TECHNICAL INNOVATION

Viability markers for determination of desiccation tolerance and critical stages during dehydration in *Selaginella* species

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Abstract

While most plants die below a threshold of water content, desiccation-tolerant species display specific responses that allow them to survive extreme dehydration. Some of these responses are activated at critical stages during water loss and could represent the difference between desiccation tolerance (DT) and death. Here, we report the development of a simple and reproducible system to determine DT in *Selaginella* species. The system is based on exposure of excised tissue to a dehydration agent inside small containers, and subsequent evaluation for tissue viability. We evaluated several methodologies to determine viability upon desiccation including: triphenyltetrazolium chloride (TTC) staining, the quantum efficiency of PSII, antioxidant potential, and relative electrolyte leakage. Our results show that the TTC test is a simple and accurate assay to identify novel desiccation-tolerant *Selaginella* species, and can also indicate viability in other desiccation-tolerant models (i.e. ferns and mosses). The system we developed is particularly useful to identify critical points during the dehydration process. We found that a desiccation-sensitive *Selaginella* species shows a change in viability when dehydrated to 40% relative water content, indicating the onset of a critical condition at this water content. Comparative studies at critical stages could provide a better understanding of DT mechanisms and unravel insights into the key responses to survive desiccation.

Keywords: Critical stage, desiccation tolerance, *Selaginella*, tissue viability.

Introduction

Desiccation tolerance (DT) is defined as the ability of an organism to dry to equilibrium with moderately dry air [≤50% relative humidity (RH)] and to resume its metabolism when rehydrated (Alpert and Oliver, 2002; Alpert, 2005). Drying cells to 50% RH, corresponding to a water potential of about −100 MPa, leads to metabolic arrest, since cellular water content is below the level required to form an aqueous monolayer around macromolecules (Alpert, 2005; Leprince and Buitink, 2015). Essential features of DT include the ability of plants or tissues to limit the damage to a repairable level, to maintain cellular and subcellular physiological integrity in the dry state, and to repair damage upon rehydration (Bewley, 1979; Alpert and Oliver, 2002). In most early-diverging clades of land plants (bryophytes), DT exhibits a high degree of plasticity and is dependent upon both external and internal environmental factors (Oliver *et al.*, 2020). Nevertheless, many bryophytes exhibit DT characterized by constitutive protection mechanisms that allow them to survive desiccation regardless of the drying rate (from a few minutes to several hours), whereas DT in tracheophytes is mainly based on the activation of protection mechanisms that lead to DT only if water loss is gradual (from several hours to several days) (Oliver *et al.*, 2000).

During dehydration, the plant cell volume can decrease by 60–80%; therefore, to survive the mechanical stress caused by water loss, cells of desiccation-tolerant pteridophytes and eudicot angiosperms accumulate compatible solutes, increase vacuolation, and activate cell wall folding (Farrant *et al.*, 2007; Oliver *et al.*, 2020). Sensitive species do not possess these mechanisms, and consequent subcellular damage, for example plasma membrane disruption, is lethal (Farrant *et al.*, 2007). Accumulation of soluble sugars is directly related to DT in seeds and vegetative tissues (Alpert and Oliver, 2002), and their proposed functions include protection of membranes and macromolecules, filling and stabilization of vacuoles, and vitrification (a glassy state) of the cytoplasm (Hoekstra *et al.*, 2001; Dinakar and Bartels, 2013). The principal protective sugar that accumulates to high levels during dehydration is sucrose; however. carbohydrate metabolism is highly diverse among desiccation-tolerant plants, and other molecules such as trehalose, raffinose family oligosaccharides, and even unusual sugars such as octulose can also accumulate in tolerant species (Zhang *et al.*, 2016).

Although desiccation-tolerant species shut down photosynthetic activity during early dehydration, electron flow in light-harvesting reactions continues, resulting in overproduction of reactive oxygen species (ROS), which can lead to damage to macromolecules (Dinakar *et al.*, 2012; Challabathula *et al.*, 2018). Desiccation-tolerant species are classified as homoiochlorophyllous or poikilochlorophyllous, according to the strategy to either protect or dismantle their photosynthetic machinery during dehydration (Challabathula *et al.*, 2018). Poikilochlorophyllous species evolved the ability to dismantle

thylakoid membranes and degrade chlorophyll in order to reduce ROS production in the desiccated state (Dinakar *et al.*, 2012). These components are then resynthesized and reassembled during rehydration. In contrast, homoiochlorophyllous plants retain and protect their photosynthetic apparatus during desiccation, allowing a rapid reactivation upon rehydration (Dinakar *et al.*, 2012). Thus, homoiochlorophyllous species require more effective mechanisms of protection against ROS in comparison with poikilochlorophyllous species (Farrant *et al.*, 2007; Challabathula *et al.*, 2016; Georgieva *et al.*, 2017).

Desiccation-associated damage is lethal for most plants, and the understanding of how protective mechanisms in desiccation-tolerant species allow them to survive extreme dehydration will be important for the future breeding of drought-tolerant crops. Therefore, it is necessary to develop accurate indicators to determine the critical stages and define viability during the desiccation process. The maintenance of antioxidant potential (specifically glutathione redox potential) in desiccation-tolerant organisms has been proposed previously as a marker of viability for evaluation of long-term desiccation (Kranner and Birtić, 2005). Indicators of metabolically active tissues, such as triphenyltetrazolium chloride (TTC) which is reduced to an insoluble red compound (triphenylformazan) by the activity of the mitochondrial respiratory chain (Ruf and Brunner, 2003), could also be used as viability markers. The TTC assay is commonly used to evaluate seed viability (Lopez Del Egido *et al.*, 2017), but its use to determine viability in vegetative tissues is still limited. Here we report a dehydration system based on the use of excised tissue (denominated as explants) and compare several methodologies to measure tissue damage and recovery with the objective of selecting reliable viability markers for DT studies. The establishment of these markers was carried out in *Selaginella*, a globally distributed genus which occupies a broad diversity of habitats ranging from tropical rainforests to deserts (Zhou *et al.*, 2015). The methodology developed allows determination of the type of DT responses shown by desiccation-tolerant *Selaginella* species and the specific water contents at which a sensitive species could lose tissue viability. We also show that our dehydration system coupled with these viability markers can be applied successfully in desiccation-tolerant species other than *Selaginella*. Our methodology represents a simple and robust tool to determine DT ability and identify critical stages during the dehydration process.

Materials and methods

Plant material

All the experiments described below were performed on the desiccation-tolerant species *S. lepidophylla* and *S. sellowii*, as well as on the desiccation-sensitive species *S. silvestris* and *S. denticulata*. The DT ability of a further 12 *Selaginella* species was determined by TTC assays and whole-plant experiments. The TTC staining method was also evaluated in two moss

(*Braunia secunda* and *Pogonatum comosum*) and three fern (*Myriopteris aurea*, *Pleopeltis mexicana*, and *Pleopeltis plebeia*) species. A list of species and collection sites is provided in Supplementary Table S1. Specimens were taxonomically determined according to the key of Mickel and Smith (2004), and voucher specimens were deposited at the MEXU herbarium, UNAM. Plants were transferred to pots or trays (depending on size). The tolerant species *S. lepidophylla* and *S. sellowii* were maintained in the dried state at room temperature until used. The sensitive species *S. denticulata* and *S. silvestris* were maintained in a growth chamber (22 °C, 16 h light period). All samples used to determine DT ability were maintained in hydrated conditions in a greenhouse under ambient light conditions until used.

Explant desiccation treatments

Desiccation-tolerant species (*S. lepidophylla* and *S. sellowii*) were watered and maintained in a hydrated condition in a growth chamber (22 °C, 16 h light period) for at least 5 d before DT experiments. Desiccation treatments involving explants placed in small closed containers are described in detail in Supplementary Protocol S1. The desiccated state was evaluated 1 week after explants were placed in the drying system (25 °C, 16 h light period). The rehydrated state was evaluated by adding 5 ml of deionized water to desiccated tissue and analyzing after 24 h.

Electrolyte leakage measurements

Explants with an initial fresh weight of 100 ± 1 mg were briefly rinsed in deionized water to remove the exudates of damaged cells, and excess water was removed by blotting. Desiccated explants were transferred to 50 ml polypropylene tubes and rehydrated. When the rehydration step was completed, deionized water was added to exactly 25 ml and the samples were incubated for 4 h at room temperature in an orbital shaker (160 rpm). Well-watered samples were included to evaluate explants under hydrated conditions. Electrolyte leakage (C_i) was measured with a HI2003 conductivity meter (Hanna Instruments, USA). Subsequently, samples were placed in boiling water for 30 min and, after cooling, the conductivity was measured to obtain a value representing the total amount of ions in the sample (C_{max}). The conductivity of deionized water was also measured (C_w) and relative electrolyte leakage (REL) was calculated using the following formula:

$$\text{REL} = \left[\frac{(C_i - C_w)}{(C_{max} - C_w)} \right] \times 100$$

Chlorophyll content and maximum quantum efficiency

Explants for chlorophyll quantification were transferred to 15 ml polypropylene tubes, frozen in liquid nitrogen, and stored at −80 °C until used. Chlorophyll extraction and quantification (total chlorophyll, Chl *a*, and Chl *b*) were carried out according to Richardson *et al.* (2002). The incubation time with DMSO (65 °C) was modified to 45 min for the tolerant species. Chlorophyll quantification was adapted for measurement in 96-well microplates.

