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Changes in photosynthetic rate and stress volatile emissions through desiccation-rehydration cycles in desiccation tolerant epiphytic filmy ferns (Hymenophyllaceae)

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Abstract

Exposure to recurrent desiccation cycles carries a risk of accumulation of reactive oxygen species that can impair leaf physiological activity upon rehydration, but development of filmy fern stress status through desiccation and rewetting cycles has been poorly studied. We studied foliage photosynthetic rate and volatile marker compounds characterizing cell wall modifications (methanol) and stress development (lipoxygenase (LOX) pathway volatiles and methanol) through desiccation/rewetting cycles in lower-canopy species *Hymenoglossum cruentum* and *Hymenophyllum caudiculatum*, lower- to upper-canopy species *H. plicatum* and upper-canopy species *H. dentatum* sampled from a common environment and hypothesized that lower canopy species respond more strongly to desiccation and rewetting. In all species, rates of photosynthesis and LOX volatile emission decreased with progression of desiccation, but LOX emission decreased with a slower rate than photosynthesis. Rewetting first led to an emission burst of LOX volatiles followed by methanol, indicating that the oxidative burst was elicited in the symplast and further propagated to cell walls. Changes in LOX emissions were more pronounced in the upper-canopy species that had a greater photosynthetic activity and likely a greater rate of production of photooxidants. We conclude that rewetting induces the most severe stress in filmy ferns, especially in the upper canopy species.

Keywords

emission bursts; Hymenophyllaceae; lipoxygenase pathway volatiles; methanol; desiccation kinetics; recovery

Introduction

Resurrection plants are characterized by remarkable capacity of toleration of multiple desiccation/rehydration cycles and by rapid adjustments of foliage physiological characteristics upon changes in water availability (Beckett, Loreto, Velikova, Brunetti, Di Ferdinando, Tattini, Calfapietra & Farrant, 2012, Beckett, Minibayeva, Lühje & Böttger, 2004, Farrant, 2000, Proctor, 2000, Proctor & Tuba, 2002). This allows them to colonize microhabitats where water availability is highly variable such as tree stems and rocky substrates in otherwise humid ecosystems.

Among resurrection plants, epiphytic filmy ferns are characteristic components of temperate to tropical humid forests that can form a dense cover on protruding roots and tree stems (Dubuisson, Hennequin, Bary, Ebihara & Boucheron-Dubuisson, 2011, Dubuisson, Schneider & Hennequin, 2009, Parra, Acuña, Corcuera & Saldaña, 2009, Saldaña, Parra, Flores-Bavestrello, Corcuera & Bravo, 2014). In epiphytic filmy ferns, leaf lamina typically consists of only one cell layer and consequently, stomata cannot curb leaf water loss (Hietz, 2010). Desiccation stress in filmy ferns leads to major reductions in foliage photosynthetic characteristics, even up to complete cessation of photosynthesis once the minimum frond relative water content is achieved (Bravo, Parra, Castillo, Sepúlveda, Turner, Bertín, Osorio, Tereszczuk, Bruna & Hasbún, 2016, Hietz & Briones, 2001, Proctor, 2003, Proctor, 2012). Physiological changes are further accompanied by cell shrinkage and ultrastructural alterations in cell walls and chloroplasts as well as in chemical modifications in cell wall polysaccharides and associated proteins (Bravo *et al.*, 2016). Once rewatered, both physiological, ultrastructural and chemical changes are rapidly, within tens of minutes to a few hours, reversed (Bravo *et al.*, 2016, Garcés, Ulloa, Miranda & Bravo, 2017), implying that the filmy ferns have a very high constitutive capacity to cope with recurrent desiccation/rewatering cycles, a strategy called homoiochlorophyllous strategy (Bravo *et al.*, 2016, Garcés Cea, Claverol, Alvear Castillo, Rabert Pinill & Bravo Ramírez, 2014).

Filmy ferns colonize a spectrum of habitats from high-light lower-humidity upper-canopy environment to lower-light higher-humidity lower-canopy environments, but it is currently unclear how the extent of physiological modifications varies across species from different habitats. Furthermore, homoiochlorophyllous strategy poses potential risks of overenergization of photosynthetic machinery and enhanced rate of formation reactive oxygen species during desiccation, especially in species colonizing higher light habitats in the upper canopy where the excess excitation energy is the greatest when photosynthesis becomes inhibited in desiccated leaves. To our knowledge, changes in leaf oxidative status through desiccation/rewatering cycles have not been studied in filmy ferns, but enhanced leaf oxidative status upon rewatering has been observed in mosses (Mayaba, Minibayeva & Beckett, 2002, Minibayeva & Beckett, 2001).

