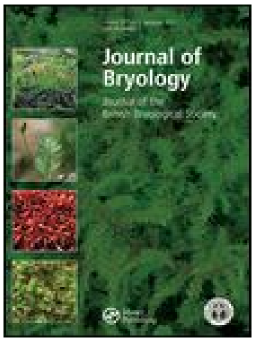

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Prehydration mitigates damage accrued from prolonged periods of desiccation in cultured shoot apices of *Syntrichia ruralis*

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ABSTRACT

Introduction. We hypothesised that a prehydration period (exposure to humid air) should mitigate the damaging effects of short or long periods of desiccation in a desert moss, *Syntrichia ruralis*.

Methods. Cultured uniclonal shoots of *S. ruralis* were dried rapidly, equilibrated at 42% relative humidity (RH), allowed to remain at this RH for 1–50 days, and either (1) prehydrated (24 h at 100% RH) then rehydrated with liquid water, or (2) rehydrated directly using liquid water without a prehydration treatment, and assessed using chlorophyll fluorescence and visual damage.

Key results. At both 20 min and 24 h postrehydration, all chlorophyll fluorescence measures (F_m, F_v/F_m, Φ_{PSII}, qP) were higher in shoots that were prehydrated. Prehydrated *S. ruralis* shoots also had less visual leaf damage 7 d postrehydration. Duration-dry (*DD*) had an overall negative effect for all fluorescence measures on shoots immediately after rehydration, an effect that dissipated at 24 h postrehydration in three of the four fluorescence measures (F_m, F_v/F_m, Φ_{PSII}). Leaf damage was not influenced by Duration-dry. Although no interaction was detected between Prehydration and Duration-dry treatments, the benefits of prehydration were accentuated based on fluorescence metrics F_v/F_m and Φ_{PSII}, when shoots are exposed to longer drying periods.

Conclusions. A prehydration treatment implemented just prior to hydration with liquid water significantly mitigated shoot damage accrued from a prior rapid drying event and *DDs* of up to 50 d at 42% RH. Based on photosynthetic efficiency, prehydration conferred greater effects at longer *DDs*. Experiencing humid conditions prior to rainwater mitigates damage incurred during prolonged desiccation.

KEYWORDS
Chlorophyll fluorescence; constitutive; desiccation tolerance; equilibrating relative humidity; inducible; suprasaturation

Introduction

Among poikilohydric plants (those which equilibrate their tissue water content with ambient relative humidity), vegetative desiccation tolerance (DT) describes a trait that allows an individual to recover from equilibration with potentially dangerously dry air (≤85% relative humidity, (RH); Glime 2019). At about this RH (equating to a water potential ~ –20 MPa at 20°C) most metabolic activities of the cell cease (Walters et al. 2005) and tissue water content (WC, dry weight basis) is ~30% (Schonbeck and Bewley 1981a), the threshold for bulk water availability (for membranes and cytoplasm, Hoekstra et al. 2001). This 30% WC condition may correspond to even higher equilibrating RHs, as shown for *Physcomitrella patens* (Hedw.) Bruch & Schimp. to be closer to 95% RH (Koster et al. 2010). The trait of DT is experienced across at least four environmental dimensions (factors), which include, along a time sequence, (1) the rate at which the plant is dried (rate of desiccation, or *RoD*); (2) the equilibrating atmospheric relative humidity (*RHeq*), which determines tissue water content; (3) the duration over which the plant is desiccated (duration-dry, or *DD*); and (4) the rate at which the plants are rehydrated (rate of rehydration, or *RoR*), which may include an exposure to humid air prior to hydration with liquid water (prehydration). DT is achieved through the production, during the gradual drying of tissues and/or upon rehydration, of sugars, protective and repair proteins, protective osmolytes, and scavenging enzymes for reactive oxygen species (mechanisms reviewed in Proctor, Oliver, et al. 2007, and conceptualised in Cruz de Carvalho et al. 2014).

Exploring the interrelationships between and among the four factors of DT (*RoD*, *RHeq*, *DD*, *RoR*) is seldom attempted because the factors are neither independent nor coincident in time. As the plant dries, the sequence of these factors experienced by the plant in a wet/dry/wet cycle is *RoD* → *RHeq* → *DD* → *RoR*. For example, before an equilibrating RH can be achieved, the plant will have been previously desiccated at a particular rate. Similarly, assessments of duration-dry will be dependent upon both the rate at which the plants were previously dried as well as the prevailing RH during the dry period. Therefore,

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a study of the effect of different rates of rehydration is only possible in the context of the previous drying conditions. One way to explore the interacting effects of the factors is to hold two factors constant and assess the other two factors. Here, we hold the *RoD* and *RHeq* constant and determine the influence of *RoR* (as prehydration) on the ability to tolerate different *DDs* (durations-dry).

Duration-dry (*DD*)

As noted by Hoekstra (2005), 'the maximum duration of the dry period at a given temperature can be expected to be ecologically important', with ageing 'mainly the result of free radical damage'. A multitude of studies has assessed duration-dry (*DD*) among bryophytes. At least 84 species have been subjected to various tests of *DD* (summarised in Stark et al. 2017, with *DD* variously known as 'desiccation time', 'desiccation period', or 'longevity') with the experimental duration-dry ranging from hours to years. Even very short *DDs* in mosses may elicit damage. For example, an increase in *DD* of only one day resulted in greater ion loss upon rehydration (2 vs. 1 d; Brown and Buck 1979). With very short *DDs*, the two factors of *RHeq* and *DD* overlap, with an equilibration period normally from 12–48 h.

The ability to tolerate longer periods of dryness is directly influenced by hardening, as evidenced by the seasonality of DT experienced within the same populations (Dilks and Proctor 1976). Although shoots of mosses are most often the subject of *DD* studies, diaspores including gemmae, meiospores, and fragments indicate germination percentage declines over a matter of days (Cleavitt 2002; Stieha et al. 2014) to weeks (Löbel and Rydin 2010) depending on the species and the diaspore size. During a *DD*, a lit environment while dry is more damaging to plants than a darkened environment, with results more marked after longer *DDs* (Moore et al. 1982; Alpert 1988). Michael C. F. Proctor and his colleagues documented the effect of *RHeq* on *DD* in a number of bryophytes, with the experimental design of multiple *RHeqs* × multiple *DDs* × multiple species (e.g. Proctor 2003; Proctor, Ligrone, et al. 2007; Léon-Vargas et al. 2006), revealing that vulnerabilities and optimal tolerances of *RHeq* are remarkably species specific.

