A field portable method for the semi-quantitative estimation of dehydration tolerance of photosynthetic tissues across distantly related land plants

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Desiccation tolerant (DT) plants withstand complete cellular dehydration, reaching relative water contents (RWC) below 30% in their photosynthetic tissues. Desiccation sensitive (DS) plants exhibit different degrees of dehydration tolerance (DHT), never surviving water loss >70%. To date, no procedure for the quantitative evaluation of DHT extent exists that is able to discriminate DS species with differing degrees of DHT from truly DT plants.

We developed a simple, feasible, and portable protocol to differentiate between DT and different degrees of DHT in the photosynthetic tissues of seed plants and between fast desiccation (<24 h) tolerant (FDT) and sensitive (FDS) bryophytes. The protocol is based on (1) controlled desiccation inside Falcon tubes equilibrated at three different relative humidities that, consequently, induce three different speeds and extents of dehydration and (2) an evaluation of the average percentage of maximal photochemical efficiency of PSII (F_v/F_m) recovery after rehydration.

Applying the method to 10 bryophytes and 28 tracheophytes from various locations, we found that (1) imbibition of absorbent material with concentrated salt-solutions inside the tubes provides stable relative humidity and avoids direct contact with samples; (2) for 50 ml capacity tubes, the optimal plant amount is 50–200 mg fresh weight; (3) the method is useful in remote locations due to minimal instrumental requirements; and (4) a threshold of 30% recovery of the initial F_v/F_m upon reaching RWC ≤30% correctly categorises DT-species, with three exceptions: two poikilochlorophyllous species and one gymnosperm.

The protocol provides a semi-quantitative expression of DHT that facilitates comparisons of species with different morpho-physiological traits and/or ecological attributes.

Abbreviations – DHT, dehydration tolerance; DS, desiccation sensitive; DT, desiccation tolerant; DW, dry weight; FDT, fast desiccation (≤24h) tolerant; FDS, fast desiccation sensitive; F_v/F_m, maximal photochemical efficiency of photosystem II; FW, fresh weight; RH, relative humidity; RWC, relative water content; TW, turgor weight; WC, water content.

Introduction

Desiccation tolerance (DT) is the ability of some organisms to resume normal metabolic activity upon rehydration after being dehydrated to an absolute water content below 0.1 g H_2O g^{-1} dry weight (DW; Bewley 1979, Alpert 2005, Fernández-Marín et al. 2016, Farrant et al. 2017), a water potential ≤-100 MPa, or to a relative water content (RWC) ≤30% (Zhang and Bartels 2018). It is frequently considered as a qualitative trait, and plants are accordingly classified as desiccation tolerant (DT) or desiccation sensitive (DS). DT is common in reproductive structures such as seeds or pollen (i.e., it is estimated that around 90–95% of seeds are DT;

Hong et al. 1998, Gaff and Oliver 2013), but its occurrence among photosynthetic tissues has a much more restricted distribution, particularly among tracheophytes (Alpert 2006, Fernández-Marín et al. 2016, López-Pozo et al. 2018). DT in photosynthetic tissues is quite frequent among lichens and bryophytes, uncommon among pteridophytes, not reported in gymnosperms, and is very rare (135 species) among angiosperms, the latter referred to as "resurrection plants" (Gaff 1977, 1989, Porembski 2011, Gaff and Oliver 2013, Fernández-Marín et al. 2016, Farrant et al. 2017, López-Pozo et al. 2018). Only a few monocots among DT plants degrade chlorophyll and dismantle the photosynthetic apparatus in a reversible manner during dehydration, the so-called poikilochlorophyllous species (Tuba and Lichtenthaler 2011, Fernández-Marín et al. 2016). Upon rehydration, poikilochlorophyllous plants have to reconstruct the photosynthetic apparatus and thus naturally take longer to regain full photosynthetic capacity (Sherwin and Farrant 1996, Farrant et al. 2015).

The mechanism of DT relies mostly on three aspects: (1) the ability to withstand mechanical stress (i.e., preservation of plasmalemmae, cell wall interaction, cell to cell connections through the plasmodesmata, and intracellular compartmentalization into organelles), as well as the ability to preserve ultrastructure and function of macromolecules (i.e., enzymes) at very low intracellular water contents; (2) the ability to reversibly slow down metabolism to the equivalent of a quiescent state; and (3) the ability to cope with high oxidative stress exacerbated during desiccation and rehydration processes (Fernández-Marín et al. 2016). Protection of the subcellular organization is proposed to occur through complex interactions of stress-associated molecules such as LATE EMBRYOGENESIS ABUNDANT (LEAs) and HEAT SHOCK PROTEINS (HSPs), sucrose and osmoprotectants such as raffinose family oligosaccharides (RFOs), as well as the presence of extensive and robust antioxidant systems (Sherwin and Farrant 1998, Hoekstra et al. 2001, Dinakar and Bartels 2012, Farrant et al. 2017, Giarola et al. 2017, Fernández-Marín et al. 2018, Verhoeven et al. 2018). Conversely, DS species may show different degrees of dehydration tolerance (DHT), ranging from the loss of viability after loss of 1% total water content (e.g., as in many succulents) to a maximum of 69% in a few species (Höfler 1941). In this sense, DHT can be defined as a continuum from DS plants with very little tolerance, through to plants with intermediate tolerances, to truly DT plants. This continuum has not been characterized thus far.

The process of rehydration is potentially more damaging than desiccation. It can exacerbate mechanical and metabolic stresses and result in the elevated production of reactive oxygen species (ROS; Kranner et al. 2008). In DT species, rehydration is accompanied by the repair of damage incurred on drying, regulated cell wall unfolding, and the re-establishment of cellular integrity and metabolic activity (Moore et al. 2013, Fernández-Marín et al. 2016).

Consequently, rewatering of desiccated tissues is also a critical component of the experimental protocols developed to characterise the responses of DHT in plants (Slate et al. 2018).

The attainment of tolerance to desiccation depends on both internal (biological) and external (environmental) factors. For example, leaf age or collection season are among the biological factors that influence DT capability in a few species (Farrant et al. 2009, Griffiths et al. 2014), while irradiance and dehydration recurrence as external factors can enhance antioxidant concentration and protective mechanisms in photosynthetic tissues (Lizarazo et al. 2010, Fernández-Marín et al. 2010). Among the external factors, the rate of dehydration (speed), the extent (final water content) and the length of time the tissue is maintained in the desiccated state are particularly relevant (Hoekstra 2005, Koster et al. 2010, and very nicely reviewed recently by Stark 2017a). Thus, even among DT-species, rapid drying can be more damaging as it precludes sufficient time to furnish adequate protection. This general rule applies for most groups of photosynthetic DT organisms, including mosses (Fernández-Marín et al. 2013, Cruz de Carvalho et al. 2012, 2017), green algae (Gasulla et al. 2009, Guéra 2009), lichens (Fernández-Marín et al. 2010, Gauslaa et al. 2012), angiosperms (Farrant et al. 1999, Fernández-Marín et al. 2011, 2018), and even for DT animals, such as tardigrades (Boothby et al. 2017). From this perspective, some differences can also be found within DT plants, with some species being able to withstand drying <30% RWC only when it is reached slowly and/or after acclimation processes (Farrant et al. 1999, Cruz de Carvalho et al. 2011, 2012, 2014). This is particularly true for several bryophyte species that do not survive rapid (typically ≤24 h) desiccation to ≤30% RWC, but will survive if the drying process is much slower (e.g. from several days to up to 1–2 weeks; Stark et al. 2013, Cruz de Carvalho et al. 2014, Hajek and Vicherová 2014, Xiao et al. 2018). Acclimation/deacclimation or hardening/dehardening processes can partially shift the survival response of a species to a certain desiccation regime. For instance, typical DT taxa such as *Syntrichia* species can lose their ability to survive dehydration after long-term dehardening treatment (e.g. several weeks) by incubation under controlled hydrated conditions (Schonbeck and Bewly 1981, Stark et al. 2016). Similarly, traditional DS taxa such as some *Spahgnum* species can survive desiccation provided that controlled hardening (partial dehydration) is applied (Hájek and Vicherova 2014). Depending on the species ecology, these pre-treatments may not, however, reflect what the plants actually experience in nature (Hellwege et al. 1994). While these artificial pre-treatments can be very useful for addressing specific physiological questions (Cruz de Carvalho et al. 2014, Stark et al. 2013b), it is also true that under the same conditions, some bryophyte taxa are more tolerant to fast desiccation than others. As an example, gametophores that previously experienced dehydration events (either in the field or in the lab) are able to survive rapid desiccation, as in

the case of *Syntrichia ruralis* (Schonbeck and Bewley 1981). In contrast, *Physcomitrella patens* requires a slow process of desiccation in order to ensure survival (Xiao et al. 2018).

Based on this differential sensitivity, the so-called "Austin protocol" was proposed as a method to systematically characterise DT among bryophytes (Wood 2007). This protocol was based on the assessment of the recovery of maximal photochemical efficiency (F_v/F_m) after the rehydration of bryophyte samples previously desiccated and equilibrated under two different atmospheric relative humidities (RH): 67–75% and 20–30%. In this protocol, the tested bryophytes were classified into two groups according to their survival at equilibration at either 67 or 23% RH (Wood 2007). With regards to other groups of DT organisms, different desiccation procedures have been used as a method to discriminate recovery levels, including in lichens (Gauslaa et al. 2012, Fernández-Marín et al. 2010), free-living microalgae (Candotto-Carniel et al. 2015), fern gametophytes (Riaño and Briones 2015), and angiosperm pollen (Marks et al. 2014).