High-resolution direct monitoring of leaf oxidative status and overall stress level through desiccation/rewatering cycles is not feasible. However, a plethora of volatiles is released from stressed plants (Loreto & Schnitzler, 2010, Matsui, Sugimoto, Kakumyan, Khorobrykh & Mano, 2010, Niinemets, Arneth, Kuhn, Monson, Peñuelas & Staudt, 2010), and the release of several of stress volatiles is typically quantitatively associated with the severity of

stress (Beauchamp, Wisthaler, Hansel, Kleist, Miebach, Niinemets, Schurr & Wildt, 2005, Jiang, Ye, Li & Niinemets, 2017, Loreto & Schnitzler, 2010, Niinemets, Kännaste & Copolovici, 2013). Among these volatiles, the release of lipoxygenase (LOX) pathway volatiles, a mixture of various C6 aldehydes and alcohols and their derivatives is a ubiquitous response to different abiotic and biotic stresses; LOX volatiles are typically elicited upon severe stress that leads to membrane-level damage (Beckett *et al.*, 2012, Copolovici, Kännaste, Pazouki & Niinemets, 2012, Copolovici, Pag, Kännaste, Bodescu, Tomescu, Copolovici, Soran & Niinemets, 2017, Copolovici, Väärtõu, Portillo Estrada & Niinemets, 2014, Kanagendran, Pazouki & Niinemets, 2018, Li, Harley & Niinemets, 2017). The pathway starts with the release of free polyunsaturated fatty acids from plant membranes, and production of a mixture of 9- and 13-hydroperoxy linoleic and -linolenic acids by lipoxygenases (Andreou & Feussner, 2009, Liavonchanka & Feussner, 2006). The hydroperoxy acids are further converted to C6 volatiles in a series of reactions, and immediately released or first derivatized and then released, often from organ locations distant from the immediate stress impact (Matsui, Sugimoto, Mano, Ozawa & Takabayashi, 2012, Scala, Allmann, Mirabella, Haring & Schuurink, 2013). In addition, LOX volatile release that relies on symplastic processes is often accompanied by cell-wall dependent methanol emission (Beauchamp *et al.*, 2005, Copolovici *et al.*, 2012, Li *et al.*, 2017) that results from activation of cell wall pectin methyl esterases that catalyze demethylation of galactouronan methyl esters in pectin (Micheli, 2001, Pelloux, Rustérucchi & Mellerowicz, 2007). Modification in pectin methylation level alters cell wall mechanical properties (Cosgrove, 2016), and given the major changes in filmy fern cell walls during the desiccation and rehydration (Bravo *et al.*, 2016), desiccation/rewatering cycles are expected to alter the constitutive release of methanol from filmy ferns.

To our knowledge, the effects of desiccation stress on volatile release in resurrection plants have been studied only in the angiosperm *Xerophyta humilis* where LOX emission was maximized at an intermediate level of desiccation stress, but it was not altered by rehydration (Beckett *et al.*, 2012). However, differently from mosses and filmy ferns, *X. humilis* features a poikilochlorophyllous strategy characterized by massive losses of pigments and reductions in photosynthetic capacity during desiccation (Csintalan, Tuba, Lichtenthaler & Grace, 1996, Tuba, Lichtenthaler, Maroti & Csintalan, 1993b, Tuba, Protor & Csintalan, 1998). Thus, a much stronger volatile response is expected in homoiochlorophyllous species that do maintain pigments and photosynthetic activity through desiccation.

We studied four epiphytic filmy ferns - *Hymenoglossum cruentum* (Cav.) C. Presl that has simple leaves, and *Hymenophyllum caudiculatum* Mart. var. *productum* (K. Presl.) C. Chr., *Hymenophyllum dentatum* Cav. and *Hymenophyllum plicatum* Kaulfuss (Hymenophyllaceae) that all have pinnately dissected compound leaves (Fig. 1). The studied species differ in their vertical distribution in the stem of the host trees in Chilean temperate rainforest canopy. *Hymenoglossum cruentum* and *H. caudiculatum* colonize more humid and deeper shade habitats with their cover on tree stems extending from tree base to up to 3 m in the canopy, *H. dentatum* colonizes habitats with lower humidity and greater light intensity on tree stems between 1-9 m and *H. plicatum* is a generalist with dispersal between 0-9 m (Flores-Bavestrello, Król, Ivanov, Huner, García-Plazaola, Corcuera & Bravo, 2016, Parra,

Acuña, Sierra-Almeida, Sanfuentes, Saldaña, Corcuera & Bravo, 2015, Saldaña *et al.*, 2014). We hypothesized that desiccation stress results in enhancement of stress volatile emissions and that these emissions are particularly enhanced upon rewatering. We also hypothesized that the changes in stress volatile emissions during desiccation-rewatering cycles are particularly pronounced in lower-canopy species that have adapted to less severe environmental conditions.