Length of duration-dry and recovery

Recovery in bryophytes subjected to desiccation stress has been often assessed using CO_2 assimilation, respiration, chlorophyll fluorescence, electrolyte leakage, and/or survival. By several measures, recovery is faster, for experimental plants collected in the field, where *DD* is shorter, and recovery takes longer or is not achieved where *DD* is longer (e.g. Stålfelt 1938; Hinshiri and Proctor 1971; Dilks and Proctor 1974 for 10 species; Davey 1997 for 13 species; Csintalan et al. 1999 for 3 genera; Proctor and Pence 2002; Léon-Vargas et al. 2006 for 9 species; directly related in Lüttge 2011 for cyanobacteria; Coe et al. 2012; Manukjanová et al. 2014 for 8 species; Munzi et al. 2019). Sharp differences in recovery times using F_v/F_m (maximum photochemical efficiency of dark-adapted PSII) are illustrated in shoots of *Bryum argenteum* Hedw. exposed to 7 d *DD* (5 min to recovery) vs. 1 yr *DD* (6 h to recovery; Li et al. 2014). Similarly, *Anomodon viticulosus* (Hedw.) Hook. & Taylor exposed to a shorter (8 d) *DD* recovered control levels of F_v/F_m immediately, whereas plants exposed to a longer (40 d) *DD* only recovered to 60% of control levels of F_v/F_m (Proctor and Smirnoff 2000), suggesting that extending the *DD* increases damage to the photosynthetic apparatus. Field collected plants are expected to be hardened (Bopp and Werner 1993) and this is likely responsible for the sometimes very long *DDs* tolerated in the aforementioned studies.

Constitutive desiccation tolerance

Field populations of *Syntrichia ruralis* (Hedw.) F.Weber & D.Mohr are frequent subjects of DT studies, and the species is considered constitutively protected from desiccation damage (Constitutive Desiccation Tolerance, CDT; Oliver et al. 2005). However, what constitutes an ecophysiologically constitutive response is debatable. A strict definition is a plant that does not incur damage from a drying event regardless of the rate of desiccation (*RoD*) or the depth of desiccation (*RHeq*). A looser definition of plant CDT is the ability to fully recover from a rapid-dry event at lower relative humidities. The latter definition invites questions relating both to the rate of drying equating to a rapid-dry event (minutes? hours?) and the timeline of recovery (24 h postrehydration? 3 d postrehydration?). In addition, recovery can be assessed on the tissue previously dried, or recovery can occur through regeneration of new tissues from damaged tissues. In the present study, we view a constitutive response in a bryophyte as consisting of damage protection, rather than damage repair; the latter processes are normally construed as inducible (Proctor, Oliver, et al. 2007). This distinction for CDT is not trivial, since most studies portray the bryophyte clade, or at least mosses, as species with constitutive protection from DT, while regarding DT seed plants as incorporating inducible mechanisms (e.g. Charron and Quatrano 2009; Toldi et al. 2009; Dinakar et al. 2012; Cea et al. 2014; Challabathula et al. 2016; reviewed in Stark 2017).

Syntrichia ruralis and its relatives

The moss *Syntrichia ruralis* is broadly distributed across landmasses in the non-tropical northern hemisphere, and is widely studied as a barometer of plant desiccation tolerance. Mojave Desert plants of *S. ruralis* collected and stored in an herbarium were still viable (although heavily damaged) after 20 years (Stark et al. 2017). Additionally, a Hungarian population recovered almost immediately from a *DD* of 60 d (Proctor and Pence 2002) and southern California chaparral plants dehardened for 7 d then exposed to a slow-dry event, tolerated 9 months of *DD* (dark) with full recovery in 24 h (Alpert and Oechel 1987). Proctor (2001, 2003) investigated the relationship between *RHeq* and *DD* on recovery for shoots of *S. ruralis*. Using 24 h postrehydration measurements of F_v/F_m, shoots exhibited full recovery after 60 d *DD*, but after 120 d *DD* F_v/F_m declined sharply only at the two highest *RHeqs* (43 and 74% RH), with close to full recovery at the two lowest *RHeqs* (5 and 20% RH), and optimal tolerance for dry durations at 32% RH. While Canadian populations of *S. ruralis* exhibited depressed net O_2 evolution after a *DD* of 7 d compared to a *DD* of 2 d (when rapidly dried to 22% *RHeq*), the authors found 'this trend was not consistent in other experiments, and was not explored further' (Schonbeck and Bewley 1981a), perhaps either indicative of significant ecotypic variation in this species for the DT trait *DD* (Schonbeck and Bewley 1981b), or inviting a reevaluation of the systematics of this species, as it is possible that Canadian and Mojave Desert populations of *S. ruralis* are not conspecific (unpublished data, John C. Brinda, 2020).

Other members of the genus *Syntrichia* Brid. exhibit significant variation in response to *DDs* even within the same species, and may exhibit remarkable seasonality for this trait (Dilks and Proctor 1976). After only a 5 d *DD*, carbon balance (the integrated net carbon balance following exposure to a rain event, sensu Coe et al. 2019) became negative in field-collected *S. caninervis* Mitt. shoots that had previously been dehardened (Coe et al. 2012), although light damage cannot be ruled out. Oddly, when *S. caninervis* shoots were stored for either 14 d or 1 yr at 20% RH, no regeneration differences were found (Zhuo et al. 2018); this latter result may be explained by prior field hardening. Plants of *S. ruraliformis* from the UK easily tolerated a *DD* of 12 months at 32% RH when the shoots were collected in March, but were much less tolerant (using net assimilation 24 h postrehydration) when collected in October (Dilks and Proctor 1976), the results noteworthy given the plants were dehardened for 7 d prior to test.