Maximum quantum efficiency of PSII (F_v/F_m) was determined in 15 min dark-adapted explants with a Pocket PEA chlorophyll fluorimeter (Hansatech Instruments, UK).

Antioxidant potential assays and total phenol and flavonoid content

Explants with an initial fresh weight of 100 ± 1 mg were used for the following protocols. Tissue was transferred to 2 ml polypropylene tubes containing a steel bead, frozen in liquid nitrogen, and stored at −80 °C until used. Frozen tissue was ground using a TissueLyser II (Qiagen Inc., USA) with three rounds of shaking at 30 Hz for 30 s. *In vitro* antioxidant activity was measured by the ferric ion reducing antioxidant power (FRAP) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays according to the methodology described by Yahia *et al.* (2011). Total phenolic content was determined according to Maranz *et al.* (2003) and total flavonoid content was determined according to Sakanaka *et al.* (2005). All procedures were adapted for measurement in 96-well microplates.

RNA integrity

Tissue was transferred to 2 ml polypropylene tubes containing a steel bead, frozen in liquid nitrogen, and stored at −80 °C until used. Frozen tissue was ground using a TissueLyser II (Qiagen Inc., USA) with three rounds of shaking at 30 Hz for 30 s. RNA was extracted using PureLink™ Plant RNA Reagent (Invitrogen) according to the manufacturer's instructions. RNA integrity was determined by electrophoresis on 1.2% agarose gels.

Triphenyltetrazolium staining

Determination of tissue viability using TTC staining was adapted from Lin *et al.* (2001) and Ruf and Brunner (2003). Samples were placed in 12-well plates with 2–3 ml of TTC solution (0.6% TTC; 0.05% Tween-20; 0.1 M potassium phosphate buffer, pH 7.0), vacuum infiltrated for 10 min, and incubated for 24 h at room temperature in darkness. A detailed methodology to determine viability by TTC assay and image analysis to measure the proportion of the viable area is described in Supplementary Protocol S1. Image processing was carried out using GIMP software (version 2.10.12) and pixel quantification to determine viable explant areas using the software Easy Leaf Area (Easlon and Bloom, 2014).

Whole-plant desiccation treatments

Whole-plant experiments were performed on at least three individuals (in pots or trays) per species in the greenhouse, by withholding water from plants for 30 d. Plants were watered and evaluated 2 d after rehydration. Species in which at least half of the tissue of each individual retained their green color at rehydration were classified as desiccation tolerant.

Statistical analysis

Results are expressed as mean values ±SD, and the number of replicates is given in the figure legends. Statistical analysis of the data was performed using R 4.1.1. One-way ANOVA ($P<0.05$) and Tukey's HSD test were applied to determine groups for the analysis of drying rates. For maximum quantum efficiency, chlorophyll content, and electrolyte leakage measurements, the significance of differences between control (hydrated, unstressed tissue) and each water state was analyzed using Student's *t*-test.

Results

Drying rate establishment and evaluation by physical appearance

Desiccation experiments to evaluate DT-related traits in different *Selaginella* species were carried out in a simplified system based on explants rather than whole plants (Fig. 1A). For the drying system, a saturated salt solution or silica gel is placed in the bottom of a small plastic container. A piece of foam rubber is then placed on top as a support for an open Petri
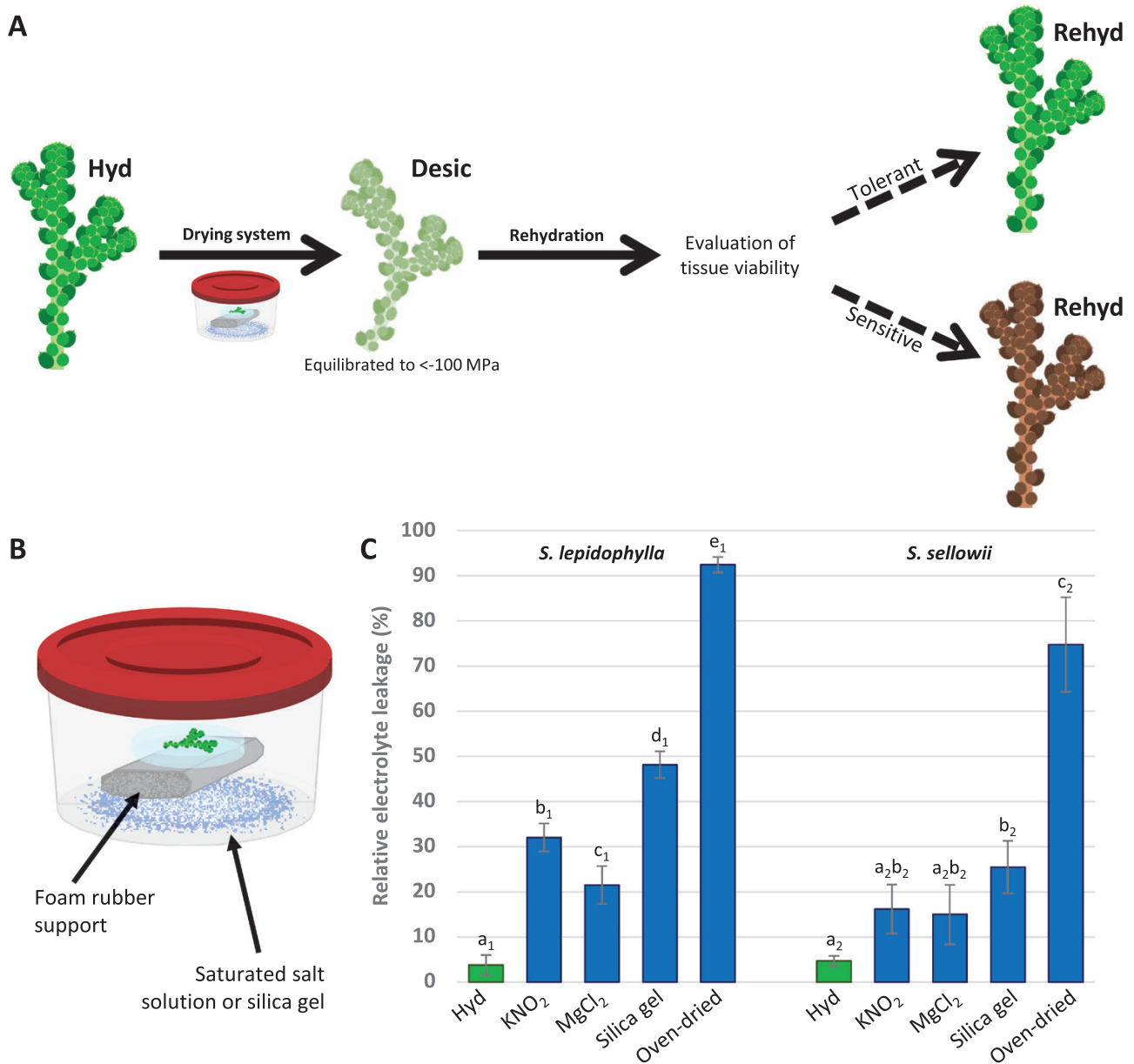
Fig. 1. Evaluation of desiccation tolerance at the explant level. (A) Schematic diagram of the experimental design using explants. Stages evaluated included hydrated (Hyd), desiccated (Desic), and rehydrated (Rehyd) conditions. (B) Representation of the drying system: the positions of the tissue (open Petri dish over a support) and a humidity control agent are shown. (C) Relative electrolyte leakage of *S. lepidophylla* and *S. sellowii* explants exposed to different drying rates (blue bars) produced by saturated salt solutions or silica gel. Hydrated explants (green bars) were used as control unstressed tissue. Oven-dried explants (65 °C) represent a state of irreparable damage. Each bar represents the mean of three replicates and error bars indicate the SD. Different letters indicate significant differences (ANOVA test, $P<0.05$) per species (distinct subscripts).

dish containing the explants, and the plastic container is closed tightly (Fig. 1B; Supplementary Protocol S1). Explant desiccation treatments (and technical replicates) were performed in individual desiccators for each condition in order to obtain reproducible results. Explants of desiccation-tolerant *Selaginella* species do not survive if dehydration is too fast to allow DT responses to occur (e.g. drying in an oven at 65 °C). Therefore, to determine appropriate rates of water loss, we evaluated three drying regimes: slow drying using a saturated solution of KNO_2 (45.5–46.5% RH), moderate drying using a saturated solution of $MgCl_2$ (32–33% RH), and rapid drying using silica gel (5–7% RH). To test the drying regimes, we used explants from two well-characterized desiccation-tolerant species *S. lepidophylla* and *S. sellowii*. Measurement of REL showed that the moderate drying rate produced the least membrane damage in both tolerant species (Fig. 1C). Therefore, the moderate drying rate ($MgCl_2$), which equilibrates at around −149 MPa (Xiao *et al.*, 2018), a stricter water potential than

the threshold for determination of plant DT (–100 MPa), was selected for the comparisons between desiccation-tolerant and sensitive species.