Material and methods

Intact ferns of *H. cruentum*, *H. caudiculatum* var. *productum*, *H. dentatum* and *H. plicatum* were collected from natural habitats in Parque Katalapi, Puerto Montt, X Región, Chile (41.52° S 72.755° W, altitude 80 m). The site supports a classic low-altitude temperate evergreen rainforest with ample moss and fern cover on the ground and on tree stems and branches (Saldaña *et al.*, 2014 for a detailed description of the sample site). To avoid confounding effects of acclimation to different light environments on the physiological characteristics studied here, all samples were taken from similar height of 0.3–1 m in a moderately open part of the stand (ca. 30% site openness). The ferns were gently removed from their substrate, put in polyethylene bags with wet filter paper and transported to the lab for physiological measurements.

Experimental design and relative plant water content

Two parallel experiments were run under identical conditions, one to estimate time-dependent changes in leaf water content upon desiccation and rehydration and the other for continuous measurements of foliage photosynthetic rate and volatile emission rates.

Prior to the start of the experiment, the plants were maintained in high humidity (100%) atmosphere in the dark at 25 °C for 12 h. Then the plants were transferred to moderately high light (150–200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for photosynthesis induction. The plants were stabilized for 20–30 min. at this light level in high humidity conditions to keep full hydration. After stabilization, the entire plant module was transferred to an illuminated (300–400 $\mu\text{mol m}^{-2} \text{s}^{-1}$) 1.2 L gas-exchange chamber of a custom-made gas-exchange system described in detail in Copolovici *et al.* (2010). The light level chosen in current experiments corresponds to light intensities observed in situ on surface of filmy ferns colonizing the upper canopy and to intensities observed during lightflecks in the lower canopy (Parra *et al.*, 2015). The chamber is made of double-walled glass with stainless steel bottom, and a preset chamber temperature is maintained by water that first passes through a thermostated water-bath and then through the space between the chamber walls. In these experiments, the chamber was operated at 25 °C and air (flow rate 1.4 L min^{-1}) with low atmospheric humidity (average $\pm$ SE vapor pressure deficit of 3.029 ± 0.002 kPa, corresponding to an average relative humidity of $4.75 \pm 0.05\%$) to simulate desiccation stress conditions as those occurring when ferns in their natural environment are rapidly exposed to sunflecks penetrating the tree canopy. Ambient chamber CO_2 concentration was 380–395 $\mu\text{mol mol}^{-1}$ and it was stabilized by passing the ambient air through a 10 L buffer volume. The chamber air was vigorously mixed with a fan installed in the system, resulting in fully turbulent conditions. After leaf insertion, the stabilization of gas flows took ca. 3 min, and thus, these initial non-steady state

CO₂ exchange and volatile emission data were discarded. The plant was maintained under these desiccation treatment conditions for 4 hr. At 4 hr since the start of the experiment, the plant was rapidly rehydrated (within ca. 10 min.) by spraying the plant uniformly with distilled water and the experiment was further continued for 1.5 h after surface water had evaporated (see below).

In parallel experimental runs, the plant mass (M_F) was estimated every 30 min. since the start of the experiments under light in analogously dry atmospheres (relative air humidity 4-6%). In the case of rehydration, the plant surface was let to air dry prior to its mass estimation (ca. 10-15 min). At the end of the experiment the plant was dried at 72 °C for 48 h and the dry mass (M_D) was estimated. From these measurements, the relative water content (RWC, %) at different time points was calculated as:

$$RWC\% = 100 \left(1 - \frac{M_{F,0} - M_F}{M_{F,0} - M_D} \right), \quad (1)$$

where $M_{F,0}$ is the fresh mass of the fully turgid leaves at the beginning of the experiment after evaporation of the surface water.

Gas-exchange and volatile emission measurements

Once the plant was enclosed in the gas-exchange chamber, CO₂ and H₂O concentrations at chamber in- and outlets were continuously measured with an infra-red dual-channel gas analyzer operated in differential mode (CIRAS II, PP-systems, Amesbury, MA, USA). Simultaneously with these measurements, parts of ingoing and outgoing air (200 ml min⁻¹) were drawn for measurements of concentrations of volatile organic compounds by a Proton-Transfer Reaction Quadrupole Mass Spectrometer (high sensitivity version of PTR-QMS, Ionicon, Innsbruck, Austria). Methanol emission was calculated from the measurements of the signal of protonated parent ion (m/z) of 33⁺, while the sum of volatile octadecanoid pathway products (LOX products) was found as the sum of signals of the key fragment and parent ions with masses 83⁺ (fragments of hexanal, (*Z*)-3-hexenol, (*E*)-3-hexenol, and (*E*)-2-hexenol, (*Z*)-3-hexenyl acetate and (*E*)-2-hexenyl acetate), 85⁺ (pentenal parent ion, and fragments of hexanol and hexyl acetate), 99⁺ (parent ions of (*E*)-2-hexenal and (*Z*)-3-hexenal) and 101⁺ (parent ions of hexenol isomers and hexanal) as described in detail by Fall et al. (1999), Copolovici and Niinemets (2010) and Portillo-Estrada (2015).