Prehydration studies

The poikilohydric nature of bryophytes allows plants to exchange water vapour with the atmosphere, and therefore tissue water content (WC) will be higher at higher equilibrating RHs (Glime 2019). Several studies find that WC of bryophyte shoots, following desiccation then exposure to various equilibrating RHs (*RHeq*), increases most sharply when *RHeq* reaches 80% and above (e.g. Anderson and Bourdeau 1955), with a marked rise in WC between 95 and 100% *RHeq* (Dhindsa 1985). Exposure of bryophytes to humid air when plants are either hydrated or desiccated, although understudied, has long been known to (1) mitigate damage incurred from desiccation (e.g. Schonbeck and Bewley 1981a for *S. ruralis*; Pardow and Lakatos 2013 for tropical bryophytes); and (2), harden bryophytes to the current or to a future drying event (Beckett 1999). The first process is known as prehydration, and the second known as partial drying. Thus exposure to a humid environment carries benefits not only for desiccation recovery, but also induces protection prior to desiccation. For example, a prehydration treatment resulted in significantly less loss of intracellular K from shoots of several bryophytes (Brown and Buck 1979), suggesting some mediation of either membrane damage during drying or membrane damage during rehydration by prehydrating shoots first. For *S. norvegica* F.Weber, as long as the *RoD* was >30 min from full turgor to leaf curling, prehydration effectively protected shoot apices from heavy damage or dying following rehydration with liquid water (Slate et al. 2018). The critical tissue WC for mitigation of desiccation-induced damage is unknown, but the time required to reach constant mass at 100% RH is about 4 h in *Syntrichia* (Slate et al. 2018; cf. Dhindsa 1985). Remarkably, prehydration of previously dried tropical cloud forest mosses at ≥85% RH activated photosynthesis (using F_v/F_m) in ~2 h (Lakatos 2011), in some cases with no differences with and without liquid water at 100% RH (Pardow and Lakatos 2013). Prehydration of tissue previously exposed to *RHeqs* ≤50%, which equates to a WC of $\sim\leq 0.1$ g g^{-1} DW, eases the membrane and cytoplasm transition from a vitrified (glass or gel) to a liquid crystalline phase, avoiding the damage incurred when liquid water is absorbed directly by cells in the gel phase (Hoekstra et al. 2001). While the majority of research on prehydration has occurred under controlled laboratory conditions, environmental monitoring has shown that conditions allowing prehydration of desiccated mosses are likely to occur in their native habitats (e.g. Zheng et al. 2017; Clark 2020), thus probing the influence of prehydration on recovery from desiccation has broad ecological relevance. While it has been suggested that prehydration mitigates cellular damage incurred during desiccation, it is still unknown how prehydration interacts with duration-dry to influence physiological recovery.

Hypotheses

We hypothesise that the ability to tolerate long periods of desiccation declines with time in cultured, fully dehardened (to desiccation) plants, and this ability can be improved when shoots are allowed to prehydrate prior to the addition of liquid water.

Materials and methods

Species notes and culturing protocol

Syntrichia ruralis is a common moss of semiarid landscapes in western North America. It has a widespread holarctic distribution and has been treated as either a single polymorphic species or as part of a complex along with several smaller, poorly defined taxa. Typical *S. ruralis* may be recognised by its unistratose, ovate-lingulate leaves with strongly differentiated hyaline basal regions. These leaves are rounded to the apex and crowned with a hyaline, spinose hair-point. They are somewhat twisted around the stem when dry and recurved to squarrose when wet. In addition, the leaf margins are tightly recurved along most of their length and the laminal cells are small, with several low, branched papillae on both surfaces. Finally, the stem lacks a central strand and the leaf costa lacks any associated hydroids. The Mojave Desert plants studied here are less robust than plants from more mesic environments, but agree in these essential characters. Shoots from a Mojave Desert collection of *Syntrichia ruralis* (*John Brinda 7192*, USA, California, San Bernardino County, Granite Mountains at Granite Pass, 27 March 2015, *MO*) were placed into culture (plastic Petri dishes, inner diameter 35 mm), decontaminated of visible algae and bacteria through successive subculturing of shoot apices, grown to maturity on locally collected fine sand (pH-neutral, sieved at 500 µm, dry-autoclaved 60 min at 121°C), and watered on alternating weeks with sterile distilled water and with a 30% inorganic nutrient solution (Hoagland and Arnon 1938). These single-clone lines were cultured in Petri dishes placed in a growth chamber set to a 12 h photoperiod (20°C light, 8°C dark, noting actual temperatures ranged from 16–19°C light and 6–9°C dark due to use of a lower shelf in the chamber) at ~90 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR). Clonal lines used in the present experiment had been subcultured and grown to maturity through at least three asexual generations. Cultures were maintained with superincumbent water (in effect a diluted nutrient or water solution), i.e. in a suprasaturated state, in order to inhibit the potential of hardening to DT under normal culturing conditions, as can occur (Hájek and Vicherová 2014), allowing us to assess the inherent DT of dehardened shoots. Shoot apices ~2 mm in length from cultures up to one year old were used in the experiment.

Table 1. Experimental design for testing the influence of prehydration on the duration-dry tolerance of shoot apices of *Syntrichia ruralis*.

Factor of desiccation tolerance	Treatment
Rate of drying (*RoD*)	<6 min from full turgor to leaf curling
Equilibrating relative humidity (*RHeq*)	42%
Duration-dry (*DD*)	3, 10, 20, 30, 40, 50 days
Rate of rehydration (*RoR*)	(a) 24 h @100% RH then liquid water, or (b) liquid water directly

Experimental design (Table 1)

Rate of drying (*RoD*) and equilibrating relative humidity (*RHeq*) were held constant, and duration-dry (*DD*) was varied from 3 to 50 days, consistent with the range in *DD* this species may encounter in nature. Following each *DD* treatment, one set of shoot apices was subjected to a 24 h prehydration period prior to rehydration with liquid water, while the other set of shoot apices was rehydrated directly with liquid water (control, the no-prehydration treatment). Dark-adapted chlorophyll fluorescence was assessed 20 min and 24 h postrehydration, and visual leaf damage was assessed 7 d postrehydration.