We first tested whether the explant system could be used to differentiate between previously characterized desiccation-tolerant and sensitive *Selaginella* species. With this aim, we subjected explants from the same two desiccation-tolerant species (*S. lepidophylla* and *S. sellowii*) and two previously characterized sensitive species (*S. silvestris* and *S. denticulata*) to desiccation. Both tolerant and sensitive *Selaginella* species reached equilibrium (desiccated state) within 48 h after explants were placed in the drying system (Supplementary Fig. S1). Evaluation of the physical appearance of explants showed complete recovery after rehydration of the tolerant species *S. lepidophylla* and *S. sellowii*, whereas explants of the sensitive species suffered severe oxidation (Fig. 2A). Tissue of desiccation-tolerant species showed morphological changes such as stem curling and/or microphyll folding which minimize the area of photosynthetic tissue exposed to light and the generation of ROS during desiccation. In contrast, microphylls of sensitive species underwent shrinkage but most of the photosynthetic tissue remained exposed to light, leaving it vulnerable to the generation of ROS in the desiccated state (Fig. 2A).

Viability evaluation through homoiochlorophyllous properties

Since tolerant *Selaginella* species maintain their photosynthetic apparatus during desiccation, determination of the maximum quantum efficiency of PSII (F_v/F_m) can be used to evaluate recovery after desiccation (Fig. 2B). Both tolerant and sensitive *Selaginella* species showed a significant decrease in F_v/F_m values following desiccation; however, only tolerant plants recovered the initial values upon rehydration (similar to hydrated, unstressed controls) (Fig. 2B). Complete recovery of F_v/F_m values could be associated with an effective protection of the photosynthetic apparatus, including pigments. Chlorophyll fluorescence, evaluated by imaging of tissue exposed to UV light, showed that for desiccation-tolerant species fluorescence was similar before and after desiccation, whereas for sensitive plants a diminished fluorescence was observed after desiccation (Supplementary Fig. S2). Total chlorophyll content for *S. lepidophylla* and *S. sellowii* in the desiccated state decreased to 82% and 80.5% respectively, in relation to fully hydrated chlorophyll content and recovered to 92.3% and 94.3%, respectively, upon rehydration (Fig. 3A). In contrast, the sensitive species *S. silvestris* and *S. denticulata* showed a decrease to 68.6% and 79.6% of the hydrated total chlorophyll content, respectively, under desiccation, and a further reduction to 52.8% and 46.1% total chlorophyll content, respectively, after rehydration (Fig. 3A).

Antioxidant potential was evaluated by FRAP and DPPH assays (Table 1). In hydrated conditions, tolerant species exhibit at least a 2- to 4-fold higher level of antioxidant capacity

for both assays in comparison with sensitive species. Although both tolerant and sensitive species showed increased antioxidant capacity in response to desiccation, the levels observed in the tolerant species *S. lepidophylla* and *S. sellowii* were significantly higher than in sensitive plants (Table 1). During rehydration, antioxidant potential in sensitive species decreased to even lower levels than in hydrated tissue. In contrast, during rehydration of *S. lepidophylla* explants, antioxidant potential remained higher than in the initial hydrated state (Table 1), but only the FRAP assay showed this behavior for explants of *S. sellowii*. Additionally, total phenol content and total flavonoids were determined and displayed similar patterns to those observed in the antioxidant potential assays (Table 1).

Retention and integrity of essential components

Membrane damage indirectly measured by REL showed a significant difference between desiccation-tolerant and desiccation-sensitive *Selaginella* species (Fig. 3B). Membrane integrity in the sensitive species is completely compromised upon rehydration (~90% of REL) compared with tolerant species (15–23% of REL). In addition, REL was lower for all drying rates in *S. sellowii* in comparison with *S. lepidophylla* (Fig. 1C), indicating a better membrane protection capacity in the former species.

The presence of ribosomes is required to reinitiate protein biosynthesis during rehydration, therefore we tested whether rRNA integrity could also be a useful indicator for DT ability. Integrity of rRNA was maintained in all *Selaginella* species in the desiccated state, at least during the short period evaluated (1 week). However, rRNA was clearly degraded in the sensitive species during rehydration, whereas the integrity of rRNA was largely intact in tolerant species (Fig. 3C).

Determination of tissue viability by analysis of respiratory chain activity

We tested whether the TTC assay could be used to determine tissue viability in *Selaginella* after a desiccation treatment. Fresh tissue showed a strong red color, indicating that this test can be used to evaluate viability in vegetative tissues. Explants were then tested using the TTC assay following desiccation (Fig. 4). Rehydrated explants of desiccation-tolerant species showed a strong red color similar to unstressed tissue. In contrast, rehydrated explants of sensitive species showed no detectable red coloration (as observed in control explants killed by boiling), indicating that these explants were not metabolically active (did not recover respiration) after the dehydration–rehydration cycle (Fig. 4).

Identification of novel desiccation-tolerant species

Determination of tissue viability by the TTC test proved to be an efficient and accurate method for determination of DT
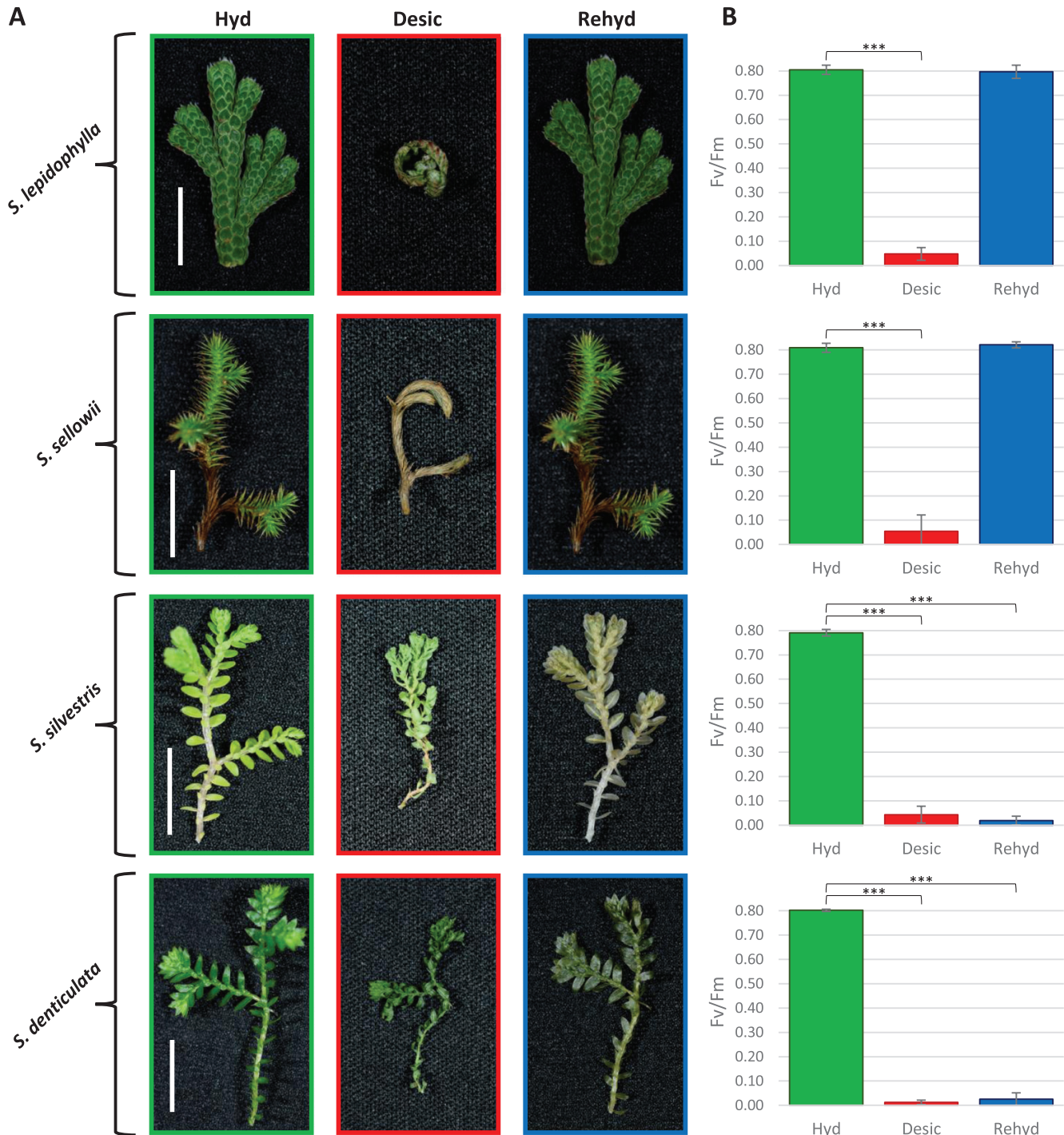
Fig. 2. Evaluation of explant recovery by visual inspection and determination of the quantum efficiency of PSII. (A) Physical appearance of *S. lepidophylla*, *S. sellowii*, *S. silvestris*, and *S. denticulata* explants during a dehydration–rehydration cycle. (B) Maximum quantum efficiency of PSII (F_v/F_m). The hydration stage is color-coded: green, red, and blue indicate the hydrated (Hyd), desiccated (Desic), and rehydrated (Rehyd) state, respectively. Error bars represent an SD of 10 points per condition. Bars with asterisks are significantly different from Hyd (Student's *t*-test, ***$P<0.001$). Scale bar=1 cm.