The measurements were conducted continuously for the whole 4 hr desiccation treatment (Fig. 2 for a sample response), and the chamber was briefly opened for leaf rehydration as during RWC estimation. To avoid water condensation in the chamber, the measurements were continued again after surface water had evaporated and gas flows stabilized (10-15 min hiatus in the measurements). To avoid mechanical stress effects on volatile emission (Portillo-Estrada *et al.*, 2015), this procedure was preferred over blotting leaves dry by a filter paper.

Fully hydrated samples were photographed and leaf area was estimated at the end of the experiment by a custom-made software (Pindala 1.0 by Indrek Kalamees), and the rate of net

assimilation per unit leaf area (A) was calculated according to von Caemmerer and Farquhar (1981) and volatile emission rates according to Niinemets et al. (2011). For the rates of net assimilation we show data averaged for every 10 min, while for the emission measurements, the data were smoothed using a moving average with 15 s averaging window.

Although RWC estimation experiment and the measurements of gas exchange and volatile emissions were run in parallel, due to a certain variation among the parallel runs, e.g. due to stabilization of gas-flows before the start of measurements, the data synchronization is accurate to 5-10 min.

Data analyses

Three replicate experiments with different fern modules were carried out for all species. We show both the representative sample kinetics for individual species as well as averages for derived characteristics. In particular, time-dependent reductions in RWC, net assimilation, and total LOX emission rates during the desiccation cycle were fitted by exponential relationships to derive the first-order rate constants of the decay (Fig. 2 for the sample fits). As net assimilation rate stabilized somewhat earlier (or the response became biphasic), a time-period of 0-1 h since the start was used for net assimilation (Fig. 2B). For LOX volatiles and RWC that declined continuously in time without evidence of multiphasic kinetics, the entire desiccation period was used to derive the decay rate constants (Figs. 3-4). Average values of decay constants for each characteristic were estimated for each species. Averages of net assimilation rate and methanol and LOX volatile emission rates at the start of the experiment, and 0.5 and 1 h after the start were also calculated. The emission bursts of LOX volatiles and methanol after rewatering were integrated from rewatering until the emissions reached a new steady state, between ca. 4-4.5 h (Fig. 4-5), and species averages were estimated. Averages of all characteristics were compared among species by one-way ANOVA followed by Tukey's post hoc test, and all comparisons were considered significant at $P < 0.05$.

Results

Time-dependent declines in fern water content and net assimilation rate

After transfer of plants to low atmospheric humidity of $4.75 \pm 0.05\%$, frond relative water content (RWC, Eq. 1) decreased in all species (Fig. 3). The average rate of reduction in water content through the desiccation period (k_{RWC}) was not significantly different among species (Fig. 6A), and all species reached a very low frond average RWC of 2-4% by the end of dehydration treatment (Fig. 3).

The decrease in RWC during desiccation was associated with concomitant changes in net assimilation rate (Fig. 3). In some cases, net assimilation rate continued at a significant level even at 4 h since the start of desiccation (e.g., *Hymenophyllum caudiculatum* and *H. dentatum* in Fig. 3). This reflected high non-uniformity of decrease in frond water content with the frond parts close to the rachis remaining visually unaffected (data not shown, Fig. 1 for images of desiccated leaves). The species significantly differed in the initial rate of reduction of photosynthesis (k_A) with the fastest rate of reduction observed in

Hymenoglossum cruentum and *H. dentatum* followed by *Hymenophyllum plicatum* and *H. caudiculatum* (Fig. 6A). No clear relationship among k_{RWC} and k_{A} was observed across species (Fig. 6B).

Changes in emissions of lipoxygenase pathway volatiles (LOX) and methanol during desiccation and their relationship to net assimilation rate

All species emitted significant amounts of lipoxygenase volatiles at the start of the desiccation treatment and the LOX emission rate decreased continuously through the desiccation treatment, (Fig. 4A), although the reduction was typically less than in RWC (Fig. 4A, Fig. 6A). The rate of reduction in LOX emission rate (k_{LOX}) was the greatest for *H. cruentum* followed by *H. caudiculatum* and *H. dentatum*, and the lowest rate of reduction was observed for *H. plicatum* (Fig. 6A). Differently from LOX emissions, methanol emission rate increased through desiccation in *H. cruentum* and *H. caudiculatum* and was relatively invariable in *H. dentatum* and *H. plicatum* (Fig. 4B).