As indicated above, vegetative tissues of DS species display different degrees of tolerance to cell dehydration (DHT). We propose that there is a continuous gradient of DHT in such species, while true "DT" can be defined as the ability to recover metabolic activity after severe dehydration to RWC <30%. This distinction is of relevance for the correct interpretation of physiological and –omics data (Zhang and Bartels 2018). Therefore, although the terms dehydration- and desiccation-tolerance have been used synonymously with respect to vegetative tissues (Blum and Tuberosa 2018), here we use DHT as a quantitative continuum trait and DT as a qualitative absolute term, the latter referring only to truly tolerant plants. It is necessary to mention that while this threshold of RWC <30% seems to be clearly defined for tracheophytes (Zhang and Bartels 2018), it may be not that accurate for bryophytes where a much higher proportion of species are known to tolerate this water content if the drying speed is sufficiently slow (>24 h and typically up to several days). Regardless, no simple screening technique for DHT is available that allows for comparisons of plants within a wide phylogenetic range, neither is there a portable protocol reliable for use in remote areas in the field. With this aim, we have developed a standard, portable, and simple procedure for the semi-quantitative evaluation of tolerance to dehydration in a wide range of embryophyte species and photosynthetic tissues in the field, providing evidence of its usefulness from two contrasting case studies with different levels of technical support and difficulty: (1) an assay of bryophytes with contrasting habitat conditions in the field in Spain, and (2) a survey of tracheophytes comprising plants from controlled growing conditions, botanical gardens and from the field in South Africa, Chile, United Kingdom and Spain.

Methods

Protocol set up

General description of the procedure

Only green photosynthetic tissues were used. Individual sample weight and F_v/F_m were recorded at three time points along the protocol, i.e., that of fully hydrated material prior to dehydration ($t_{Control}$), upon full dehydration (t_{Dh}), and upon rehydration (t_{Rh}). After collection in the field, leaf material was maintained in darkness in a saturated atmosphere for 24 h in order to assess maximal F_v/F_m and to obtain turgor weight (TW) at $t_{Control}$ (Fig. 1). Afterwards, plant samples were placed inside 50 ml-Falcon® tubes under three desiccating regimes (RHs: =80%, =50%, and <10%) and were allowed to equilibrate with their respective atmospheres for 24 h (in the case of bryophyte samples) or 48 h (in the case of tracheophytes). Three replicates were used per treatment. After desiccation, samples were removed from the tubes, weighed, and F_v/F_m was again measured (t_{Dh}). Plant samples were finally rehydrated in a saturated atmosphere (≈100% RH) and in darkness for 24 h; a time considered sufficient to obtain an almost complete recovery of F_v/F_m in many organisms (Pandey et al. 2010, Proctor 2010) and for small leaf pieces to recover turgor. F_v/F_m and weight were again measured (represented as t_{Rh}). This allowed the calculation of relative recovery from each dehydration treatment with respect to control values [e.g. for each species and desiccation treatment, we expressed Fv/Fm as the % of the initial value (Hájek and Vicherová 2014)]. Then the average F_v/F_m recovery following the three desiccating treatments was used as a proxy of overall recovery and thus as a quantitative indicator of DHT. Consequently, the majority of DT species should yield higher values (i.e., closer to 100%), while the values in most DS species are expected to be low (closer to 0%). All incubations were conducted in darkness and within a temperature range of 20±5ºC. The complete protocol is summarised in Fig. 1.

Desiccation procedure

Desiccation treatments were performed inside 50 ml-Falcon tubes (Fig. 2A) – hereafter referred as the "Falcon test"– in equilibrium with approximately 12 g of silica gel [RH <10%, which at 20ºC corresponds to a water potential (Ψ) of -310 MPa (Gaff and Oliver 2013)] or with 10 ml of concentrated solution of $MgCl_2$ (280 g of $MgCl_2$ hexa-hydrated were dissolved in 150 ml of Milli-Q water; 50% RH, Ψ≈-94 MPa) or of NaCl supersaturated solution (80% RH, Ψ ≈-30 MPa). The concentrated solution of $MgCl_2$ allowed for a relatively constant ≈50% RH in the temperature range between 15 and 30ºC (Fig. S1, Supporting information). RH is kept constant as soon as the Falcon tubes are incubated in darkness (e.g. to avoid overheating due to a greenhouse effect). One of the main problems of using salt solutions in the field is that they can

easily enter into contact with the samples, irreversibly affecting the measurements. In the present protocol, we have prevented sample moistening by salt solutions (and facilitated handling and transport) by simply absorbing the solutions to coiled pieces (3 x 17 cm) of commercial kitchen sponge (Spontex Natura ®) separated from the samples by plastic mesh (65 mm x 65 mm, 1 mm ø). Relative humidity in the atmosphere of the empty tube and in the tube containing samples was assessed with a RH probe (HMP45C, Campbell Scientific, Loughborough, UK) and recorded with a data logger (CR10, Campbell Scientific, NL; Fig. 2B). Each sensor was placed inside the tube through a hole in the lid of the tube, conveniently sealed once the wire was placed through it. Data were recorded until stabilization every 30 s and averaged every hour.

Optimization of biomass load inside the test tubes and of the time required to reach desiccation equilibrium was performed using different initial amounts (25, 50, 100, 200, or 400 mg of FW) of the moss *Syntrichia ruralis* (Hedw.) F.Weber & D.Mohr (Fig. 2). Once optimised, the same amount of plant material was used for bryophyte and tracheophyte analyses, with the tracheophytes being subjected to a longer desiccation time (48 h).

Rehydration procedure

Rehydration of samples was conducted by contact with wet tissue paper (using distilled water) and incubation in a saturated atmosphere (RH≈100%) for 24 h in darkness. The rehydration procedure was selected after three alternative procedures were tested in a representative group of bryophyte and tracheophyte species, including several bryophytes (*Bryum* sp., *Porella canariensis* (F.Weber) Underw., *Hypnum cupressiforme* Hedw., *Pseudoscleropodium purum* (Hedw.) M.Fleisch., *Plagiomnium undulatum* (Hedw.) T.J.Kop., *Polytrichastrum formosum* (Hedw.) G.L.Sm.), ferns (*Nephrolepis exaltata* (L.) Schott, *Phlebodium aureum* (L.) J.Sm., *Davallia canariensis* (L.) Sm.) and angiosperms (*Amaranthus* sp., *Olea europaea* L., *Fraxinus* sp.), collected from the experimental field of the Universitat de les Illes Balears (UIB; Figs S1 and S2). The first method involved the direct immersion of samples in distilled water. The second procedure involved the maintenance of samples in a Petri dish in contact with moistened tissue paper. The third procedure was as the second, except that the Petri dishes were maintained in a closed chamber with 100% RH. After testing a desiccation-rehydration cycle under the three described methods, it was concluded that moistening with wet tissue paper in a saturated atmosphere was the best protocol that yielded the highest recovery, and hence constituted the adopted methodology. The first procedure (water immersion) was discarded, as it tended to overestimate water content and underestimate recovery after rehydration and also caused visible necrotic lesions, particularly on the excised tissues of vascular plants, which may

affect the results. The other two methods yielded similar results in some species, but in many others the third method allowed for a better recovery.

Determination of absolute water content and relative water content

Plant material was weighed with a Mettler Toledo scale (AB104, Mettler Toledo, Barcelona, Spain) with a precision of 0.1 mg in the case study I (bryophytes), and with a COBOS scale (JT-120M, Balanzas COBOS Precision, Barcelona, Spain) with 1 mg precision for the case study II (tracheophytes). To obtain the dry weight (DW), plant material was dried at 70°C in an oven for 24 h and maintained in a closed box with silica gel until weighing. Water content (WC) was calculated as: WC=(FW-DW)/DW and expressed as g H_2O g^{-1}DW.

Relative water content (RWC) was estimated as the percentage of water content at any time, referred to the maximum (turgid) water content: RWC=(FW-DW)/(TW-DW) × 100, where TW was turgid weight (after 24 h incubation over wet tissue paper and 100% RH in darkness. Note that excess water was blotted with tissue paper before weighing).

Chlorophyll fluorescence

Maximal photochemical efficiency of photosystem (PS) II (F_v/F_m) was used as a proxy for the photosynthetic integrity of the tissue and was measured with a portable modulated PAM fluorometer (Walz, Effeltrich, Germany): PAM 2500 was used in the case study I (bryophytes) and in the protocol setup, and a Junior PAM in the case study II (tracheophytes). The maximum Chl *a* fluorescence yield (F_m) was induced with a saturating pulse, while minimum fluorescence (F_o) was recorded with low measuring light intensities after several hours of dark acclimation. The maximal photochemical efficiency of PSII (F_v/F_m) was then calculated as (F_m-F_o)/F_m. The relative rate of recovery of F_v/F_m after desiccation-rehydration (t_{Rh}) with respect to the initial values ($t_{Control}$) was used as an estimator of tolerance to the reached RH, in each case similar to Hájek & Vicherová (2014). The average F_v/F_m recovery of the three desiccating treatments at t_{Rh} was used as a proxy for the DT level of each species.