capacity in previously uncharacterized *Selaginella* species (Fig. 5A–C; Supplementary Fig. S3). Based on the TTC assay, the following species were classified as desiccation tolerant: *S.*

extensa, *S. rupincola*, *S. wrightii*, *S. nothohybrida*, *S. ribae*, *S. polyptera*, and *S. schiedeana*. The analysis also confirmed the DT status of *S. pilifera*, a previously reported desiccation–tolerant species
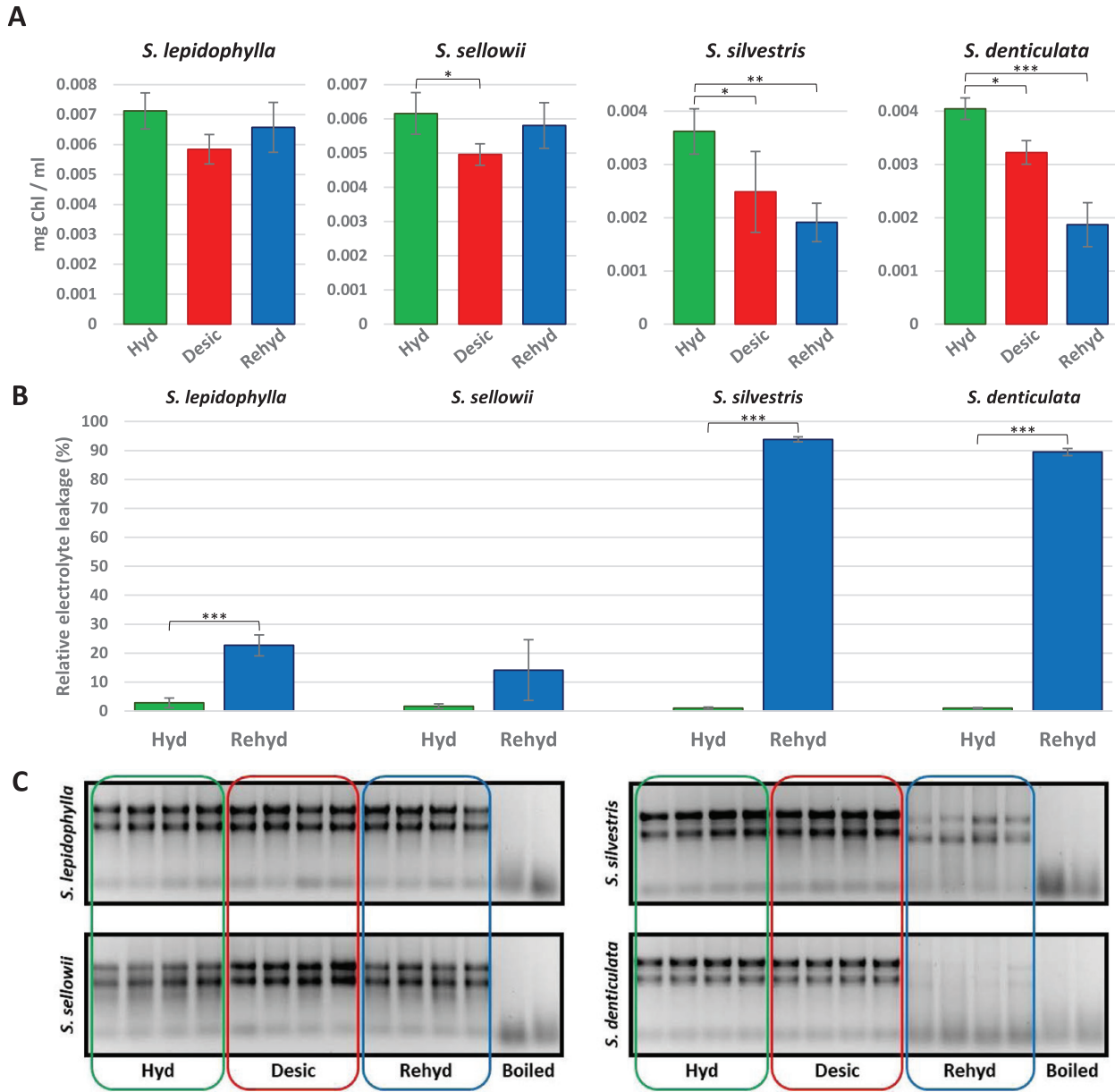
Fig. 3. Maintenance of chlorophyll content and integrity of essential components during the desiccation process. (A) Quantification of total chlorophyll (Chl *a*+*b*) under hydrated (Hyd), desiccated (Desic), and rehydrated (Rehyd) conditions. (B) Membrane damage indicated by relative electrolyte leakage before and after desiccation. (C) rRNA integrity examined by agarose gel electrophoresis. Boiled explants were included as non-viable tissues. Abbreviations and color code are similar to those in Fig. 2. Error bars represent the SD of four replicates. Bars with asterisks are significantly different from Hyd (Student's *t*-test, *$P<0.05$, **$P<0.01$, ***$P<0.001$).

(Eickmeier, 1980). Explants of the samples *S. flexuosa*, *S. lineolata*, and *S. stellata* were classified as desiccation-sensitive species. A different staining pattern was observed in *S. delicatissima* (Fig. 5B) in which only the apices of one-third of the evaluated explants remained viable after the desiccation process; therefore, this species was classified as a tissue-specific tolerant species.

To corroborate that the explant dehydration system combined with the TTC test can correctly identify desiccation-tolerant and sensitive species, we selected a tolerant and a sensitive species from those identified by the explant assay

(*S. polyptera* and *S. flexuosa*), to establish evaluation times for whole-plant desiccation assays. Plants were grown under greenhouse conditions and dehydrated by withholding water until pots reached a constant weight (30 d), then subsequently rehydrated for evaluation of DT capacity (Supplementary Fig. S4). *Selaginella polyptera* plants classed as tolerant retained their green color on rehydration, whereas the *S. flexuosa* individuals showed evident damage (tissue oxidation) and loss of turgidity on rehydration. Similar whole-plant experiments were carried out for all *Selaginella* species evaluated in this study;

Table 1. Differences in antioxidant potential between tolerant and sensitive *Selaginella* specie.

Species	Condition	FRAP (mg AA g IFW^{-1})	DPPH (mg AA g IFW^{-1})	TPC (mg g IFW^{-1})	TF (mg g IFW^{-1})
S. lepidophylla	Hydrated	1.21 (0.49)	0.63 (0.37)	6.93 (1.67)	3.60 (1.16)
S. lepidophylla	Desiccated	5.82 (0.77)	6.70 (0.42)	12.90 (2.37)	9.30 (3.13)
S. lepidophylla	Rehydrated	2.23 (0.35)	1.18 (0.28)	8.44 (0.40)	4.71 (1.66)
S. sellowii	Hydrated	3.43 (0.31)	4.40 (0.23)	14.98 (1.46)	7.42 (1.70)
S. sellowii	Desiccated	5.06 (0.60)	7.91 (0.90)	18.61 (2.18)	10.04 (2.42)
S. sellowii	Rehydrated	4.04 (0.96)	3.07 (1.15)	12.74 (2.39)	6.57 (2.89)
S. silvestris	Hydrated	0.61 (0.16)	0.07 (0.02)	3.34 (0.37)	1.62 (0.19)
S. silvestris	Desiccated	0.93 (0.18)	0.26 (0.07)	4.32 (0.50)	2.20 (0.35)
S. silvestris	Rehydrated	0.23 (0.09)	-0.05 (0.01)	2.22 (0.21)	1.28 (0.11)
S. denticulata	Hydrated	0.74 (0.12)	-0.02 (0.01)	1.60 (0.12)	1.80 (0.32)
S. denticulata	Desiccated	1.78 (0.19)	0.90 (0.06)	3.70 (0.32)	3.39 (0.58)
S. denticulata	Rehydrated	0.61 (0.18)	-0.11 (0.02)	1.44 (0.17)	1.56 (0.20)