The kinetics of LOX emission and net assimilation rate were qualitatively similar through the desiccation treatment (Fig. 5A), but across species, k_{LOX} was in all cases less than k_{A} (Fig. 6A). *Hymenoglossum cruentum* stood out from the other fern species by having greater rate of reduction in k_{LOX} at given k_{A} (Fig. 6C). Faster reduction in RWC during desiccation (Fig. 6B) and greater LOX emission rate (Fig. 6D) were associated with slower rate of reduction in LOX emission rate during desiccation (Fig. 6D). Methanol emission rate was not associated with either net assimilation rate (Fig. 5B) or with LOX compound emission rate (Fig. 5C) through the desiccation treatment.

Alterations in RWC, net assimilation rate and volatile emissions upon rewatering

Rewatering resulted in a rapid increase in frond RWC and almost simultaneous increase in net assimilation rate (Fig. 3). Rewatering also led to major bursts in LOX volatile (Fig. 4A) and methanol (Fig. 4B) emissions. The emission burst of LOX volatiles preceded the maximum in net assimilation rate in *H. caudiculatum*, LOX burst occurred simultaneously with peaking of net assimilation rate in *H. cruentum*, and it occurred slightly after the photosynthetic maximum in *H. dentatum* and *H. plicatum* (Fig. 5A). Methanol emission burst typically occurred after peaking of net assimilation rate, except for *H. caudiculatum* (Fig. 5B). In all cases, the LOX emission burst occurred earlier than the methanol emission burst (Fig. 5C). After reaching the maxima of LOX and methanol emissions, both LOX and methanol emission rates decreased with decreasing frond RWC (Fig. 4). By the end of measurements at 5.5 h, LOX emission rates reached levels similar to those during desiccation experiment at ca. 0-2 h since the start of desiccation, except for *H. cruentum* and *H. caudiculatum* where LOX emission rates declined almost to a baseline level (Fig. 4A). Methanol emission rates were typically higher at the end of the measurements than before rewatering, except for *H. cruentum* where methanol emission reached similar values than before the rewatering and in *H. caudiculatum* where methanol emission reached close to the background values. The total amount of LOX volatiles (Fig. 7A) and methanol (Fig. 7B) released during the rewatering emission burst was greater in *H. plicatum* and *H. dentatum* than in *H. cruentum* and *H. caudiculatum*.

Discussion

Responses of relative water content and photosynthetic rate of filmy ferns to desiccation and rewetting

Epiphytic filmy ferns (Hymenophyllaceae) exposed to recurrent desiccation events in their natural habitats have a high desiccation tolerance and capacity to rapidly respond to rehydration by profound plastic modifications in cell wall characteristics and maintenance of photosynthetic capacity and pigment contents through desiccation (homiochlorophyllous desiccation tolerance strategy). Filmy ferns have only one cell layer and thus, their photosynthetic capacity is generally low, between ca. $1\text{--}4\ \mu\text{mol m}^{-2}\text{ s}^{-1}$ (Parra *et al.*, 2009, Parra *et al.*, 2015) as was also observed in the current study in fully hydrated samples (Table 1, Fig. 3). Due to lack of stomata and water-permeable cuticular layer, the relative water content (RWC) of filmy ferns rapidly decreases in dry atmospheres, resulting in concomitant reductions in foliage photosynthetic characteristics (Flores-Bavestrello *et al.*, 2016, Proctor, 2012, Saldaña *et al.*, 2014) as observed in our study (Fig. 3, Table 1). Numerically, the rate of initial decrease in RWC and net assimilation rate (A) was similar in *H. cruentum* and *H. dentatum*, but RWC decreased somewhat faster than A in *H. caudiculatum* and *H. plicatum* (Fig. 6A). Greater desiccation resistance of photosynthesis at given RWC might be indicative of species differences in cellular environment such as differences in the content of membrane-stabilizing sugars (Garcés Cea *et al.*, 2014). In addition, after the initial rapid decline, the reduction in A was further slowed down in all species compared with RWC reduction, resulting in a multiphasic reduction pattern (Fig. 3). Such delayed responses have been observed also in other filmy ferns (Bravo *et al.*, 2016, Garcés Cea *et al.*, 2014, Saldaña *et al.*, 2014), and suggest a certain heterogeneity in drying across the fern leaf. Heterogeneity in drying is consistent with our visual observations of less dehydrated frond areas close to the major veins that likely still rely on water supply from veins and rachis during the relatively short-term desiccation treatment, and with the evidence that major lateral water potential gradients can develop within fern fronds (Pittermann, Brodersen & Watkins, 2013). Analogously, desiccation develops faster in marginal areas of moss leaves (Cruz de Carvalho, Catalá, Marques da Silva, Branquinho & Barreno, 2012).