Water content

Shoot water content (WC) on a dry weight (DW) basis was determined for shoots taken directly from cultures (*suprasaturation*), blotted of visible external water (*full turgor*), at equilibration with 42% RH (*RHeq* 42%, selected to be a few points below 50%, i.e. below the phase change of water to gel, water potential ~117.1 MPa, 20°C), and after a 24 h equilibration with 100% RH (*prehydration*), as follows. A group of 3–4 shoot apices each ~3–5 mm in length was clipped directly from cultures and weighed such that the weight included any free water elevated above the substrate surface, blotted for ~10 s on a chemical wipe until free water was not detected at 60× magnification, weighed quickly (seconds) to the nearest µg, placed in a desiccator targeting 42% RH and allowed to equilibrate 7 d, weighed quickly, and then placed at 100% RH (Figure 1) for 24 h, and weighed quickly once again. The shoot group was then oven-dried for 3 d at 80°C and reweighed for the final time. WC was calculated as follows: [(Experimental Wt – Oven-dry Wt)/(Oven-dry Wt)] × 100, yielding a percentage DW for each of the 'experimental wt' states (suprasaturation, full turgor, *RHeq* 42%, and prehydration).

Desiccating technique

One or two shoots of *S. ruralis* were removed from cultures that were up to one year old and the shoot apices cut to ~2 mm in length (Figure 1B). Cut shoot apices were cleaned, blotted on a chemical wipe until free visible external water dissipated from the apex as viewed at 60× magnification, and placed into a Petri

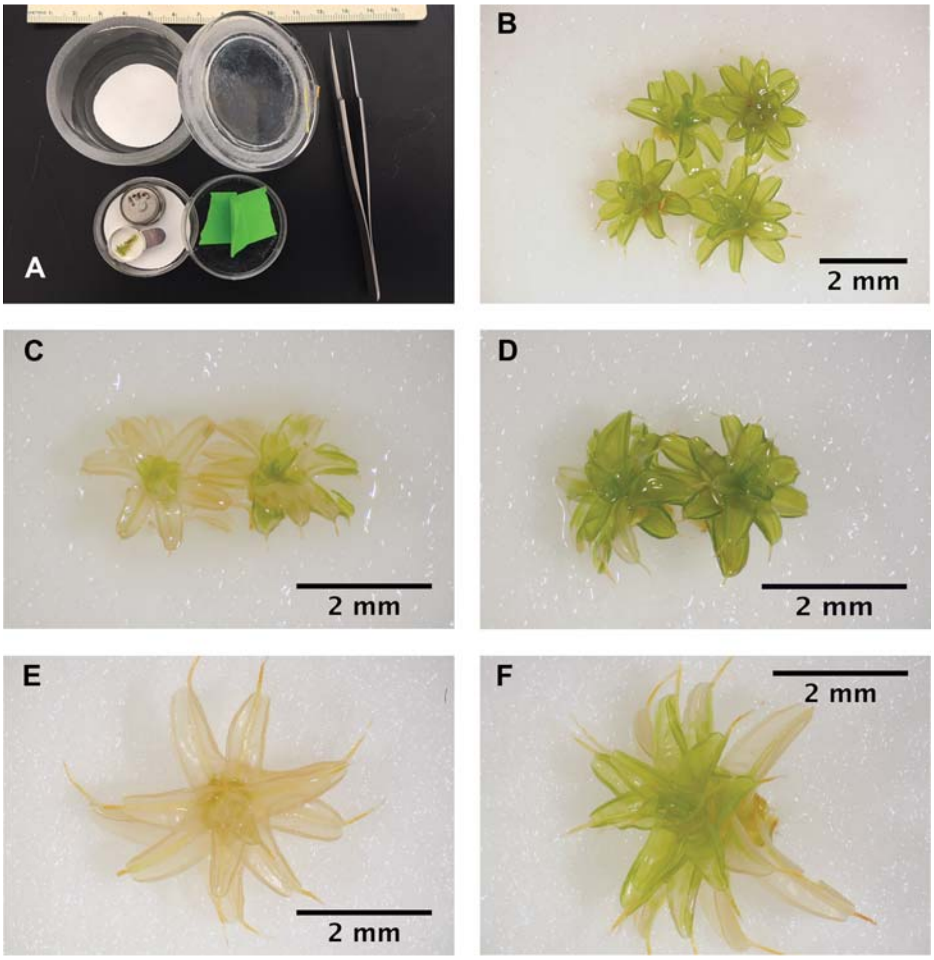

Figure 1. (A) Prehydration setup consisting of a lidded Petri dish inside a lidded glass jar, each with saturated filter paper, with shoots on unsaturated filter paper in weigh boat alongside an *iButton*. (B) Control shoots that have never dried and cultured in a suprasaturated condition. Representative variation in visual shoot damage photographed 7 d postrehydration from (C) a 3 d duration dry (*DD*) without prehydration, (D) a 3 d *DD* with prehydration, (E) a 40 d *DD* without prehydration, and (F) a 40 d *DD* with prehydration. Preceding each *DD* the shoots were rapidly dried to equilibration with 42% relative humidity.

dish containing 1 sheet of filter paper (Whatman #1) and a Thermochron *iButton* (Data logger temperature & RH, model DS 1923, Embedded Data Systems, Lawrenceburg, KY, USA) positioned between the shoots. This Petri dish (unlidded) was moved into a desiccator set to 42% RH above a saturated solution of potassium carbonate (K_2CO_3). A transparent lid was used on the desiccator, and by viewing under the lowest light setting under a dissecting microscope the time (to the nearest minute) to leaf curling was recorded until it was clear that all shoots dried in this fashion exhibited leaf curling in <6 min (a standard rapid-dry, RD, event). Desiccators were kept at continuous low light (~3 µmol m^{-2} s^{-1} PAR) in an environmental room set to 20°C for up to 50 days. Control plants were assessed directly from the cultures (never dried).