Antioxidant potential measured by FRAP (ferric-ion reducing antioxidant power) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays expressed as ascorbic acid (AA) equivalents. TPC (total phenol content) and TF (total flavonoid) contents are expressed as caffeic acid and catechin equivalents, respectively. Data represent the mean value of four replicates; values in parentheses are the SDs. Equivalents are expressed per g of the sample initial fresh weight (IFW).

we corroborated that in all cases the explant assay was representative of the whole-plant phenotype, confirming the DT capacity of the following species: *S. extensa*, *S. nothohybrida*, *S. polyptera*, *S. ribae*, *S. rupincola*, *S. schiedeana*, and *S. wrightii* (Supplementary Fig. S5). However, the whole-plant analysis may incorrectly classify a specimen such as *S. delicatissima* as a sensitive species because almost all tissue showed excessive oxidation, but a few apices can recover under prolonged evaluation times (Supplementary Fig. S5). In contrast, the TTC assay has the advantage of indicating that this species possesses a DT mechanism specific to apical tissue (Fig. 5B).

To test the general applicability of the dehydration system in combination with the TTC assay to determine tissue viability in other known desiccation-tolerant models, we tested the DT ability of the fern *Myriopteris aurea*, and the mosses *Braunia secunda* and *Pogonatum comosum*. We were able to confirm the DT of all three species using the TTC assay (Fig. 5D–F). The TTC assay was also tested in the fern species *Pleopeltis plebeia* and *P. mexicana*; however, due to the size of these samples, tissues were dried using a different dehydration system (desiccator jar with a saturated solution of MgCl$_2$) (Supplementary Fig. S6). The staining pattern of *P. plebeia* fronds clearly indicated that this species is desiccation tolerant as previously reported (Hietz, 2010), whereas *P. mexicana* showed viable as well as non-viable sections within the same frond. The results for *P. mexicana* could be due to the dehydration conditions used in these experiments (i.e. the very rapid drying rate produced by the MgCl$_2$ for this species).

Loss of tissue viability at specific water contents

The viability of *S. silvestris* during dehydration was determined at different levels of water content using the TTC assay (described in detail in Supplementary Protocol S1). At specific relative water

contents (RWCs), explants were removed from the drying system and immediately rehydrated. An initial analysis of tissue viability by TTC staining revealed a change in viability between explants dehydrated to 40% and 20% RWC (Fig. 6A). A procedure to determine the proportion of viable tissue by image analysis of the red-stained areas within explants was implemented. The main steps for the quantification of the viable areas are presented in Fig. 6B and details of image analysis are described in Supplementary Protocol S1. A detailed analysis of samples exposed to these water contents determined that the viable area of explants decreased by 13.5% at 40% RWC and further decreased by 67.8% at 20% RWC (Fig. 6C). Although most of the explant area remains viable at 40% RWC, measurement of quantum efficiency indicated that the tissue was under stress with a F_v/F_m of 0.53 (Supplementary Fig. S7), compared with 0.8 in unstressed tissue in both desiccation-tolerant and sensitive species (Fig. 2B). Furthermore, quantum efficiency showed an even lower value at 20% RWC (F_v/F_m 0.47; Supplementary Fig. S7).

All explants of *S. silvestris* dehydrated to 80% and 60% RWC can recover and re-establish growth in substrate (i.e. explants can create a new individual), indicating that if tissue damage occurred at these water contents it was limited to a repairable level. At 40% RWC, most of the explant area is viable according to the TTC test (Fig. 6C), but not all the explants were capable of continued growth in substrate and most of them had large sections that suffered visible damage (Supplementary Fig. S7). After 4 weeks in substrate, explants that survived dehydration to 40% RWC showed green sectors with clear growth and the emergence of at least one rhizophore (a specialized structure which produces roots). The failure of some explants to re-establish growth (~18%) suggests that irreversible damage begins when the tissue is dehydrated to 40% RWC. Moreover, the TTC staining pattern of *S. silvestris* at 20% RWC revealed that in some explants the apical region was still viable, which
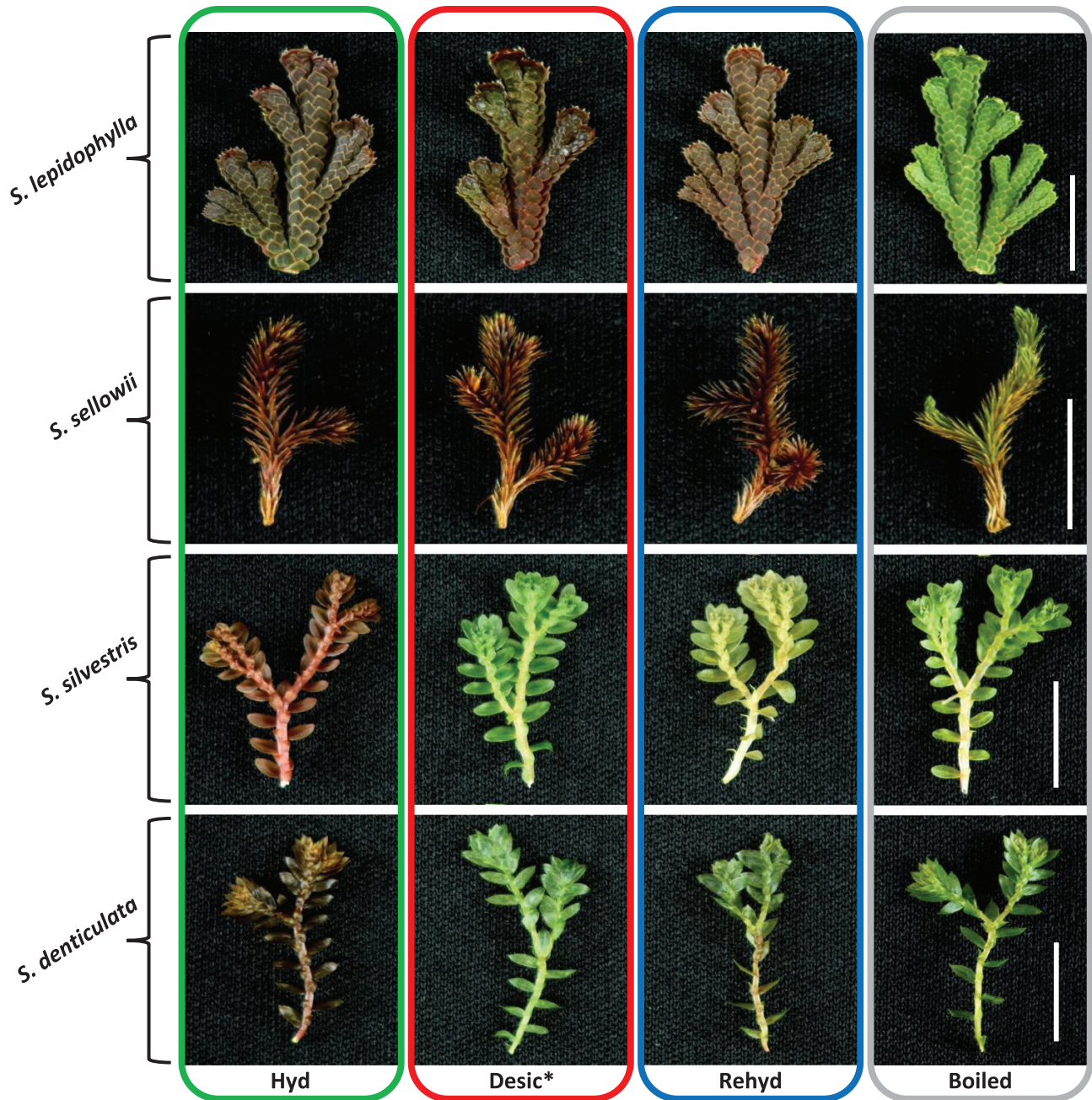
Fig. 4. Determination of tissue viability based on respiratory activity. The respiratory chain produces an insoluble pink/red compound by the reduction of triphenyltetrazolium chloride (TTC). Desiccated tissue was directly submerged in TTC solution (Desic*). No TTC reduction was observed in boiled explants included as non-viable tissues (gray box). Abbreviations and color code are similar to those in Fig. 2. Scale bar=1 cm.

also indicated that the apical region was the last part of the explant to lose viability. However, these small viable sectors showed a much slower growth (limited to a green tip and only a few developed rhizophores) compared with explants dehydrated to 40% RWC (Supplementary Fig. S7). Our results indicate 40% RWC as the onset of a critical threshold during dehydration for *S. silvestris* that causes irreversible damage in large sectors of most explants.