On the basis of changes in foliage dark-adapted quantum yield (F_v/F_m), certain differences in desiccation tolerance of photosynthesis in dependence on canopy location have been shown in temperate Chilean Hymenophyllaceae (Garcés Cea *et al.*, 2014, Saldaña *et al.*, 2014). However, in our study where the plants were sampled from a common tree height of 0–1 m, differences in desiccation responses among species were not clearly linked to canopy height. We did not observe significant differences in the rate of RWC reduction upon desiccation among the four studied species (Fig. 6), but the initial rate of decrease in A was slower in *H. plicatum* (generalist species, extending from tree bottom to the height of 9 m) and *H. caudiculatum* (lower canopy species, 0–3 m) than in *H. cruentum* (lower canopy species 0–3 m) and *H. dentatum* (upper canopy species 1–9 m, Fig. 6). Analogously a faster rate of reduction in foliage dark-adapted quantum yield was observed in desiccated *H. dentatum* than in *H. caudiculatum* in the study of Garcés Cea *et al.* (2014).

Rehydration resulted in a rapid recovery, within ca. 30 min, of frond water content and foliage photosynthesis rate in all species (Fig. 3). Fast recovery of water content reflects high hydrophilicity and capillarity of fern fronds and is consistent with past observations (Bravo *et al.*, 2016, Garcés Cea *et al.*, 2014), whereas fast recovery of photosynthetic rates indicates that foliage photosynthetic capacity was not altered during desiccation. The maintenance of integrity of photosynthetic apparatus during moderately long desiccation treatments is in agreement with the evidence that dark-adapted chlorophyll fluorescence yield remains at a relatively high level and frond pigment content and composition are only weakly affected during moderately long desiccation treatments, a strategy called homoiochlorophyllous desiccation tolerance strategy (Bravo *et al.*, 2016, Flores-Bavestrello *et al.*, 2016). However, previous studies have indicated that the rate of recovery of photosynthetic characteristics is determined by the time of desiccation prior to rehydration; typically, the longer the period of desiccation the longer it takes to reach the pre-stress values of photosynthetic characteristics (Proctor, 2003, Proctor, 2010, Proctor, 2012). Although there is evidence of species-specific differences in the rate of recovery of chlorophyll fluorescence characteristics after rehydration, due to the specifics of the measurement protocol and time-resolution of gas-exchange measurements, we cannot address these differences in our study.

Changes in lipoxygenase pathway (LOX) volatile emissions through desiccation

In the study of the resurrection angiosperm species *Xerophyta humilis*, desiccation stress resulted in enhanced emissions of the saturated aldehyde hexanal, emissions of which peaked at ca. 40% RWC and declined thereafter together with massive losses of photosynthetic pigments (Beckett *et al.*, 2012). Surprisingly, we observed that total LOX compound emissions decreased through the desiccation period (Fig. 3). We suggest that the LOX emissions during the desiccation primarily reflect the oxidative burst generated upon transfer of ferns from lower light/high humidity atmosphere to higher light/lower humidity atmosphere and associated generation of oxidative stress due to a certain overexcitation of photosynthetic machinery. In fact, low light/high humidity to high light/low humidity transitions are common during high intensity lighflecks and result in a large share of excess quantum flux density that can lead to photoinhibition of filmy ferns (Parra *et al.*, 2015). Rapid responses to altered light and humidity conditions are consistent with previous observations on induction of LOX emission responses by higher light level (Loreto, Barta, Brilli & Nogues, 2006). Of course, drought and desiccation stresses per se can lead to enhanced cellular oxidative status (Dhindsa, 1991, Mittler, 2002, Noctor, Veljovic-Jovanovic, Driscoll, Novitskaya & Foyer, 2002, Scheibe & Beck, 2011, Smirnoff, 1993, Tambussi, Bartoli, Beltrano, Guimét & Araus, 2000), reflecting the overenergization of electron donors in chloroplasts and mitochondria and concomitant production of reactive oxygen species (ROS) when cellular water content decreases. Indeed, there is evidence of enhanced LOX emissions under drought (Copolovici, Kännaste, Rimmel & Niinemets, 2014, Pag, Bodescu, Kännaste, Tomescu, Niinemets & Copolovici, 2013). In the angiosperm *X. humilis* (Beckett *et al.*, 2012), the enhanced hexanal emission under desiccation stress was hypothesized to be associated with thylakoid disassembly into membranous vesicles (Farrant, 2000), and this was likely responsible for delayed recovery of photosynthesis upon rehydration (Beckett *et al.*, 2012, Tuba, Lichtenthaler, Csintalan, Nagy & Szente, 1996,