Studied species and collection sites

Case study I: Bryophytes

Here, 10 bryophyte species that had been previously well-characterised in their responses to desiccation were analysed, including five liverworts and five mosses, half of which are described as fast desiccation tolerant (FDT; e.g. tolerant to desiccation below 30% RWC within

a time extent ≤24 h) and the other half described as fast desiccation sensitive (FDS; see Table 1 for specific references to each species). Specimens were collected in the field in different locations along a climatic and elevational gradient (400–1300 m a.s.l.) in La Rioja (Northern Spain) in November 2015 (autumn) in the hydrated state (specifications on meteorological conditions the week before sampling are shown in Table S1). Immediately after collection, samples were stored in darkness for 24 h at a moderately variable temperature of 20±5ºC and in a 100% RH atmosphere.

Case study II: Tracheophytes

For this case study, 14 DS (e.g. not previously described as DT) and 14 known DT species were evaluated, the latter including some of the model DT-plants (i.e., *Xerophyta viscosa*, *Craterostigma plantagineum* and *Myrothamnus flabellifolius*; Table 1). The 28 evaluated species included different functional groups (angiosperms, gymnosperms, ferns) and were collected from different sites, during the summers of 2016 and 2017, as specified in Tables 1 and S1. For a number of species, both field-grown and potted plants were compared, with no significant differences in the recovery levels (in agreement with the fact that DT is an inherent character of some species); thus, data from field and potted species were pooled together in the results. The potted plants were grown with horticultural substrate Prohumin® (Projar SA, Valencia, Spain) and perlite (3:1) with additional fertilization (Multigreen®, Haifa Chemicals, Madrid, Spain): 5 g per litre of substrate. The plants were watered daily to field capacity and maintained under optimal conditions. Further details on plant conditions during the 30 days before sampling are shown in Table S1. Only photosynthetic tissue (i.e., leaf blade) of 100–200 mg initial FW, avoiding main venations, was used.

Statistical analyses

Statistical differences among treatments with respect to the WC, RWC, and F_v/F_m of the samples were analysed by one-way analysis of variance (ANOVA) after assessing data homoscedasticity. Alternatively, the Kruskal-Wallis test was used for heteroscedastic data. All statistical analyses were assessed at $\alpha = 0.05$. The SPSS v20 package (IBM Corp., Armonk, NY) was used for the statistical analyses.

Results

Protocol setup

Falcon tubes of 50 ml constituted excellent hermetic containers for individual replicates,

allowing for the handling of a relatively high number of samples in a relatively small space. Sponge and mesh efficiently prevented direct contact between the saturated salt solutions and the samples (Figs. 1 and 2A), concurrently allowing for a relatively quick equilibration of the atmosphere (Fig. 2): i.e., RH inside the tubes reached 95% of the expected equilibrium value in less than 5 h (Fig. 2B). When samples were placed inside the tubes, the equilibrium took longer, but was achieved in less than 24 h (Fig. 2B). When the desiccating tubes were recycled for a second use, equilibrium RH was maintained within the expected range. Samples incubated over silica gel did exhibit an initial slight increase in RH within the tube, but this was nevertheless maintained at < 10% RH during the second cycle.

As expected, equilibrium was reached more rapidly with the strongest desiccant. Thus, silica gel was faster than the $MgCl_2$ and NaCl solutions. After 12 h, at least 99% potential water loss had occurred in the silica gel tubes when the sample mass was ≤100 mg (Fig. 3). In contrast, after 12 h, only the smallest sample (25 mg) had reached 99% potential water loss in the NaCl treatment (Fig. 3A). After 24 h, with the only exception of 400 mg FW in NaCl, 99% potential water loss was reached for all desiccants and masses tested (Fig. 3A). Thus, a FW between 50–200 mg of hydrated bryophyte tissue and a desiccation time of at least 24 h were appropriate to obtain desiccated tissues in equilibrium with the tube atmosphere (Fig. 3B). As the desiccation treatments dried the bryophyte samples in ≤24 h, and given that recent literature indicates that slow desiccation in bryophytes occurs over several days or even weeks (Buda et al. 2013, Stark et al. 2013b, Xiao et al. 2018, Cruz de Carvalho et al. 2017), we used the nomenclature fast DT (FDT) and fast DS (FDS) to refer to the bryophyte species tested. The three RHs used successfully induced three different rates of dehydration and final water contents after 24 h (Fig. 3B). Same amount of initial FW was also suitable for photosynthetic tissue of tracheophytes (Figs S1 and S2).

Case study I: bryophytes with contrasting responses to desiccation

Initial values of F_v/F_m were close to or higher than 0.7 in most of the bryophyte species, except in two FDS species (*Marchantia polymorpha* and *Hookeria lucens*; Table 2). Initial F_v/F_m values were, on average, slightly and significantly lower in FDS than in FDT species: 0.659 ± 0.026 and 0.722 ± 0.013 (average ± SE, respectively). The final WC reached after desiccation treatments did not differ significantly when comparing FDT and FDS species (Fig. 4A) being, on average (± SE), 0.22 ± 0.06 g H_2O g^{-1} DW at RH ≈ 80%, 0.08 ± 0.00 at RH ≈ 50%, and 0.02 ± 0.00 at RH <10%. In contrast, final RWC was slightly but significantly higher in the FDT than in the FDS species at the end of the dehydration treatments (Fig. 4B). The F_v/F_m recovery rate after rehydration clearly discriminated between FDS and FDT species at all RHs, being on

average 18% for DS and 96% for DT species (Fig. 5). FDS bryophytes only showed an intermediate recovery (49%) under the mildest dehydration treatment (RH≈80%; Fig. 5). When considered individually, and for the RH≈80% only, some of the FDS species showed a $\%Fv/Fm_{NaCl}$ higher than 30%: *F. antipyretica*, *H. lucens*, *L. cruciata*, *S. undulata* (Table S2).

Case study II: tracheophytes from different worldwide origins

Initial values of F_v/F_m showed no significant differences between DT (0.736 ± 0.025 on average) and DS (0.757 ± 0.050) tracheophyte species (Table 2). The strength of the different desiccants was strongly related with both WC and RWC (Fig. 4). No differences in WC or RWC were observed between DS and DT species (Fig. 4A). On average, dehydration treatments resulted in 29.6% RWC at 80%RH, 16.4% at 50%RH and 8.4% at RH <10% (Fig. 4B). DT species yielded higher F_v/F_m recoveries after dehydration than DS species, independently of the treatment applied (Fig. 5). On average, recovery was 58% in DT and 24% in DS species.

Overall evaluation of DT

The average value of F_v/F_m after rehydration (used as a proxy for DHT) was calculated for each species. A value ≥30% F_v/F_m recovery was the threshold that best discriminated between FDT and FDS in the case study I and between DT and DS in the case study II (highlighted in Fig. 6), and this was improved further when only samples that had desiccated at RWC≤30% were included in the F_v/F_m average calculation (Fig. 6b, Table S3). The difference between DS and DT was clearly delineated by a gap of between 25 and 92% among bryophytes (Fig. 6). In contrast, recovery values represented a continuum among tracheophytes, ranging from 0% in *Helianthus annuus* to 96% in the filmy fern *Hymenophyllum dentatum* (Fig. 6). Interestingly, several DT-ferns yielded the highest recoveries, whereas two angiosperm 'resurrection plants' (*X. viscosa* and *Barbacenia purpurea* Hook.) presented very low recovery values within the DT group. This was particularly evident in *B. purpurea* (with 9.3% F_v/F_m recovery, which was similar to the lowest values obtained in DS species) when compared to the average of the remainder of DT angiosperms studied (37.9% F_v/F_m recovery). This is likely due to the fact that these two species are poikilochlorophyllous and thus take longer to recover photosynthesis. Conversely, four DS species, namely the angiosperm *Triticum aestivum*, the ferns *Davallia canariensis* and *Blechnum magallanicum,* and the gymnosperm *Juniperus oxycedrus* presented recoveries slightly above 30% (Fig. 6A). Only *J. oxycedrus* maintained this trend, when samples that dried at ≥30% RWC were not included in the F_v/F_m recovery calculation (Fig. 6B).

When all species among each group were considered together, the Falcon test was able to discriminate between FDT and FDS among bryophytes and between DT and DS species among

tracheophytes (Fig. 5). Nevertheless, the average F_v/F_m recovery was much higher in FDT bryophytes than in DT tracheophytes (Figs 5 and 6). Even the highest values of recovery among tracheophytes, observed in ferns, were lower than the lowest values in FDT bryophytes (Fig. 6). This occurred despite that WC and RWC values at the end of the desiccation treatments were remarkably lower (around 4-fold) in the bryophytes for all RHs (Fig. 4, Tables S2 and S3).