Chlorophyll fluorescence

Groups of 1–2 shoots (the same shoot groups described under Desiccating technique above) were rehydrated (or used directly from cultures if controls) in a drop of sterile water, placed in a fluorescence clip on a chemical wipe dipped in water allowing the shoots to remain hydrated (suprasaturated sensu Stark 2017), dark-adapted for 20 min, and assessed using a modulated chlorophyll fluorometer (FMS2, Hansatech, King's Lynn, UK) at 20 min and 24 h postrehydration (following the 'Austin Protocol': Wood 2007). 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 (~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 centres 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_v/F_m) followed by an assessment of the effective quantum yield of PSII photochemistry (Φ_{PSII}) and photochemical quenching (*qP*), the latter two measurements determined as described in Genty et al. (1989). F_v/F_m is a measure of general physiological condition of the photosynthetic apparatus; Φ_{PSII} represents the fraction of excitation energy flowing through PSII and hence is an indicator of active photosynthesis (Green and Proctor 2016); and *qP* is a 'measure of the oxidation state of the first electron acceptor Q_A' (Proctor, Oliver, et al. 2007), and represents the proportion of PSII reaction centres that are open (Maxwell and 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 and Vicherová (2014).

Shoot damage

Following the assessment of chlorophyll fluorescence, shoots were placed on 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. Visual leaf damage was assessed on day 7 by examining the shoots at 60× magnification and assigning each (of 10–20) leaves along a shoot to one of three categories: entirely chlorophyllous (green, value = 1), partially chlorophyllous (partial cell damage, value = 0.5), or entirely chlorotic (brown, value = 0).

Statistics

Generalised linear models (GLMs) were used to compare the individual and interacting effects of varying duration-dry (*DD*) and exposure to prehydration on shoot physiological condition. Fluorescence parameters were modelled individually (F_m, F_v/F_m, Φ_{PSII}, and *qP*) as dependent variables and separately for the 20 min and 24 h postrehydration measurements with *DD* and prehydration modelled as fixed factors (8 GLMs). A GLM was also used to assess the individual and interacting effects of varying *DD* and exposure to prehydration on leaf damage where leaf damage was modelled as a dependent variable with *DD* and prehydration modelled as fixed factors. Prior to analyses, normality of each dependent variable was assessed with histograms of the residuals and homoscedasticity with scatterplots of the residuals and estimated fitted values. In all cases, our data met the assumptions of a linear model. GLMs were used to account for our unbalanced sample sizes. X^2- and *p*-values were estimated using the Anova function in the car package (Fox and Weisberg 2011) and Tukey HSD post-hoc contrasts were conducted with the emmeans package (Lenth 2018). All analyses were conducted in R version 3.5.1 (R Core Team 2018).

Results

Water content, equilibrating relative humidities and fluorescence controls

Water content (WC) of shoot apices of *S. ruralis* ranged from 11.9 ± 1.3% at a *RHeq* = 42% to 2390 ± 165.2% in the suprasaturated state (Table 2). Control values of chlorophyll fluorescence (Table 3) were derived from plants grown continuously in culture in a suprasaturated condition.

Prehydration, duration-dry, and their interactions

At the first postrehydration measurement (20 min postrehydration to allow for dark adaption), chlorophyll fluorescence (F_m, F_v/F_m, Φ_{PSII}, and *qP*) values were significantly higher in shoots that were prehydrated 24 h (Figure 2, Supplemental Table 1). These overall positive effects of prehydration on shoot fluorescence parameters were still evident after a 24 h recovery period (Figure 2, Supplemental Table 1). Prehydrated *S. ruralis* shoots also had less leaf damage 7 d postrehydration than non-prehydrated shoots (Figure 3, Supplemental Table 3). In contrast to the effects of prehydration, *DD* had an overall negative effect on shoot damage that was evident immediately after rehydration (20 min) for all fluorescence parameters but dissipated with 24 h in three of the four fluorescence parameters (F_m, F_v/F_m, and Φ_{PSII};

Table 2. Tissue water content (WC) on a dry weight basis (DW) for shoots of *Syntrichia ruralis* taken directly from culture (suprasaturated), blotted of visible external free water (full turgor), equilibrated for 3 days at 42% relative humidity (RH), and held at 100% RH for 24 h (prehydrated).

Condition of shoot	Tissue water content (% DW)
Suprasaturated	2390.7 ± 165.2
Full turgor	406.1 ± 25.4
Equilibrated with 42% RH	11.9 ± 1.3
Prehydrated at 100% RH	207.0 ± 29.4

WC was calculated as [(Experimental Wt – Oven-dry Wt)/(Oven-dry Wt)] × 100, yielding a percentage DW, *N* = 3.

Table 3. Control values for selected chlorophyll fluorescence parameters of shoot apices of cultured uniclonal *Syntrichia ruralis* shoots either subjected to a 24 h prehydration treatment (prehydrated) or rehydrated with liquid water directly (not prehydrated).

Parameter	Prehydrated	Not prehydrated
F_m	400.3 ± 42.5	376.7 ± 49.4
F_v/F_m	0.567 ± 0.032	0.578 ± 0.030
Φ_{PSII}	0.515 ± 0.038	0.520 ± 0.035
qP	0.845 ± 0.019	0.830 ± 0.022

Plants were dark-adapted for 20 min, values represent Means ± one SE, *N* = 23. See Methods for tissue preparation details.