Tuba, Lichtenthaler, Csintalan & Pocs, 1993a, Tuba *et al.*, 1993b). In Hymenophyllaceae, chloroplast movements toward the cell walls and chloroplast size reduction have been reported (Garcés *et al.*, 2017), and both these changes can contribute to avoidance of overexcitation of thylakoids. However, modifications in chloroplast ultrastructure are much less pronounced in filmy ferns (Bravo *et al.*, 2016, Garcés Cea *et al.*, 2014). Furthermore, in filmy ferns, photosynthetic recovery is much faster under a moderately long desiccation stress (Fig. 3), suggesting that despite the desiccation stress resulted in LOX release, it did not lead to sustained membrane-level damage.

The reduction in LOX emission rate through desiccation was qualitatively similar to the reductions in RWC (Fig. 3) and in foliage photosynthetic activity (Fig. 4A), indicating that the reduction of LOX emissions paralleled the overall decrease in fern leaf physiological activity due to reduction in the number of active cells. However, the reduction in LOX emission rate was slower than in RWC and photosynthetic activity (Fig. 6A, Table 1), indicating that LOX activity was much less sensitive to the reduction in frond water status.

Across the species, the reduction in LOX emission was the strongest in the lower-canopy (0-3 m) species *H. cruentum*, followed by the lower canopy (0-3 m) species *H. caudiculatum* and the upper canopy (1-9 m) species *H. dentatum* and finally by the generalist (0-9 m) species *H. plicatum* (Fig. 6A). In addition, *Hymenoglossum cruentum* clearly stood out when both the time constants for reductions in photosynthesis and LOX emission were considered (Fig. 6B), and the upper canopy species *H. dentatum* and generalist species *H. plicatum* had greater LOX emission rates remaining at different stages of desiccation (Fig. 4A, Fig. 6D, Table 1). The rate of emission and total amount of LOX products emitted has been associated with the severity of stress (Niinemets *et al.*, 2013 for a review), and it might be predicted that species adapted to harsher environments sustain a lower stress level at given set of environmental conditions. However, the evidence in our study suggests that, counterintuitively, the species colonizing upper canopy environments had greater and more sustained LOX emissions than the lower canopy species as the desiccation stress progressed. Given that upper canopy species had a greater photosynthetic capacity (Table 1), it is likely that they had a greater rate of production of reactive oxygen species once the use of reductive and energetic equivalents became to a halt in desiccated leaves. Thus, inherently greater physiological activity in non-stressed conditions can ultimately be responsible for greater LOX production in upper canopy species.

Emission bursts of LOX volatiles upon rewetting

We observed that there was a major emission burst of LOX volatiles upon rewetting in all fern species (Fig. 4A). The emission burst of LOX volatiles occurred simultaneously with the peak in photosynthesis or it was slightly delayed (Fig. 4A). The burst of LOX volatiles suggests a rapid enhancement of ROS production upon rewetting of filmy fern leaves. Such an enhanced ROS production might be indicative of accumulation of overenergized pigments and electron donors and concomitant production of singlet oxygen, which can attack nearby membrane lipids and peptides producing free radical molecules (Rinalducci, Pedersen & Zolla, 2004). These free radicals can further contribute to formation of water-soluble ROS, in particular H_2O_2 , leading to a fast oxidative burst once the leaf water content

increases during rehydration. Indeed, in several moss species, a major fast oxidative burst, strongly exceeding the ROS production during desiccation has been observed upon rewatering (Beckett *et al.*, 2004, Cruz de Carvalho *et al.*, 2012, Mayaba *et al.*, 2002, Minibayeva & Beckett, 2001). However, differently from our study, in the angiosperm *X. humilis*, rewatering resulted in only a minor enhancement of hexanal emissions (Beckett *et al.*, 2012). However, desiccation in *X. humilis* is associated with major losses of photosynthetic capacity and photosynthetic pigments (Csintalan *et al.*, 1996, Tuba *et al.*, 1993a, Tuba *et al.*, 1993b), reducing the ROS formation in desiccated leaves and likely explaining why oxidative burst and associated LOX emission burst were absent in this angiosperm. In contrast, as discussed above, homoiochlorophyllous filmy ferns maintain high photosynthetic pigment content through the desiccation period, and this is also the case with desiccation tolerant mosses (Tuba *et al.*, 1998) that do exhibit the oxidative burst upon rewatering (Beckett *et al.*, 2004, Cruz de Carvalho *et al.*, 2012, Mayaba *et al.*, 2002, Minibayeva & Beckett, 2001). Again, the burst of LOX volatiles was greater in the upper canopy species *H. dentatum* and *H. plicatum* (Fig. 4A and Fig. 7), suggesting a greater accumulation of ROS during desiccation, consistent with their greater capacity of photosynthetic electron transport.