Discussion

The method described here, based on different rates of desiccation and equilibrium at different water contents, was able to (1) quantitatively categorise species based on their photosynthetic tissue tolerance to dehydration and (2) discriminate DT species by the threshold value of 30% recovery of initial F_v/F_m, with a few exceptions, notably those DT angiosperms displaying a poikilochlorophyllous strategy. This is very likely because poikilochlorophyllous species take a prolonged time, some 48 h beyond full hydration, to restore the photosynthetic apparatus. Such species break down thylakoid membranes and chlorophyll during dehydration and thus require some time to reconstitute this apparatus to reflect full photosynthetic capability (Farrant et al. 2017). Recovery times longer than 48 h might have thus increased the accuracy of the Falcon test in detecting DT-poikilochlorophyllous species. Nevertheless, poikilochlorophylly represents a minority strategy among DT plants, and the use of much longer incubation times of the detached tissues may have also increased the rate of false negatives among homoiochlorophyllous species. Homoiochlorophyllous plants are able to reinstate photosynthesis more rapidly upon rehydration, as they retain and protect their photosynthetic apparatus (i.e. all DT-bryophytes evaluated in this study). Thus, the Falcon test overall presents a good balance between feasibility, simplicity, portability, broad screening possibilities, and accuracy.

When only two desiccants (NaCl and silica gel) were included in the DT evaluation (data not shown), the results closely matched those using three desiccants. From this perspective, the protocol could be simplified by removing the intermediate desiccant ($MgCl_2$, RH=50%). However, this third desiccant provides a "fine-tuning" that allows for the discrimination of "intermediate" species, that is, with different DHT within the DT group. As an example, some FDT species, such as *Rhizomnium punctatum*, failed to show a complete recovery (under the rehydration conditions of the test) after desiccation at RH <10%, while some FDS species showed a certain degree of recovery after being equilibrated at RH = 80% (i.e., *F. antipyretica*, *H. lucens*, *L. cruciata*, *S. undulata*; Table S2).

The effects of acclimation

The environmental conditions prior to sampling may create a "historical stress memory" that can persist for several days, thereby shifting the speed or extent of the desiccation that a species can tolerate (Stark et al. 2013a). Thus, some DS species, typically bryophytes and ferns, may activate inducible DT mechanisms in response to dry conditions (Cruz de Carvalho et al. 2011), while typical DT species may suffer a de-acclimation to DT when artificially kept under conditions of suprasaturation (Stark et al. 2017b). Conversely, time-lags for the reestablishment of photochemical activity after moistening can be much longer for DT angiosperm species collected from arid habitats or during dry periods (Lidén et al. 2010, Proctor 2010). To minimize such effects, in the present study, bryophytes were collected in autumn during a rainy period. However, this is a factor that should be always taken into account in field studies. Regardless, the preliminary tests in repeated species under both field and laboratory conditions suggest that the results of the Falcon test reasonably reflect the inherent and species-specific character of DT. As an example, despite the initial F_v/F_m value of *M. caffrorum* collected from the field (0.53, Table 2) being below the optimum recorded in the bibliography for this species (around 0.75 was reported in Farrant et al. 2009), it was correctly categorised as DT by the Falcon test (Fig. 6). Thus, a comparison of the final F_v/F_m values recorded for each species with those obtained initially provides a relatively reasonable buffering for slight field-acclimation effects. In addition, the comparison of initial F_v/F_m values to those found in the literature for the same species (when available) can provide an indication of the potential acclimation factors involved in the photochemical stage of the studied specimen. A more complex version of the test that focuses on species-specific plasticity in response to environmental conditions could be developed in future studies.

False negative and false positive exceptions

While the protocol described here was able to discriminate, without exception, between FDT and FDS bryophytes, there was some degree of overlap in the case of tracheophytes. In this group, two main error types may affect the sensitivity of the method to discriminate between DT and DS species: (1) the occurrence of false positives (i.e., species that show apparent recovery of F_v/F_m being severely damaged by desiccation) and (2) the occurrence of false negatives (i.e., species that do not recover F_v/F_m after the experimental desiccation/rehydration described here, but that are able to survive desiccation under natural conditions). False positives may occur in those species, such as spinach, that once dehydrated are able to maintain charge separation in PSII and a reduction in the primary electron acceptor of PS II (Q_A), but not the functionality of the complete electron transport chain (Heber and Shuvalov 2005, Kopecky et al. 2005). Another source of false positives would be xerophytic species (sclerophyllous or succulent plants) that, because of their leaf architecture, would require more time to achieve

equilibrium with the desiccating atmosphere. This error can be avoided by including only species that have been desiccated below a certain threshold of RWC around 30%, which corresponds to the boundary between dehydration and desiccation described by Zhang and Bartels (2018) based on physiological and molecular changes in the tissues, and indirectly suggested in previous works (Farrant and Moore 2011, Ginbot and Farrant 2011, Farrant et al. 2015). Indeed, when we applied this criterion to our results, we obtained 90% success within tracheophytes and 93% overall success in the classification of DT within the analysed species (Fig. 6B). Tracheophytes assessed in our study were dried out ≤ 30% on average (Fig. 4). However, some of the analysed species, such as *Blechnum magellanicum*, *Davallia canariensis,* and *Triticum aestivum,* maintained RWC ≥ 30% under the NaCl desiccation treatment. This may explain their average F_v/F_m values being over 30% (Fig. 6) and reinforces the usefulness of a threshold of 30% RWC to truly distinguish DT from merely DHT plants (Fig. 6B). This boundary RWC, however, may be slightly wider or different among species and for DT photosynthetic organisms other than angiosperms (Zhang and Bartels 2018).

False negatives can occur in species that require a method (or time) of rehydration different to that reported here for a complete recovery. This can be the case in tracheophytes that need to regain water through the xylem to achieve a safe and organised leaf unfolding (Vicré et al. 2004). In agreement with this, *M. flabellifolius*, one of the DT angiosperms with a lower recovery in our study (37%, Fig. 6), is a woody plant with a more complex requirement for xylem refilling and leaf unfolding than herbaceous plants (Wagner et al. 2000). A specific rehydration method would also be needed in the case of poikilochlorophyllous species, where a longer time or even a proper light/dark photoperiod might be required for a full restoration of the photosynthetic capacity even after full rehydration is achieved (Sherwin and Farrant 1996, Pérez et al. 2011). This is in agreement with the low recovery values found under the "Falcon test" experimental conditions in the poikilochlorophyllous species *X. viscosa and B. purpurea* (Fig. 6). The last case of false negatives related to an inappropriate rehydration method could be relevant for species, such as *Sporobolus stapfianus*, in which only intact but not detached leaves are tolerant to desiccation (Gaff and Loveys 1992, Whittaker et al. 2004). However, this is not known to be a common phenomenon. Despite the limitations of our method, no false negatives were encountered in bryophytes, where all species previously described as FDT recovered to values higher than 90% F_v/F_m. We did not detect any false positives in bryophytes, as all species described as FDS recovered less than 25% of the control F_v/F_m (Fig. 6). Additionally, the low recovery values obtained at 80% RH can indicate DS in species such as *M. polymorpha,* which, in contrast to others (e.g. *F. antipyretica*), was unable to recover even following less severe dehydration (Table S2).

Is the method useful for tracheophytes?

For tracheophytes, F_v/F_m recovery values showed a continuum from DS species belonging to different phylogenetic lineages to DT ferns. Some DS angiosperms gave values close to those of DT angiosperms, implying the risk of finding false positives, while a couple of resurrection plants gave false negatives, as the abovementioned case of *M. flabellifolius*. Overall, the thresholds of ≤ 30% RWC achieved upon desiccation and ≥ 30% recovery of average F_v/F_m upon rehydration allowed for the establishment of a clear separation between DT and DS. As originally proposed, this method is not fully precise, but the proportion of false positives or negatives appears to be very low (3 species out of 38≈ 7% error, Fig. 6B), which favours its use as a course, portable method for rapid and wide screening under remote field conditions.

If we assume that DT is a complex phenomenon resulting from the interaction of constitutive and inducible processes (Carvalho et al. 2014), the recovery rates observed using the proposed test are mostly reflecting the constitutive component of the DT strategy. Interestingly, when focusing on tracheophytes only, some of the highest F_v/F_m recovery values for DS plants were obtained in gymnosperms, and in ferns for DT plants. High resistance to cavitation in both groups (Pittermann et al. 2011) may be part of their distinguishable response to dehydration/rehydration cycles.

In addition to the abovementioned advantages, the method can be used with comparable precision both in the laboratory and in the field, as demonstrated with the case study II, provided some precautions are taken. Two limitations of biological field studies include (1) the maintenance of experimental conditions within the desired ranges and (2) the availability of scientific instruments. Here, we were able to generate desired RHs inside the Falcon tube atmospheres by absorbing saturated salt solutions in sponges while at the same time preventing accidental moistening of samples during transportation and handling. Falcon tubes prepared in this manner can be re-used at least three times without changes in the RHs. Additionally, the method can be adapted for use in remote locations due to minimal instrumentation requirements: a scale with at least three decimal digits (mg range), a box with some Falcon tubes (that must be incubated in darkness), a portable chlorophyll fluorometer, and an enclosed space, such as a building or car, where temperature can be maintained within a reasonable range of 20±5ºC (or even ±10ºC). Provided these instrumental requirements are met, this method allows for the exploration of remote biomes in instances where technical facilities of any type are unavailable. Furthermore, once back in the laboratory, biological data can be completed by biochemical analyses of samples collected in the field and preserved in silica gel (Esteban et al. 2009), or by morphological studies of tissues fixed in situ as previously described for several species of different groups (Tosens et al. 2012, Carriquí et al. 2015).