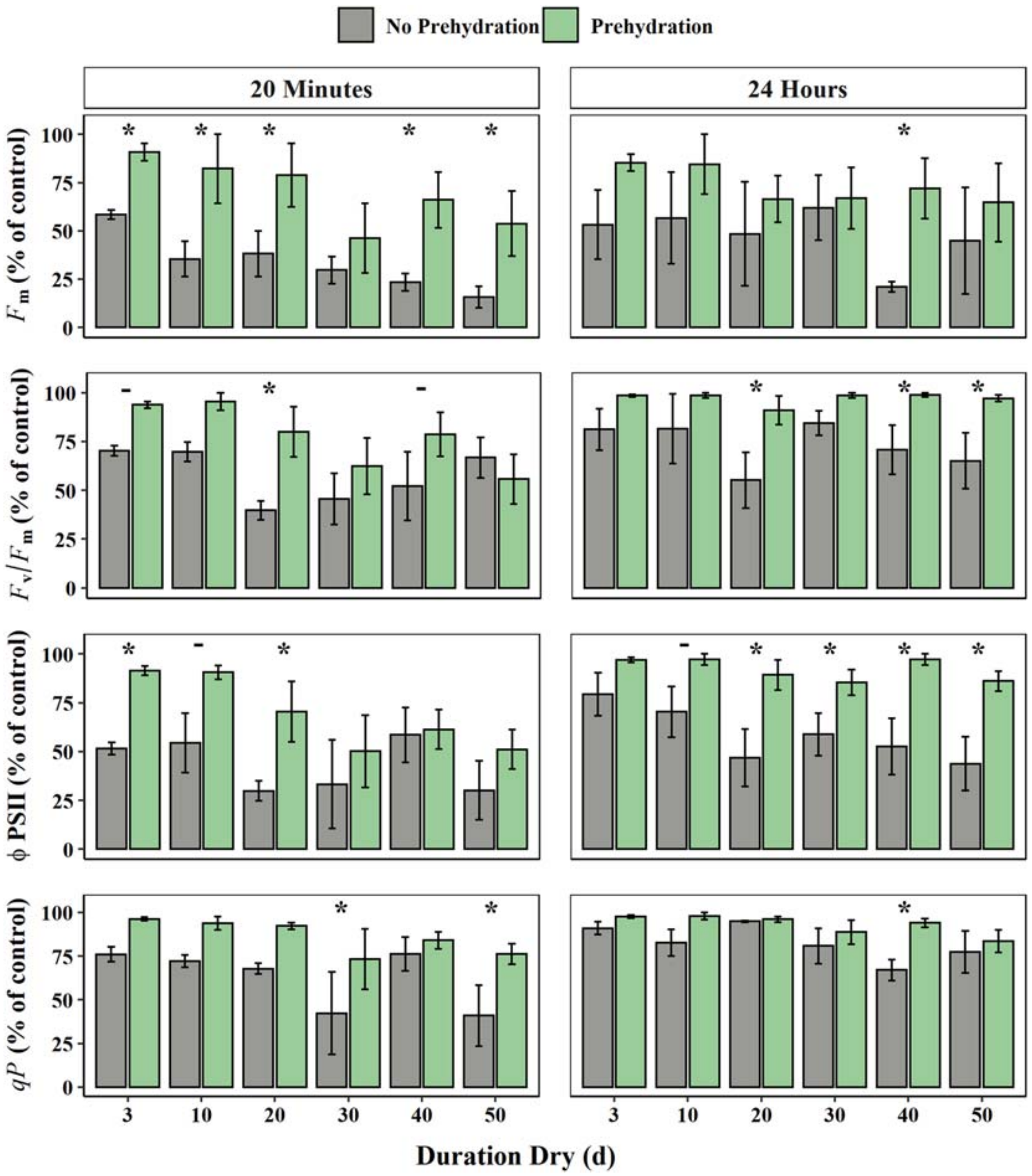

Figure 2. Recovery of *Syntrichia ruralis* cultured shoots subjected to a range of duration-drys (*DD*) and either prehydrated for 24 h prior to adding liquid water or rehydrated with liquid water directly. Prior to the *DD*, shoots were rapidly dried to equilibration with 42% RH. Shoot tips were assessed 20 min and 24 h postrehydration for maximal fluorescence (F_m), maximum photochemical efficiency of dark-adapted PSII (F_v/F_m), effective quantum yield of PSII photochemistry (Φ_{PSII}), and photochemical quenching (qP), shown as a percentage of control plants. Control readings were obtained from a group of 1–2 shoots, and these same shoots were dried and assessed (means ± one SE, $N = 3–4$, for percentages exceeding 100 used 100%). Asterisks indicate differences between treatments when $P < 0.05$; dashes indicate differences between treatments when $P < 0.08$.

Supplemental Table 1). Leaf damage 7 d postrehydration was not influenced by *DD* (Supplemental Table 3).

Prehydration and *DD* did not interact in their overall effect on *S. ruralis* shoots for any fluorescence value immediately (20 min) or 24 h postrehydration (Supplemental Table 1) or influence leaf damage 7 d postrehydration (Supplemental Table 3). However, differences in fluorescence values and leaf damage among prehydrated and non-prehydrated shoots within each *DD* were variable, did not always follow the expected pattern of higher stress with longer *DDs*, and differed among fluorescence parameters (Supplemental Tables 2, 4). For instance, immediate prehydration benefits (20 min postrehydration) on shoots dried for three days were no longer apparent 24 h postrehydration for three of four fluorescence metrics (F_m, F_v/F_m, and Φ_{PSII}; Supplemental Table 2). In contrast after a 40 d DD, the benefits of prehydration (20 min postrehydration) were still evident 24 h postrehydration for two of the four fluorescence metrics (F_v/F_m and F_m; Supplemental Table 2). Yet in other treatments, prehydration benefits that were not immediately evident after rehydration (20 min postrehydration) become so after 24 h (e.g. F_v/F_m at 50 d *DD*

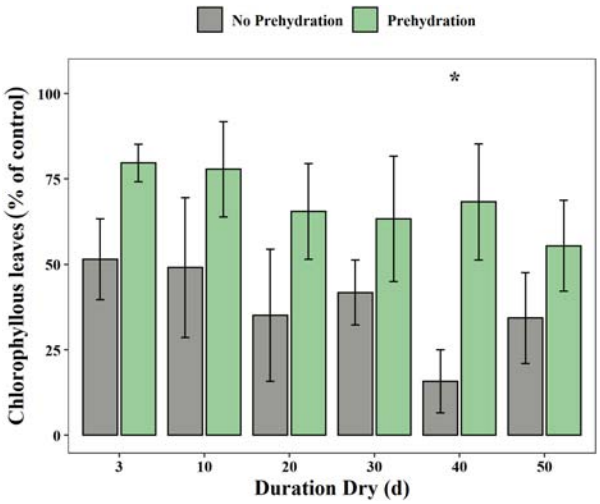

Figure 3. Visual leaf damage on shoot apices of *Syntrichia ruralis* subjected to a range of duration-drys (*DD*) and either prehydrated for 24 h prior to adding liquid water or rehydrated with liquid water directly. Prior to the *DD*, shoots were rapidly dried to equilibration with 42% RH. Shoots assessed 7 d postrehydration by assigning values to each leaf of 1 (fully chlorophyllous), 0.5 (partially chlorophyllous), and 0 (entirely chlorotic). Control readings were obtained from a group of 1–2 shoots, and these same shoots were dried and assessed (means ± one SE, $N = 3–4$). Asterisks indicate differences between treatments when $P < 0.05$.

and Φ_{PSII} at 30, 40, and 50 d *DD*; Supplemental Table 2). Leaf damage was highly variable between prehydrated and non-prehydrated shoots within each *DD*. Leaves of shoots dried for 3 or 40 d were less damaged when shoots were prehydrated prior to rehydration but leaves of shoots dried for 10, 20, 30, or 50 d did not benefit from prehydration (Supplemental Table 4). Nevertheless, mean damage was less at all *DDs* in the prehydration treatment (Figure 3).