Discussion

The dehydration techniques and methods of assessing recovery used for plant DT studies can be quite variable, complicating cross-species comparisons. Desiccation experiments are commonly performed at the whole-plant level under greenhouse or growth chamber conditions. However, whole-plant procedures rely on carefully controlled conditions (especially
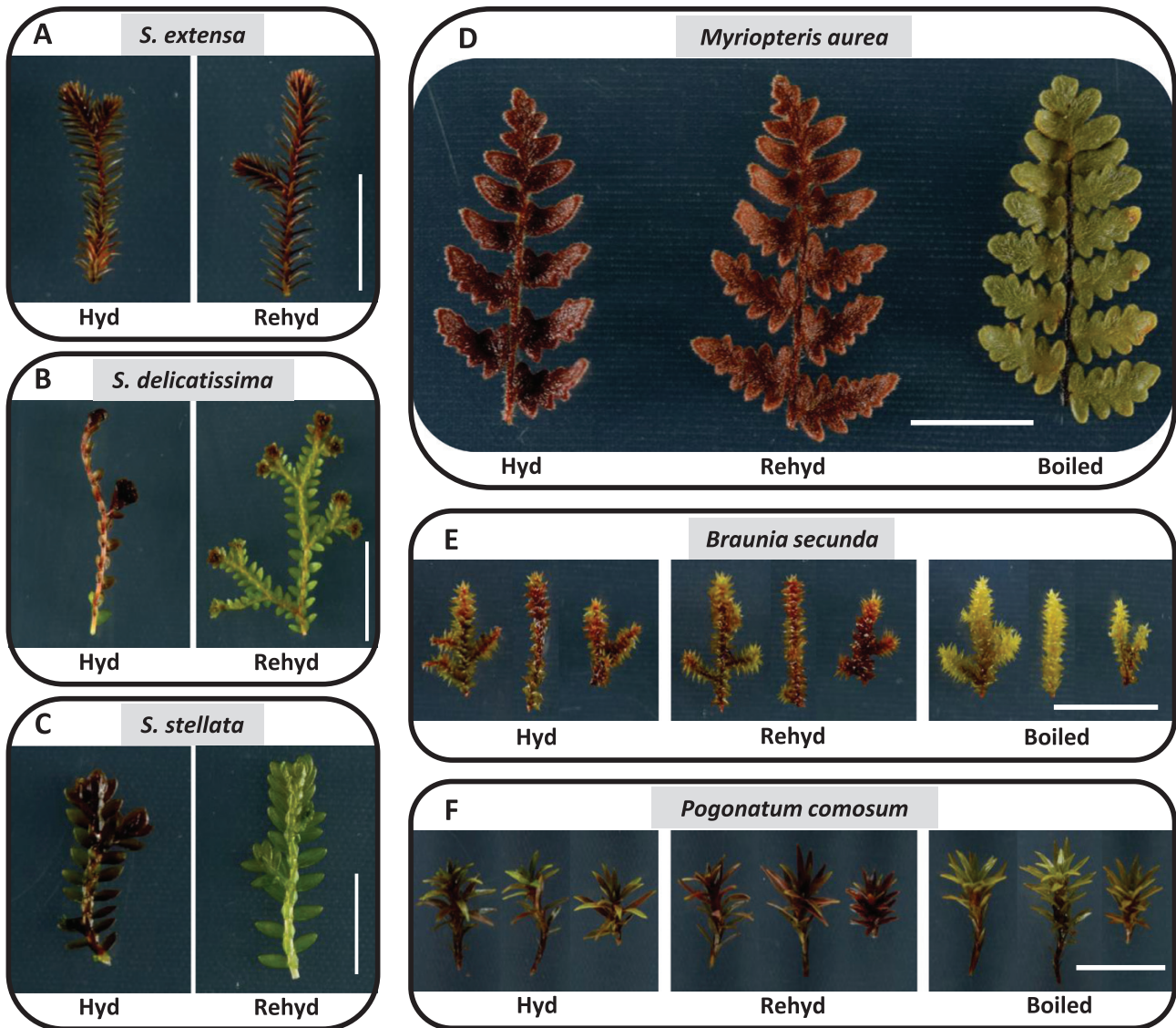
Fig. 5. Triphenyltetrazolium chloride (TTC) test to determine desiccation tolerance in several species. Photographs of representative *Selaginella* explants exposed to desiccation and subsequent rehydration (Rehyd), and then classified as desiccation-tolerant (A), tissue-specific tolerant (B), or desiccation-sensitive species (C). Tissue viability using the TTC test in other plant models: *Myriopteris aurea* (D), *Braunia secunda* (E), and *Pogonatum comosum* (F). Similar or stronger staining (red color) of tissue in hydrated (Hyd) conditions indicated viability. Non-viable controls (Boiled) are shown for other plant models. Scale bar=1 cm.

humidity) that have a direct effect on the drying rate and thus the reproducibility of the experiments. Experiments using excised tissue inside a small, closed container harboring a dehydration agent constitute a simple and more controlled method to expose tissue of different species to the same drying conditions. Additional advantages of this drying technique include a reduced evaluation period and the potential for use under field conditions that would avoid the removal of the individual from its habitat. The use of excised tissues to perform comparative desiccation studies can be applied to homoiochlorophyllous species whose detached tissues retain DT, since these tissues can exhibit the same capacity for DT as that of the whole plant (Mitra *et al.*, 2013).

Although DT in *Selaginella* involves some constitutive protection components, species of this genus also require time to activate protection mechanisms induced during water loss (Liu *et al.*, 2008; Yobi *et al.*, 2012). Leakage measurements revealed that explants exposed to rapid drying showed significant membrane damage in *S. lepidophylla* and that moderate drying rates produced lower membrane damage in tolerant *Selaginella* species (Fig. 1C). Contrary to the assumption that a slow drying rate would produce the least damage, our results showed that the slow regime results in higher electrolyte leakage than moderate drying. Slower dehydration rates may result in prolonged exposure to intermediate water contents, leading to greater damage, and this may account for the higher
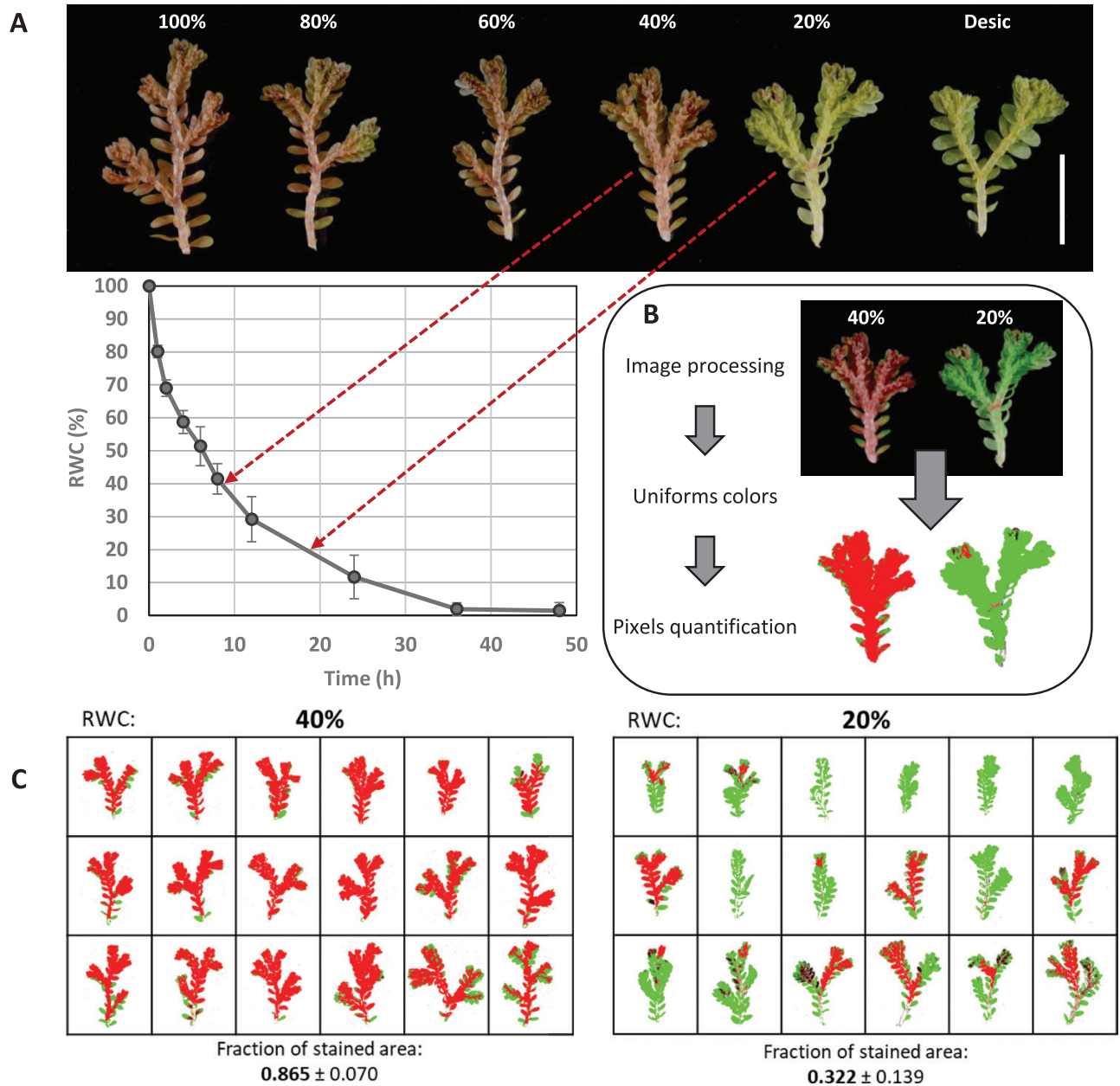
Fig. 6. Loss of viability in *S. silvestris* explants. (A) Explant drying at specific relative water contents (RWCs) and respiratory chain activity determination by the triphenyltetrazolium test. (B) Main steps of the image analysis for determination of viable area (stained tissue) at each RWC. (C) Proportion of viable area of explants dehydrated to 40% and 20% RWC. Each of the points of the drying curve correspond to six different replicates; error bars represent the SD. Scale bar=1 cm.