Conclusion

The protocol described here was able to discriminate between DT and DS bryophytes and quantitatively classify DHT within tracheophytes. Awarding that RWC ≤ 30% is reached during the three desiccation treatments and an average recovery value of $F_v/F_m \geq 30\%$ is achieved upon rehydration, the Falcon method appears as a relatively coarse but reliable and highly portable procedure for the rapid and wide screening of DHT, which could be used even under remote field conditions in both non-vascular and tracheophyte species.

Author contributions

J.I.G-P. and B.F-M. conceptualised the study and initiated the protocol set up. JMF advised on choice of DT angiosperm plants. M.L-P., M.C., A.P-C., E.N-O., JMA, A.H., U.A., J.I.G-P. and AV conducted the bryophyte experiment. M.L-P. was responsible for the bryophyte experiment. J.F., Ja.G., M.N., J.B., Jo.G., M.J.C-M. conducted the tracheophyte experiment. B.F-M., A.P-C. and M.N. performed the statistical analyses. B.F-M. and J.I.G-P. drafted the manuscript. All co-authors contributed to the final version of the work.

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Supporting Information

Table S1 Meteorological information on the collecting sites.

Table S2 Details on final water content after desiccation treatments and recovery of F_v/F_m after rehydration for each species in Case Study I.

Table S3 Details on final water content after desiccation treatments and recovery of F_v/F_m after rehydration for each species in Case Study II.

Fig. S1. F_v/F_m kinetic of 12-studied species during the "Falcon test" using three different rehydration procedures, as described in the text box.

Fig. S2. Weight kinetic of 12-studied species during the set up of the "Falcon test" procedure using three different rehydration treatments, as described in the text box.

Fig. S3. RH achieved by the concentrated $MgCl_2$ solution with regards to temperature (T).

Figure legends

Fig. 1. Diagram illustrating the proposed protocol for the comparative estimation of DT in the photosynthetic tissues of plants belonging to different clades or functional groups (see Methods for details). Samples are surrounded with wet paper tissue during hydration and rehydration. In addition, the sponge (represented as a green square in the picture) is moistened with distilled water to allow for an atmosphere at RH≈100 %.

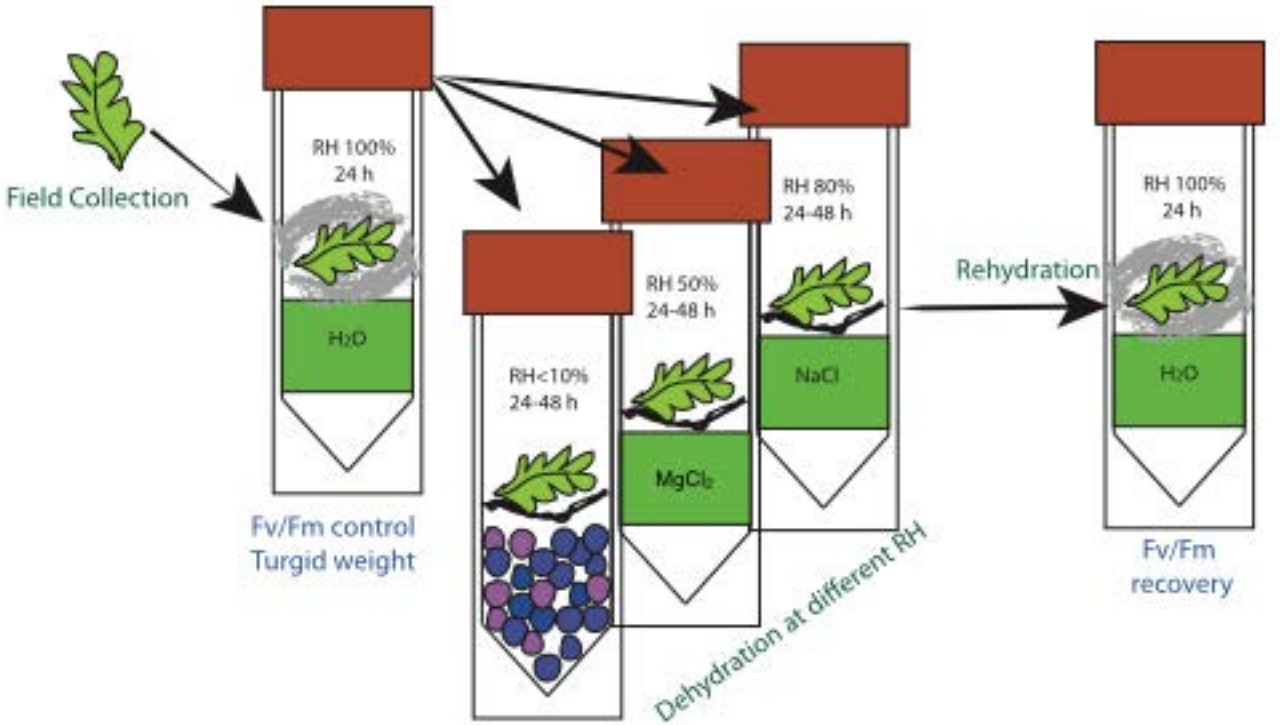
Fig. 2. Description of the Falcon tubes and monitoring of the RH inside them. (A) Falcon tube prepared for desiccation tests: in the bottom of the tube, a coiled kitchen sponge absorbs 10 ml of a supersaturated salt solution of NaCl (80% RH) or a solution of $MgCl_2$ (280 g of $MgCl_2$ hexa-hydrated dissolved in 150 ml of Milli-Q water; 50% RH), preventing leaks of liquid droplets. No sponge is used in the case of silica gel treatment. A mesh prevents direct contact between the sample and the desiccant. (B) Time course of changes in RH inside the desiccating tubes. First, RH was monitored during 5 h in empty tubes, and then in two consecutive desiccation cycles of 48 h (containing a first plant sample) and 24 h (containing a second plant sample).

Fig. 3. (A) Effect of the initial amount of plant material (*Syntrichia ruralis*) placed in the tube (in mg FW) on the final tissue water content (g H_2O g^1 DW) after 24 h of desiccation under the three RHs tested. (B) Effect of the time of desiccation on tissue water content (g H_2O g^{-1} DW) under the three RHs tested for a sample with initial FW of 200 mg. Means ± SE are shown (n=3).

Fig. 4. (A) Average tissue water content (g H_2O g^{-1} DW) and (B) average RWC (% turgid WC) after dehydration under the three RHs tested in the FDT and FDS bryophytes (Case study I) and in the DT and DS tracheophytes (Case study II; see Table 1). Each bar shows the mean ± SE for bryophytes (n=5 species) and tracheophytes (n=14). Lowercase letters above the bars indicate significant differences among treatments. When significant, differences between FDT and FDS (Case study I) or between DS and DT (Case study II) are depicted with capital letters (P<0.05).

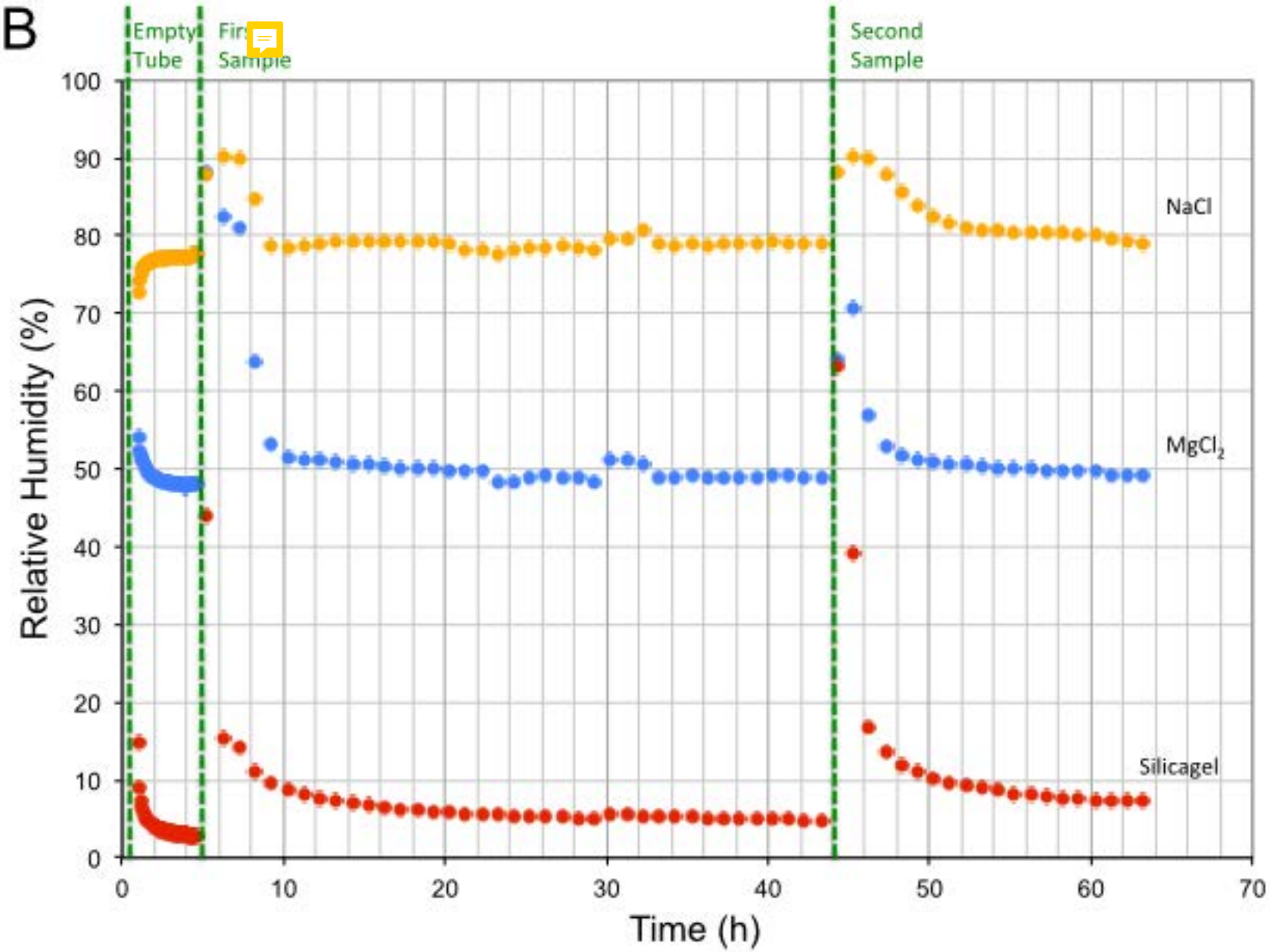
Fig. 5. Average recovery of F_v/F_m (% of control values) under the three RHs tested in the FDT and FDS bryophyte (Case study I) and in the DT and DS tracheophyte (Case study II) species (see Table 1). Each bar shows the mean ± SE (n=5 species for bryophytes and n=14 for tracheophytes). Lowercase letters above the bars indicate significant differences among treatments. When significant, differences between FDT and FDS (Case study I) or between DS and DT (Case study II) are depicted with capital letters ($P<0.05$).