Discussion

We found that recovery of all fluorescence parameters at T0 (20 min postrehydration) depended on *DD* (Supplemental Table 1), where we observed an inverse relationship between qP, F_v/F_m, F_m, and Φ_{PSII} on *DD*, but this effect disappeared when shoots were prehydrated. In other words, photosynthetic recovery parameters were unaffected by *DD* when shoots were prehydrated, indicating a potential positive effect of prehydration on recovery of the photosynthetic apparatus. This result partially supported our hypothesis that tolerance of long *DDs* is limited, but this can be mitigated, at least at initial stages of rehydration, by a crucial prehydration phase. At 24 h postrehydration, relationships with *DD* that existed when shoots were assessed at T0 were for the most part no longer apparent, potentially indicating repair of the photosynthetic apparatus had been achieved across *DD* and prehydration treatments at that point. Taken in concert, these results suggest that prehydration allows for structural or functional recovery of the components of the photosynthetic apparatus involved in efficiency of light absorption and transduction, and that these effects are especially important at early stages of rehydration following a desiccation event.

Factors causing damage during a duration-dry

Photodamage to chlorophyll accumulates during desiccation as electrons continue to flow through photosystems, with this excitation energy producing a variety of free radicals (reactive oxygen species, (ROS), primarily produced in mitochondria and chloroplasts in plant cells). Though mosses possess a plethora of antioxidant defence mechanisms, these gradually break down during extended dry periods, with ROS damage ultimately leading to chlorophyll breakdown (Kranner et al. 2008; Jia et al. 2018), bleaching of tissues, and tissue death (Kranner et al. 2002; Moore et al. 2009).

Duration-dry and recovery

Recovery from long periods of desiccation is dependent at least in part on the ability of cells and tissues to reinstate 'species-specific enzyme activities [especially antioxidant levels] during rehydration' (Kranner et al. 2008 and references therein). Thus, tolerance of *DD* is linked to rehydration recovery, with longer *DDs* necessitating longer recovery times until the plant cannot overcome the damage inflicted during an extended *DD*. For extremely sensitive species, a difference in *DD* tolerance can be detected in a single day (loss of intracellular K from membrane damage is greater from mosses exposed to a *DD* of 48 h vs. a *DD* of 24 h at $RHeq = 52\%$; Brown and Buck 1979). Based on tests of a variety of bryophytes, as *DD* is increased, recovery time also increases (e.g. Dilks and Proctor 1974; Csintalan et al. 1999; Proctor and Smirnoff 2000; Proctor 2003; Léon-Vargas et al. 2006; Fernández-Marín et al. 2013; Munzi et al. 2019). When recovery times are on the order of minutes (or seconds) following a *DD*, this result is likely due to field hardening, with the (potentially erroneous) interpretation of such data as indicative of a constitutive strategy of DT among bryophytes (Stark et al. 2014). Tolerance of *DD* is influenced by the equilibrating RH at which plants are maintained (*RHeq*). For example, *S. ruraliformis* (Besch.) Mans. tolerated a *DD* of 100 days better at *RHeqs* of 32 and 54% compared to 76% (Dilks and Proctor 1974). However, tropical cloudforest bryophytes survived best over a *DD* to 19 days at higher *RHeqs* (74 and 85%, Léon-Vargas et al. 2006). In the present study, the overall influence of *DD* on recovery using F_v/F_m, Φ_{PSII}, F_m, and qP was negative immediately following rehydration, but this negative effect dissipated within 24 h postrehydration for all metrics other than *qP* (Figure 2). This may indicate that *S. ruralis* is capable of tolerating longer *DDs* than the longest duration (50 d) incorporated in our design. Additionally, the 24 h recovery shown by *S. ruralis* suggests that this species is capable of rapid repair, yet this is dependent on a 24 h hydration period following rehydration (or 7 d hydration period if considering visual leaf damage), which may or may not be realistic in nature (discussed further below). Importantly, the general decline in fluorescence values with increasing *DD* suggests that as the *S. ruralis* photosynthetic apparatus suffers more severe damage, extended hydration periods for repair become more important. When considered more closely, our results also reveal that damage incurred during increases in *DD* can be variable in intensity (e.g. F_v/F_m values were higher after a 50 d *DD* than a 20 d *DD*). The reason for this is unclear but suggests a need for further research and additional response metrics that better elucidate exactly what is being damaged as mosses experience extended *DDs*.

Comparing *Syntrichia ruralis* to other studies and relatives

Dehardened field-collected *S. ruralis* shoot tips from chaparral habitat tolerated 9 months of dark desiccation following a gradual *RoD*, recovering to near-normal levels of photosynthesis at 24 h postrehydration (Alpert and Oechel 1987). The much greater tolerance of long *DDs* in these conspecific plants (relative to findings here) may be due to the slower *RoD* (hours vs. minutes) coupled with the light regime during the *DDs* (dark vs. low light). Material of *S. ruralis* from Spain recovered well after a 1 week *DD*, but incurred significant damage when *DD* was ≥5 weeks at $RHeq = 0\%$ (Fernández-Marín et al. 2013). Material of *S. ruralis* from Hungary behaved broadly similar to our findings, although still tolerating a 60 d *DD* with minimal damage and preferring lower *RHeqs* (Proctor 2003). *Syntrichia ruralis* shoot tips appear more tolerant of longer *DDs* than those of the close relative *S. caninervis*. In the latter species, a *DD* of 5–10 days resulted in a C deficit (Coe et al. 2012), while *S. ruralis* cultured shoots in the present study exhibited >50% recovery of Φ_{PSII} and F_v/F_m up to a *DD* of 40 days (Figure 2, Φ_{PSII} T24, non-prehydrated shoots). However, light intensity during the *DD* was greater in the *S. caninervis* study (250 vs. 3 μmol m^{-2} min^{-1}), which may contribute to damage, and the postrehydration period was shorter than 24 h (unpublished data, KKC).