electrolyte leakage observed under the slow regime. A possible scenario related to this hypothesis stems from the observation that carbon fixation in desiccation-tolerant species ceases at around −2 MPa, but light-harvesting reactions of photosynthesis continue until −15 MPa (Oliver *et al.*, 2020), producing high-energy intermediates that can result in the production of toxic levels of ROS.

Measurement of the photosynthetic parameter F_v/F_m has previously been used to determine recovery after desiccation in *Selaginella* (Pandey *et al.*, 2010; Xu *et al.*, 2018;

Alejo-Jacuinde *et al.*, 2020). Desiccation-tolerant species completely recovered F_v/F_m values after rehydration, whereas sensitive species exhibited values similar to the desiccated state, indicating that the tissues were still under considerable stress (Fig. 2B). As a viability marker, recovery of F_v/F_m clearly differentiates between desiccation-tolerant and sensitive *Selaginella* species. Similar results were previously reported for desiccation-tolerant bryophyte and fern species, where most of them show almost complete recovery of F_v/F_m after the desiccation–rehydration process (López-Pozo *et al.*, 2019).

However, in the same study, the threshold of F_v/F_m recovery established to define a desiccation-tolerant species was less clear for homoiochlorophyllous angiosperm species for which the F_v/F_m values ranged between 23% and 95% recovery depending upon the dehydration agent used (NaCl, MgCl$_2$, or silica gel), complicating the use of F_v/F_m as the only parameter to assign desiccation tolerance. This pioneer work for a standardized field portable method to evaluate DT is complemented by the additional markers reported in the present work. During rehydration, chlorophyll content almost fully recovered in desiccation-tolerant species but not in sensitive species (Fig. 3A). Our results are in agreement with those previously reported indicating that the desiccation-tolerant species *S. bryopteris* showed a similar pattern of F_v/F_m and chlorophyll content in response to desiccation (Pandey *et al.*, 2010). The tolerant species, *S. lepidophylla* and *S. sellowii*, can tolerate and recover from the leakage of 22.7% and 14.1% of their total electrolytes, respectively, but membrane integrity is completely compromised upon rehydration for the desiccation-sensitive species *S. silvestris* and *S. denticulata* (REL of ~90%) (Fig. 3B). These results, obtained using explants, are comparable with those obtained for whole plants of *Selaginella* species (Agduma and Sese, 2016).

Maintaining the integrity of cellular components such as ribosomes is essential to survive desiccation. A comparative analysis between species of the Linderniaceae family showed that levels of total and polysomal RNA from desiccation-tolerant plants were maintained during the whole dehydration treatment, whereas a sensitive species showed RNA degradation when dehydration was severe (Juszczak and Bartels, 2017). In our experiments, the integrity of rRNA was maintained in desiccation-tolerant *Selaginella* species during both the desiccation and rehydration processes, whereas in sensitive species rRNA integrity was maintained in the desiccated state but significant rRNA degradation was detected during rehydration (Fig. 3C). Cells undergo mechanical, structural, and metabolic stresses during water loss (Oliver *et al.*, 2020), but cellular damage can also occur during the influx of water at rehydration (Alpert and Oliver, 2002; Oliver *et al.*, 2005). The precise stage at which most of the cellular damage occurs (during dehydration or rehydration) and whether cellular components differ in their tolerance capacity remain uncertain.

Excessive ROS accumulation results in cell death; therefore, antioxidant protection systems play an essential role in DT (Proctor and Tuba, 2002; Challabathula *et al.*, 2018; Oliver *et al.*, 2020). Antioxidant potential in *Selaginella* explants (determined by FRAP and DPPH assays) was greater in desiccation-tolerant than sensitive species under hydrated conditions (Table 1). High antioxidant levels under normal conditions suggest that *S. lepidophylla* and *S. sellowii* are primed to tolerate forthcoming desiccation events, as described for the DT grass *Sporobolus stapfianus* (Oliver *et al.*, 2011). Antioxidant capacity increased in all *Selaginella* species in response to desiccation,

although desiccation-tolerant plants accumulate antioxidants to a greater extent. Antioxidants are also required to scavenge ROS at the rehydration stage (Farrant *et al.*, 2007; Oliver *et al.*, 2020), which is consistent with our observation that desiccation-tolerant *Selaginella* species maintained higher antioxidant potential during rehydration (Table 1). Other antioxidant compounds such as polyphenols and flavonoids have a putative membrane protective role during desiccation (Georgieva *et al.*, 2017). Metabolic analysis of hydrated and dehydrated plants determined a higher level of several amino acid-derived flavonoids in *S. lepidophylla* compared with the sensitive species *S. moellendorffii* (Yobi *et al.*, 2012). Additionally, a higher fraction of genes involved in flavonoid metabolism is observed in response to dehydration in *S. lepidophylla* and *S. sellowii* in comparison with *S. denticulata* (Alejo-Jacuinde *et al.*, 2020). This is consistent with our findings that tolerant plants showed higher levels of flavonoids in hydrated conditions and accumulated more flavonoids during desiccation than sensitive species (Table 1).

Our results also provide valuable insights from comparisons between tolerant species. Although both *S. lepidophylla* and *S. sellowii* have high antioxidant levels at the desiccated stage, in response to desiccation *S. lepidophylla* showed a significant increase in antioxidant potential, whereas *S. sellowii* explants showed only a slight increase in the antioxidant parameters. These results suggest that antioxidant protection in *S. lepidophylla* is inducible whereas in *S. sellowii* it is largely constitutive. The ability of some bryophytes to survive rapid desiccation is associated with a constitutive protection mechanism (Oliver *et al.*, 2000; Alpert and Oliver, 2002), which also involved a rapid recovery of photosynthesis (Challabathula *et al.*, 2018). A major constitutive component in the DT in *S. sellowii* correlates with lower membrane damage induced by different drying rates and faster recovery of photosynthesis during rehydration compared with *S. lepidophylla* (Alejo-Jacuinde *et al.*, 2020).

The TTC test was successfully adapted to vegetative tissue of *Selaginella*, providing a simple identification of viable tissue after desiccation treatments. The efficiency of the TTC assay as a viability marker for DT was shown by the identification of novel desiccation-tolerant species (Fig. 5A; Supplementary Fig. S3). We were able to establish that *S. extensa*, *S. nothohybrida*, *S. polyptera*, *S. ribae*, *S. rupincola*, *S. schiedeana*, and *S. wrightii* are desiccation-tolerant species. Previous studies had described some of these species as 'resurrection species' but no data delineating their DT capacity had been reported. This assay also revealed that only the apices of the explants of *S. delicatissima* exhibited DT (Fig. 5B) which could enable this species to re-establish growth and create a new individual after an extensive dry period. Further studies on this *Selaginella* specimen could reveal insights into why only the apical tissue of some explants has the capability for DT.