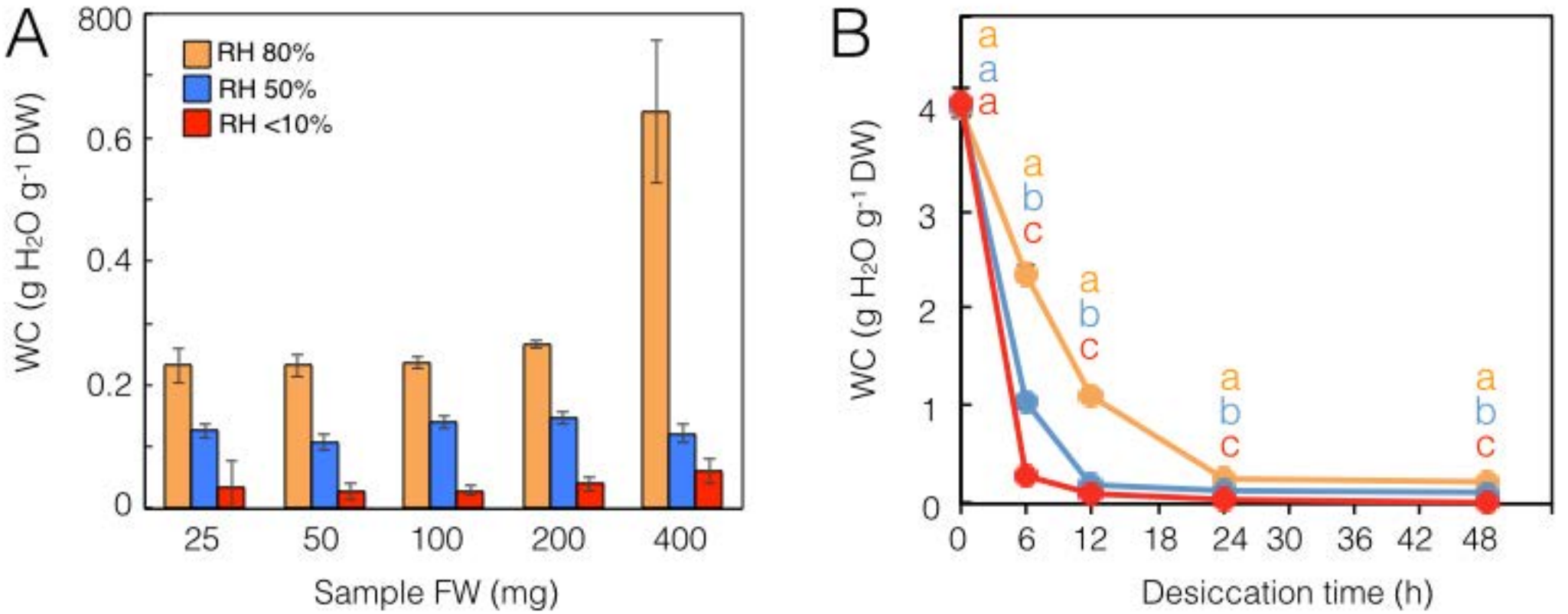
Fig. 6. (A) Average recovery of F_v/F_m after rehydration of each species. (B) Average recovery of F_v/F_m after rehydration of each species estimated by using only those samples that achieved RWC ≤30% upon desiccation. (F)DS species are depicted as white bars and (F)DT as solid bars. For further details regarding the species see Table 1. Each bar shows the mean of the three desiccation treatments for each species (n=9). F_v/F_m recovery for *M. polymorpha* and *H. annuus* was 0%. Bars highlighted in red depict the species erroneously classified by the Falcon test according to the currently available literature (see Table 1).


Field Collection
RH 100%
24 h
H2O
Fv/Fm control
Turgid weight
RH<10%
24-48 h
RH 50%
24-48 h
MgCl2
RH 80%
24-48 h
NaCl
Dehydration at different RH
Rehydration
RH 100%
24 h
H2O
Fv/Fm
recovery