Initial depression of fluorescence at T0 vs. T24

When dehardened or cultured mosses recover from a *DD*, it is typical for photosynthesis rates or fluorescence levels to be initially lower upon rehydration as compared to 24 h postrehydration (e.g. Penny and Bayfield 1982). Such a result implicates cellular repair processes active during the first 24 h of rehydration (Oliver 1991) and perhaps lends credence to the hypothesis of bryophytes employing constitutive protection (CDT) coupled with rehydration repair (Proctor, Oliver, et al. 2007). However, the degree of constitutive protection probably varies widely among species, and finding depressed initial readings of chlorophyll fluorescence in dehardened plants suggests that the majority of bryophytes incorporates a strategy of inducible desiccation tolerance (IDT; see references in Stark 2017). We postulate that initial fluorescence readings (our time zero, T0) reveal damage, while fluorescence readings 24 h postrehydration (our time 24, T24) indicate the degree of postrehydration repair. This hypothesis is dependent upon the plants under study being dehardened to DT; otherwise any physiological hardening present in plants will show up as elevated T0 readings. If we accept the premise that T0 readings indicate damage, then a strictly constitutively protected species (CDT) would not be expected to exhibit depressed T0 levels of (especially) F_v/F_m and Φ_{PSII}. This pattern is not as strong for *qP*, suggesting that photochemical quenching may be constitutive so that it activates immediately following rehydration to minimise oxidative damage. *Syntrichia ruralis* assessed herein uniformly exhibited depressed T0 readings, and thus cannot be considered as strictly CDT.

Prehydration effects

Subjecting desiccated plants to humid air for a set period of time prior to adding liquid water (prehydration), is seldom incorporated into rehydration protocols despite evidence for mitigating effects on plant health (Brown and Buck 1979; Schonbeck and Bewley 1981a; Slate et al. 2018) and evidence of its likeliness and frequency in nature (Clark 2020). Unlike *S. norvegica*, where beneficial prehydration effects largely disappeared when plants were dried rapidly (<20 min, full turgor to leaf curling, to equilibration with 0% RH; Slate et al. 2018), *S. ruralis* shoots, although dried rapidly (<6 min, to equilibration with 42% RH) still experienced a broadly positive prehydration effect, with elevated levels of most chlorophyll fluorescence measures over the entire course of the 50 d *DD*. Further, an even stronger beneficial prehydration effect is expected when slower (and more realistic) *RoDs* are tested. This result highlights the biologically significant effects of prehydrating, and encourages further study of this often overlooked phenomenon. In addition, the differences between these two related species in their response to rapid-drying indicates an ecological strategy of DT that is more inducible for *S. norvegica* and less inducible (i.e. with more constitutive protection) for *S. ruralis*. Alternatively, these species differences may instead derive from differences in the *RHeq* in each experiment (0 vs. 42%). The benefit of prehydration derives from a moderation of the membrane phase change from gel to liquid crystalline, in which membranes and cytoplasmic contents are not exposed to liquid water while in the gel (rigid) state (Tetteroo et al. 1995). Prehumidification of tissues protects the membranes from a rapid phase change (from gel to liquid crystalline) during imbibition, mitigating the damage associated with (presumably) the inrush of liquid water against rigid plasma membranes in the gel phase (Hoekstra et al. 1999).

As *RHeq* of the prehydrating environment increases above 80%, and especially above 90% RH (at 20°C), sharp increases in WC of the shoots occur, which is species-specific, to a maximum prehydrated WC at 100% *RHeq* (Anderson and Bourdeau 1955; Egunyomi 1979; Dhindsa 1985). In this partially hydrated state positive carbon balance is not possible in *S. ruralis* though respiration and metabolism are activated (Dhindsa 1985). However, in some bryophytes, carbon balance can be positive (Lange 1969) and recovery complete, i.e. at a level equating to the addition of liquid water (Lakatos 2011). Oddly, in a prehydrated condition the leaves of Canadian *S. ruralis* remained curled around the stem (the 'desiccated morphology', Schonbeck and Bewley 1981a), whereas the California plants tested here unfurled more or less completely in a few hours when maintained at 100% RH. The benefits of a 24 h prehydration exposure in *S. ruralis* indicate, in comparison to non-prehydrated shoots, a more efficient recovery of photosynthesis (Figure 2).

Conclusions

Our hypothesis that a prehydration treatment of desiccated shoots improves their ability to tolerate extended dry periods was generally sustained, although the *DD* effect on plant response was not strongly developed. The beneficial influence of a prehydrating period for mosses has likely been underestimated in studies examining desiccation and rehydration, an understandable oversight given the common methodology of hydrating dried study plants directly with liquid water. Even at the longest *DD* in the experiment (50 d), shoots from both control and prehydration treatments, while damaged, were still alive following a rapid-dry at low humidity. This longevity is noteworthy given that the shoots tested here were grown in a suprasaturated condition—with liquid water present at all times in the shoot apex region. This continuous condition inhibits even the slightest amount of hardening in culture, and points toward the presence of some constitutive processes in the plants. We postulate that (1) depressed time-zero (T0) fluorescence readings taken immediately upon rehydration can be interpreted as the degree of damage suffered by the plants; and (2) 24 h postrehydration (T24) fluorescence readings can be interpreted as the strength of recovery processes. Considering both points (1) and (2) above, *S. ruralis* is assessed as a (strongly to moderately) inducibly desiccation tolerant species (IDT), i.e. with constitutive elements imbedded within its IDT strategy.

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