Phylogenetic analyses have suggested multiple origins of DT within the *Selaginella* genus (Korall and Kenrick, 2002), and

most of the desiccation-tolerant species identified in this study belong to clades containing previously reported tolerant species. Interestingly, *S. extensa*, a desiccation-tolerant species with isophyllous morphology, was collected from a population located in a cloud forest with an annual rainfall of ~2028 mm. Most of the isophyllous *Selaginella* species occupy xeric habitats (Arrigo *et al.*, 2013) and at least eight of these have been classified as desiccation tolerant (Proctor and Pence, 2002). We identified three additional desiccation-tolerant isophyllous species (*S. extensa*, *S. rupincola*, and *S. wrightii*). All these species

belong to the same clade within the *Selaginella* phylogeny (Homoeophyllae according to Zhou *et al.*, 2015; Rupestrae according to Weststrand and Korall, 2016), suggesting that all species within this clade are likely to be desiccation tolerant (between 45 and 60 species), including those that naturally grow in very moist habitats.

Additionally, our results demonstrated that the TTC assay could also be applied to other DT models (Fig. 5). This technique correctly indicated tissue viability after desiccation in the desiccation-tolerant ferns *Myriopteris aurea* (previously
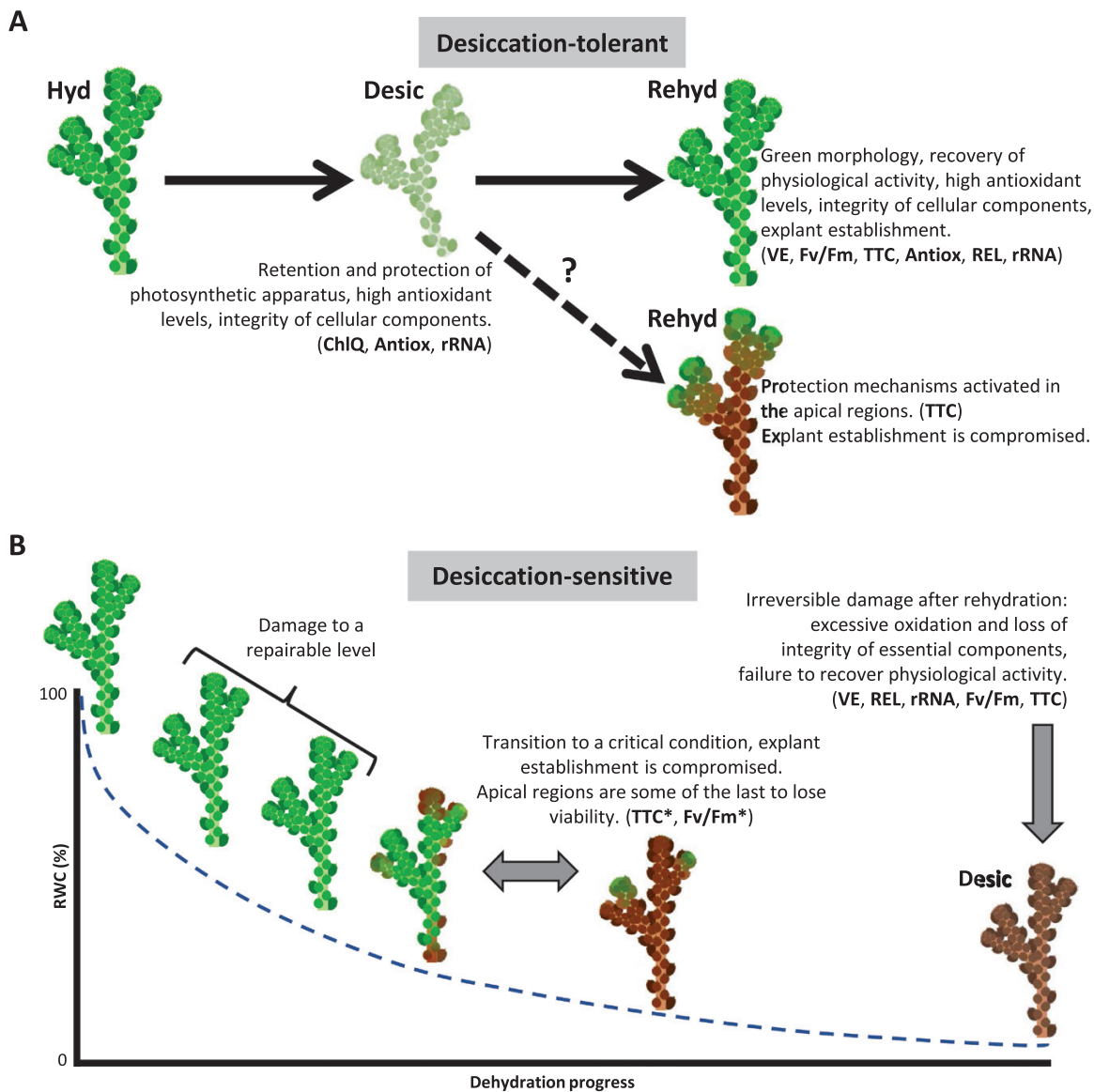
Fig. 7. Desiccation tolerance-associated characteristics and critical stage determination in *Selaginella* species. (A) Overview of measurable responses, components, or activities in desiccation-tolerant species. Tissue-specific desiccation tolerance is indicated as an alternative mechanism (dashed arrow). (B) Identification of a critical condition by viability loss in sensitive species; excessive oxidation is depicted in brown. Methodologies proposed to evaluate each characteristic are indicated in bold (in parentheses): antioxidant potential (Antiox), chlorophyll quantification (ChlQ), maximum quantum efficiency of PSII (F_v/F_m), relative electrolyte leakage (REL), rRNA integrity (rRNA), triphenyltetrazolium chloride (TTC), and visual evaluation (VE). Tissue dehydrated and subsequently rehydrated (*).

Cheilanthes bonariensis) (Iturriaga *et al.*, 2000) and *Pleopeltis plebeia* (Hietz, 2010). The experimental conditions tested here produced tissue damage in the desiccation-tolerant fern *P. mexicana* (Hietz, 2010), indicating the importance of establishing an appropriate drying rate or the duration of the desiccation phase for each plant species. Additionally, TTC analysis of the mosses *Braunia secunda* and *Pogonatum comosum* indicated that these species tolerate equilibrium with 32–33% RH (Fig. 5). The scope of the present study did not include bryophytes since standardized experimental protocols to evaluate DT are already well established for this group of plants (Wood, 2007). Although we successfully used a week-long desiccation phase to demonstrate the usefulness of the TTC method to evaluate DT in mosses and ferns, it is possible that 1 week desiccation phase could be excessive for some plant species. Therefore, using different durations of the desiccation phase could be tested in order to evaluate the degree of DT.

A whole-plant study reported that the desiccation-sensitive species *S. moellendorffii* cannot recover from dehydration to 40% RWC or below (Yobi *et al.*, 2012). Other reports have suggested that dehydration below 40% RWC produces extensive cellular damage resulting in death of most plants (Mitra *et al.*, 2013). An extensive literature analysis by Zhang and Bartels (2018) proposed a boundary between dehydration and the desiccation response at 40–30% RWC. The TTC assay proposed as viability marker was particularly useful to identify critical points during the dehydration process in a sensitive species. Our data confirmed that viability loss initiates at 40% RWC in the sensitive species *S. silvestris*, causing extensive and irreversible damage in a large portion of the tissue, and the majority of the explant area did not survive below 40% RWC (Fig. 6).

The use of explants in desiccation experiments has several advantages, and our results corroborate that the response to desiccation in explants is representative of that observed in whole-plant analysis. Our main findings are summarized in Fig. 7, including the methodologies proposed to evaluate each characteristic associated with DT. The system reported here can be exploited for the characterization of desiccation-tolerant species and to compare tolerance mechanisms between them. The main viability marker proposed in this study (TTC) represents a simple and robust method to determine DT capacity and critical stages during dehydration. Our data also uncovered a potentially novel DT mechanism in *S. delicatissima*, specific to the apical portion of the tissue.

Supplementary data

The following supplementary data are available at *JXB* online.
Table S1. Collection sites of the species included in the study.
Protocol S1. Drying system, biological material, TTC test, and image analysis of viable areas.
Fig. S1. Drying curves of *Selaginella* species.
Fig. S2. Chlorophyll retention after desiccation in tolerant species.

Fig. S3. Viability test to determine desiccation tolerance in several *Selaginella* species.
Fig. S4. Standardization of evaluation of desiccation tolerance at the whole-plant level.
Fig. S5. Whole-plant level evaluation of desiccation tolerance in several *Selaginella* species.
Fig. S6. Morphological changes in response to water availability and the triphenyltetrazolium chloride (TTC) test in *Pleopeltis* species.
Fig. S7. Recovery of *S. silvestris* explants at specific water contents.

Author contributions

GA-J and LH-E: design; GA-J: conducting most of the experimental research; GA-J and TK-G: electrolyte leakage analysis; NM-G and JPD-F: antioxidant analysis; GA-J, TK-G, KM, and DT-D: obtaining plant material and classifying novel tolerant species; GA-J, LH-E, JS, and MO: data analysis; GA-J, JS, and LH-E: writing. All authors read and approved the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Data availability

All data supporting the findings of this study are available within the paper and within its supplementary data published online.

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