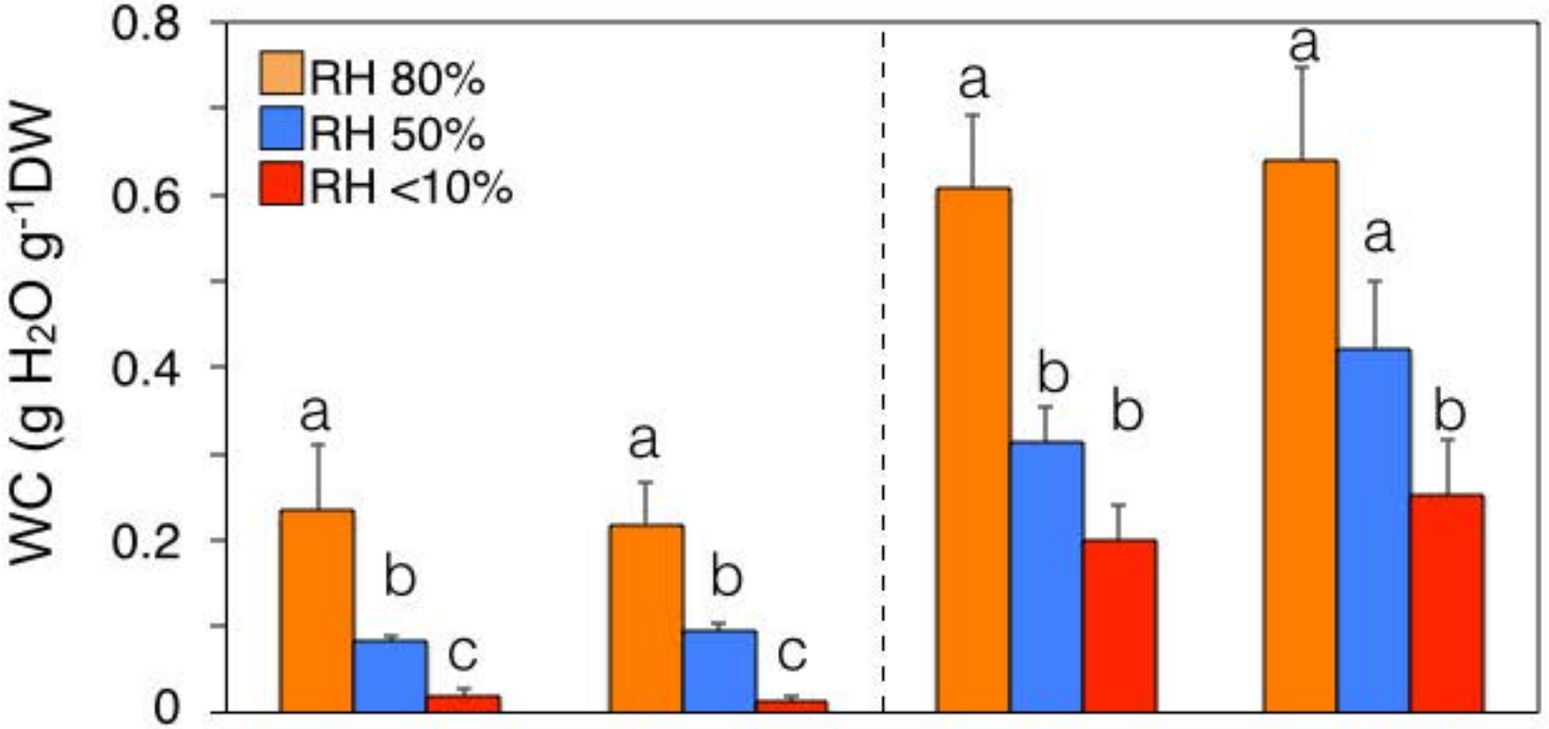
A

B

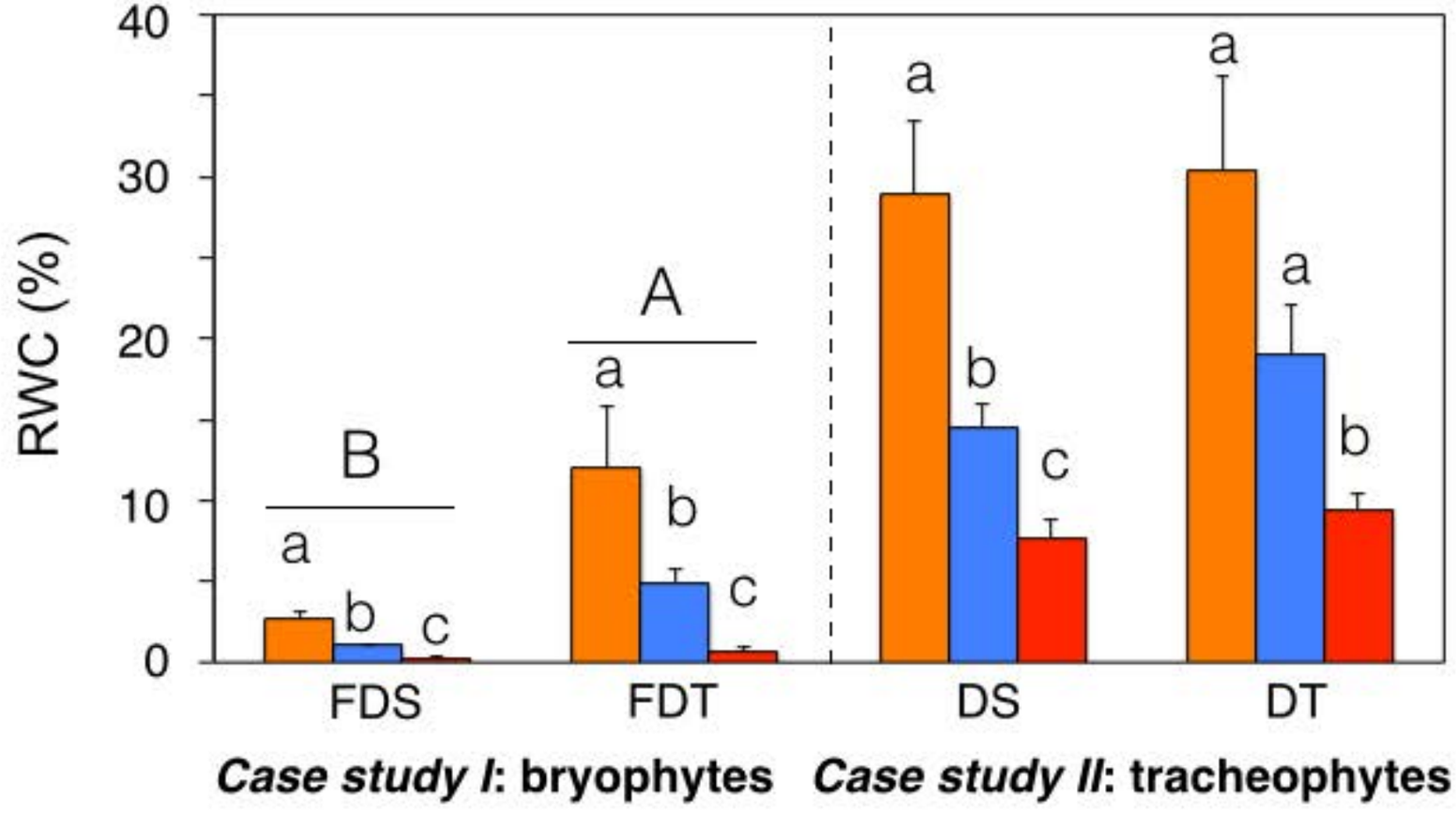

Empty Tube
First Sample
Second Sample
NaCl
MgCl2
Silicagel
Relative Humidity (%)
Time (h)
0
10
20
30
40
50
60
70
80
90
100

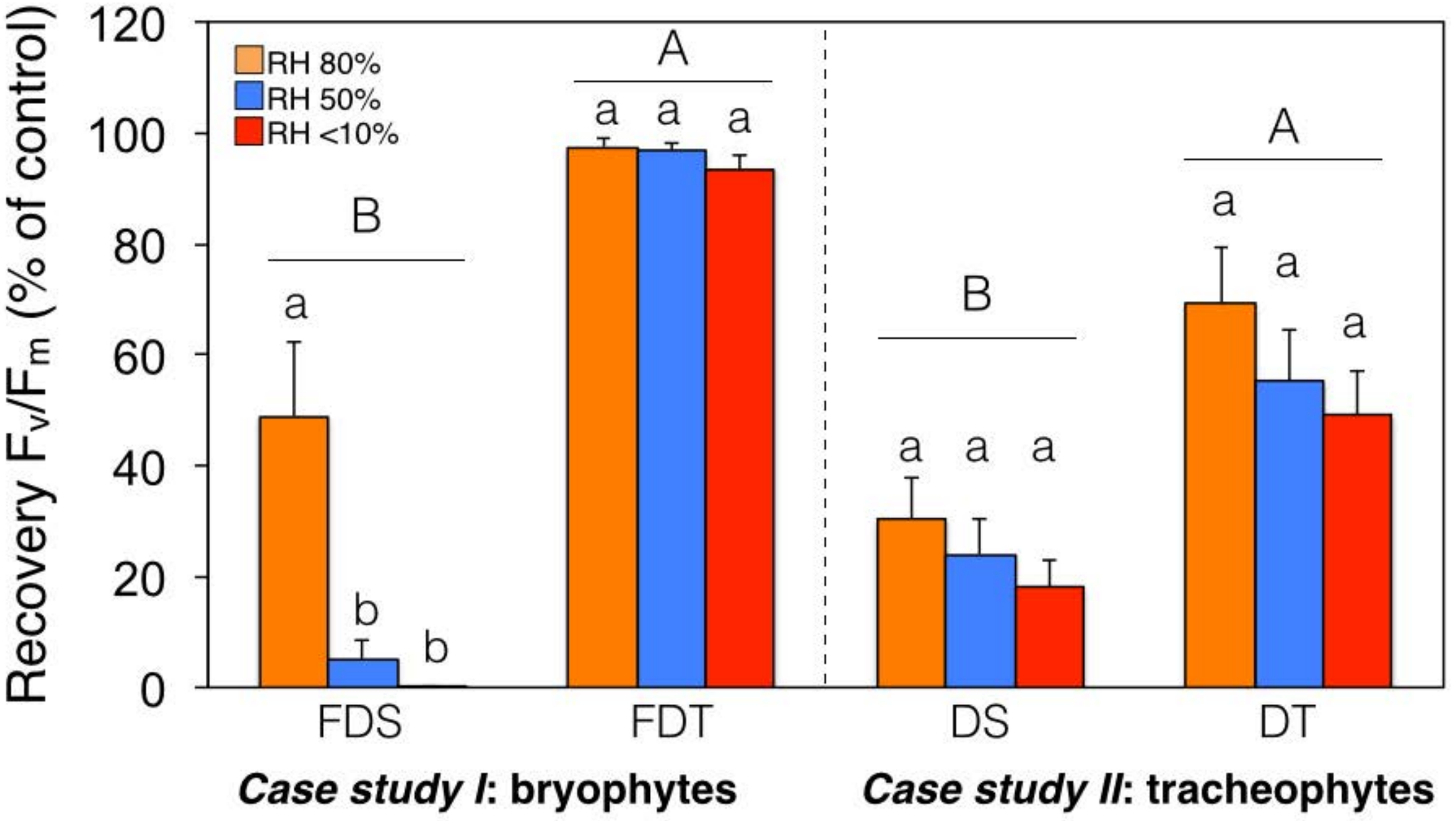

A
800
0.6
0.4
0.2
0
RH 80%
RH 50%
RH <10%
WC (g H2O g-1 DW)
25
50
100
200
400
Sample FW (mg)
B
4
3
2
1
0
a
a
a
a
b
c
a
b
c
a
b
c
a
b
c
WC (g H2O g-1 DW)
0
6
12
18
24
30
36
42
48
Desiccation time (h)

