equilibration with 100% RH. The functions of the two processes or states are quite different. Partial drying, beginning at full turgor, stimulates mechanisms to protect tissues from desiccation (IDT), as discussed earlier and demonstrated as early as Beckett (1999), our early pilot studies on *Physcomitrium* (unpublished data LRS, 2017), and lately in Rathnayke et al. (2019) for *Physcomitrium*. The plant response to partial drying is biological and inductive. On the other hand, prehydration (of dried plants) mitigates the phase change of biological glass to liquid water, greatly lessening the damage incurred. The plant response is physical and mechanical. Despite the disparity in function between prehydration and partial drying, water contents achieved for each are similar when plants are allowed to equilibrate with 100% RH at 20°C, in the neighborhood of 200% (e.g., Koster et al. 2010; Slate et al 2020), but may vary from ~100% (Anderson & Bourdeau 1955) to exceeding 300% as observed in *Funaria* of the current experiment.

Sensitive species, ecotypic variation, and the species effect on methods of drying. *Funaria hygrometrica* shoots tested here were derived from a residential habitat in the Sierra Nevada Mts of California (U.S.A.), and did not fully recover from desiccation regardless of the methods employed. However, this highly variable and cosmopolitan species is likely to exhibit ecotypic variation in the trait DT, as evidenced by the full recovery shown in plants derived from mountains of Arizona (U.S.A.) when recovered from a slow-dry to equilibration with 33% RH using the *Wetted substrate* method (McLetchie & Stark 2019). We found statistically significant simple main effects of drying method for F_m, F_v/F_m, and Φ_{PSII} on all species at both T0 and T24 with only one exception (*Funaria*). This indicates that species of bryophytes will show great variation in optimal drying preferences, and an optimal response from a very sensitive species or ecotype may vary among the three methods explored here, i.e., one may perform better using the *Stepdown* drying method, whereas another may perform better using the *Wetted substrate* method

Weaknesses of approach and future directions. The approach used here standardized the duration of each experiment to an exposure of four days to allow comparisons of induction, damage, and recovery across methods. If hardening is restricted, as per the *Subturgor hypothesis*, to the time where plants experience subturgor, then it becomes difficult to explain the success of the *Step-down* method (24 h at subturgor), where recovery 24 h postrehydration was often found to be similar to the *Partial drying* method (72 h at subturgor). Clearly, followup experiments should vary the time at subturgor for both of these methods. In addition, the success of the *Step-down* method may be influenced by exposure to intermediate water contents following a subturgor period and may be improved by interpolating intermediate *RHeqs* between 100% and 50%, e.g., exposure of plants to 100% *RHeq* (inducing subturgor processes), 95% *RHeq*, 85% *RHeq*, etc., in a more gradual removal of water from plants prior to exposure to *RHeqs* below 50%. Although metabolism is highly restricted once a plant is exposed to *RHeqs* <85%, a more gradual removal of water from tissues may lessen physical damage and thereby improve recovery. While four of the most basic, stand-alone techniques for drying bryophytes were explored and compared here, formulating a technique that represents a hybrid of two or more of these methods tested may serve to further enhance the inductive signal of DT (especially in sensitive species and given the wide variation among species tested) by maximizing gene expression during drying. For example, inserting a more gradual drying regime after a partial drying period may not only be more simulative of environmental conditions but also maximize the DT signal. Some drying methods discussed here may present more challenges in the experimental setup than others. For example, preventing condensation on plants during the *Partial drying* method is a concern that should be addressed experimentally in order to standardize the approach. In addition, focusing only on parameters such as F_v/F_m or Φ_{PSII} may overestimate recovery 24 h postrehydration: we found that tissue damage (necrosis) was present in *Phascum* and *Syntrichia* that was not predicted from fluorescence data. Visual estimates of tissue necrosis leave room for error, especially in lab-to-lab (and person to person) replication; a more quantitative approach is preferred. The blotting technique employed here approximates full turgor conditions without external free water. However, it was difficult to accurately blot protonemal mats, which can be sensitive to blotting too long. Finally, the use of a more accurate method (than *iButtons*) to assess RH may be preferred, because at high RHs close to 100% at 20°C values appear inflated by a few points, and these RHs may be critical for inferring differences among the Methods of drying.