A

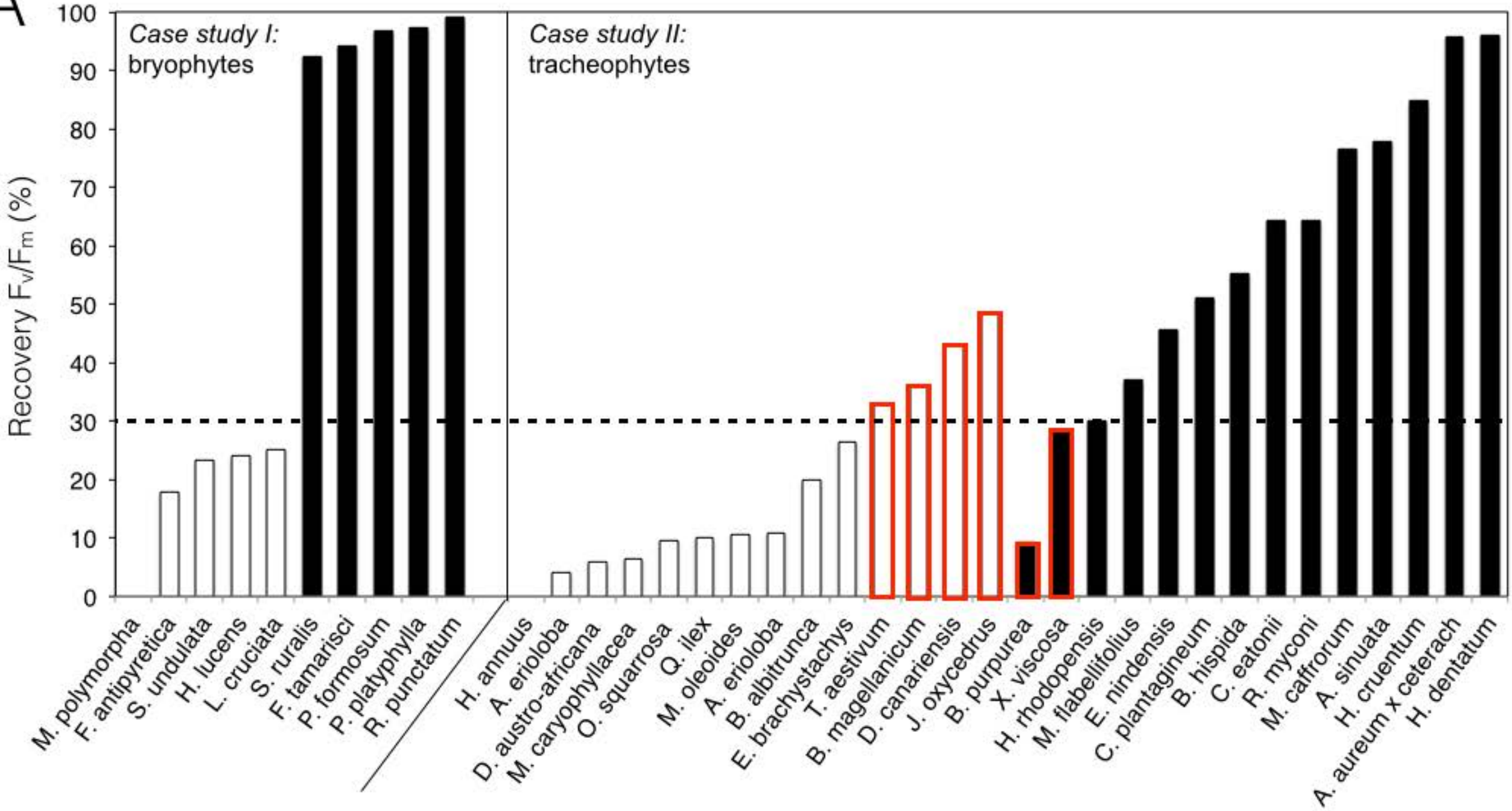

WC (g H2O g-1DW
0.8
0.6
0.4
0.2
0
RH 80%
RH 50%
RH <10%
a
b
c
a
b
c
a
b
b
a
a
b

B

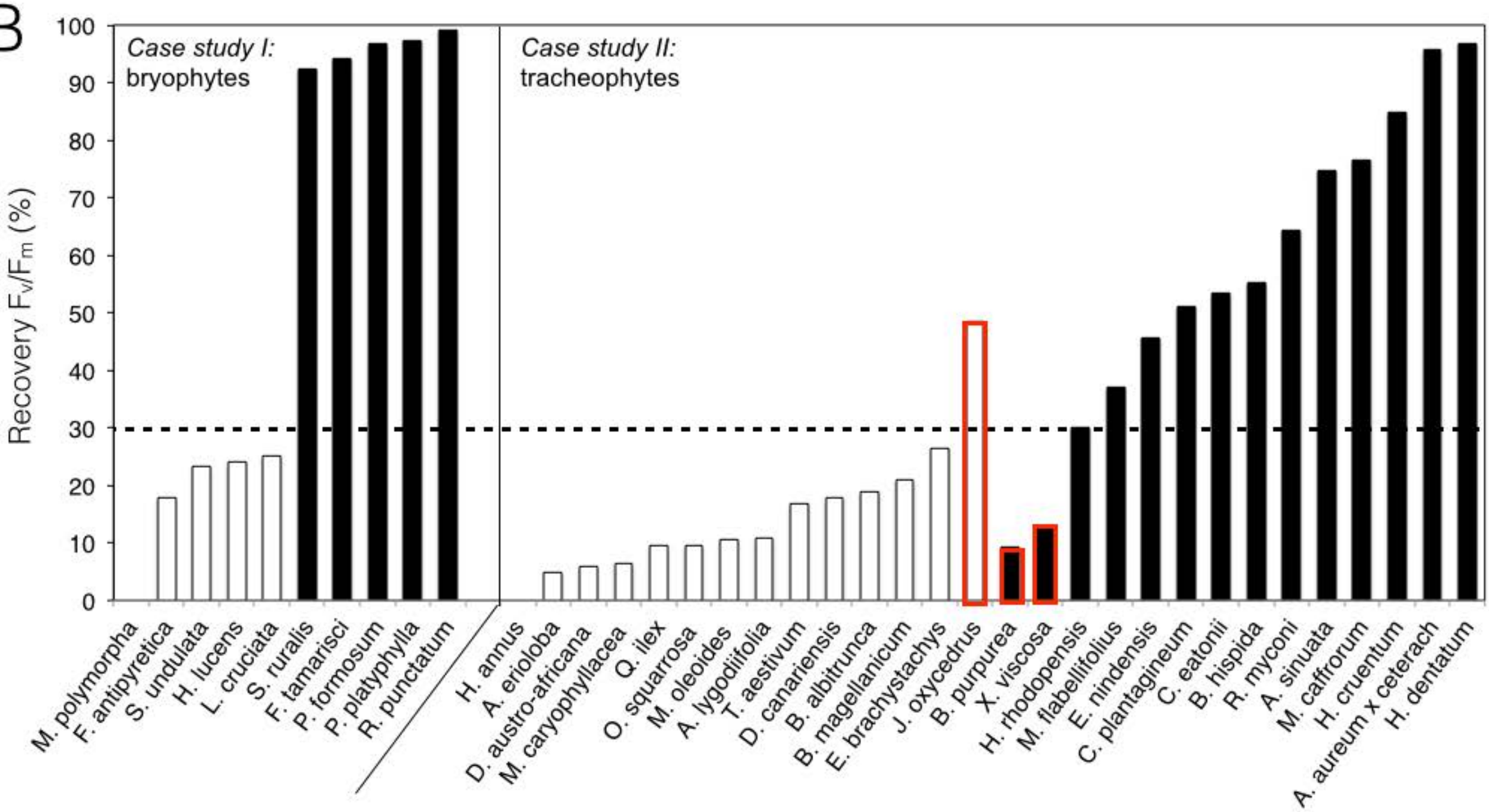

RWC (%)
40
30
20
10
0
B
A
a
b
c
a
b
c
a
b
c
a
a
b
FDS
FDT
DS
DT
Case study I: bryophytes
Case study II: tracheophytes


Recovery F_v/F_m (% of control)
120
100
80
60
40
20
0
RH 80%
RH 50%
RH <10%
A
a a a
B
a
b
b
a a a
B
a a a
A
FDS
FDT
DS
DT
Case study I: bryophytes
Case study II: tracheophytes

A


Case study I:
bryophytes
Case study II:
tracheophytes
Recovery F_v/F_m (%)
100
90
80
70
60
50
40
30
20
10
0
M. polymorpha
F. antipyretica
S. undulata
H. lucens
L. cruciata
S. ruralis
F. tamarisci
P. formosum
P. platyphylla
R. punctatum
H. annuus
A. erioloba
D. austro-africana
M. caryophyllacea
O. squarrosa
Q. ilex
M. oleoides
A. erioloba
B. albitrunca
E. brachystachys
T. aestivum
B. magellanicum
D. canariensis
J. oxycedrus
B. purpurea
X. viscosa
H. rhodopensis
M. flabellifolius
E. nindensis
C. plantagineum
B. hispida
C. eatonii
R. myconi
M. caffrorum
A. sinuata
H. cruentum
A. aureum x ceterach
H. dentatum

B


Case study I:
bryophytes
Case study II:
tracheophytes
Recovery F_v/F_m (%)
100
90
80
70
60
50
40
30
20
10
0
M. polymorpha
F. antipyretica
S. undulata
H. lucens
L. cruciata
S. ruralis
F. tamarisci
P. formosum
P. platyphylla
R. punctatum
H. annus
A. erioloba
D. austro-africana
M. caryophyllacea
Q. ilex
O. squarrosa
M. oleoides
A. lygodiifolia
T. aestivum
D. canariensis
B. albitrunca
B. magellanicum
E. brachystachys
J. oxycedrus
B. purpurea
X. viscosa
H. rhodopensis
M. flabellifolius
E. nindensis
C. plantagineum
C. eatonii
B. hispida
R. myconi
A. sinuata
M. caffrorum
H. cruentum
A. aureum x ceterach
H. dentatum