Revisiting hypotheses, concluding remarks and a suggested starting point for a new researcher in DT. The null hypothesis that differences in damage and recovery would be minimal among the four drying methods tested was not sustained. Our secondary hypothesis, the method which extends subturgor time the furthest (*Partial drying* technique) will result in the strongest signals of inducibility, was mostly supported when considering inferred damage. However, regarding recovery, the *Subturgor hypothesis* did not fully explain the success of the *Step-down* and *Wetted substrate* methods of drying. Finally, the commonly used *Conventional* method of drying bryophytes, in the literature often called equilibrium drying, is not recommended. The most efficient hardening protocol will require considerable fine-tuning, depending on the experimental species and the questions being asked. We hope that researchers will take these factors into account when choosing their methods and designing future experiments.

For new students of DT or scientists entering the field of DT, perhaps the following starting points may be useful. The first steps toward profiling the DT response of a species could consist of a sequence of experiments. *First, deharden your plants*. If you are culturing the species of interest, then dehardening is not normally an issue. If these are bryophytes collected from non-aquatic settings, the plants will need to have continuous access to water and low light conditions for at least 7 days. *Second, conduct a rapid-dry (RD) test*. After dehardening, expose a few shoots (fewer shoots ensures a rapid drying in minutes) or thalli to a RD at <50% RH and ~20°C, and allow plants to equilibrate for at least a day. Rehydrate and assess for damage immediately upon rehydration (T0) and 24 h postrehydration (T24) if chlorophyll fluorescence is an option, or take visual or digital measurements of tissue damage 7 d postrehydration. *Third, interpret the results of the RD test*. If plants are killed or damaged, an inducible strategy of DT is indicated, and the next step is to determine the minimum rate of drying (*RoD min*). Slow the rate of drying using either a *Partial drying*, *Wetted substrate*, or *Step-down* approach, aiming for the minimum time exposed to an atmosphere of >85% RH that elicits full recovery (e.g., 10 h). If one of the latter three inductive methods (e.g., *Wetted substrate*) results in severe damage upon rehydration even with a very slow drying, then dry the plants using an alternate method (e.g., *Partial drying*) prior to concluding desiccation sensitivity. If plants show no damage even at T0, then one can skip a *RoD min* determination and explore the depth (*WC min*) of desiccation tolerated (e.g., 25% RH at a particular temperature).

Fourth, explore other factors of DT. One step that follows a determination of *RoD min* is to determine the minimum water content tolerated (*WC min*). This is the ”depth” of drying, and requires creating a series of RH treatments using salt or acid solutions (or other setups). Once the *RoD min* and *WC min* are experimentally known, the profile can broaden into explorations of duration dry, rate of rehydration, prehydration effects, dehardening time, and other fruitful directions.

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Supplementary documents online:

Supplementary Table S1. Classification of bryophyte papers that incorporate a drying protocol or a description thereof. Data on relative humidity and factors of desiccation tolerance that are explored in studies are given where relevant. Species numbers are only approximated and do not factor in species redundancies. Note that a few papers are listed in >1 category.

Supplementary Fig. S1. Responses of the chlorophyll fluorescence parameter *qP* 10 min (0 h) and 24 h postrehydration in shoots or protonema of four species exposed to the four methods of drying for *Bryum argenteum* (*Bryum*) (**A**), *Funaria hygrometrica* (*Funaria*) (**B**), *Phascum cuspidatum* (*Phascum*) (**C**), and *Syntrichia obtusissima* (*Syntrichia*) (**D**). Conv = *Conventional* method, WS = *Wetted substrate* method, Step = *Step-down* method, PD = *Partially dry* method; means ±one SE.