As rehydration develops very fast, changes in the filmy fern proteome during rehydration are relatively minor (Garcés Cea *et al.*, 2014), but LOX released during the emission burst can be involved in starting proteome-level responses to cope with desiccation stress after effects. In fact, the fast rapid initial recovery is typically followed by a significant reduction in filmy fern physiological activity, especially after a prolonged desiccation stress (Proctor, 2003, Proctor, 2012), likely indicating the damage generated by ROS accumulated during desiccation. Thus, additional protein synthesis might be needed to activate repair mechanisms to recover from the after-stress effects.

Changes in methanol emissions during desiccation and rewatering

We observed continuous increases of methanol emission during desiccation in lower-canopy species *H. cruentum* and *H. caudiculatum* and no effect of desiccation on methanol emissions in the upper canopy-species *H. dentatum* and *H. plicatum*, and a major methanol emission burst in all species upon rewatering (Fig. 4B). Methanol release from plants results from the activity of pectin methylesterases that demethylate methyl ester groups in polygalactouronan chains in pectins (Micheli, 2001, Pelloux *et al.*, 2007). Methanol release is particularly pronounced in growing plant tissues (Harley, Greenberg, Niinemets & Guenther, 2007), but it is also activated upon different stresses (Beauchamp *et al.*, 2005, Jiang *et al.*, 2017, Li *et al.*, 2017). Demethylation of pectins plays a major role in altering cell wall rigidity, but the way demethylation of galactouronans alters cell wall rigidity is not well understood. As carboxylic acid residues in demethylated pectin chains can be cross-linked by Ca^{2+} bridges, demethylation is commonly thought to result in rigidification of cell walls (Cosgrove, 2016). However, once galactouronans are demethylated, cell walls become often actually softer, and this has been associated with possible lack of sufficient Ca^{2+} ions in cell walls, creation of acidic environment in cell walls due to increase in carboxylic acid residues with concomitant swelling of cell walls and activation of and facilitation of access to other cell-wall loosening agents such as expansins, endoglucanases and xyloglucan

endotransglycosylases/hydrolases (Cosgrove, 2016). As discussed in the Introduction, desiccation in filmy ferns leads to cell shrinkage, folding of cell walls and modifications in cell wall polysaccharide composition, and thus, different degree of methanol release during desiccation stress is likely indicative of different degrees of cell wall modification during desiccation. This is consistent with differences in the degree of frond curling among these fern species during the desiccation; specifically, *H. dentatum* and *H. plicatum* exhibited higher curling than *H. caudiculatum* and *H. cruentum* (Fig 1).

A burst of methanol emission is often observed upon oxidative stress (Beauchamp *et al.*, 2005, Jiang *et al.*, 2017, Li *et al.*, 2017). Interestingly, peaking of methanol release in stressed angiosperms occurs typically before the burst of LOX release (Beauchamp *et al.*, 2005, Jiang *et al.*, 2017, Li *et al.*, 2017), but in filmy ferns, the emission peak of methanol emission occurred 5-10 min after the peak in LOX volatile emission (Fig. 5C). This evidence further supports the idea that ROS was first generated in the symplast, in particular in photosynthetic membranes, and lead to elicitation of a symplastic LOX burst first, followed by diffusion of ROS into cell walls and generation of the methanol emission burst. As with total LOX, total methanol emission released during rehydration was greater in the upper-canopy species *H. dentatum* and *H. plicatum* (Fig. 7), again supporting the argument of the greater after-stress oxidative stress in these species.

The role of the methanol emission burst upon rewatering is currently unclear. As discussed above, it might be involved in reversion of desiccation-dependent cell wall modifications as discussed above. On the other hand, methanol released can similarly to LOX be involved in eliciting signaling responses (Dorokhov, Komarova, Petrunia, Kosorukov, Zinovkin, Shindyapina, Frolova & Gleba, 2012, Komarova, Sheshukova & Dorokhov, 2014).

Conclusions

Kinetic analysis of desiccation/rehydration relationships and quantitative description of photosynthetic and stress volatile responses provides a quantitative means to compare different species at a rigorously defined level of stress. Our study demonstrates that the rate of lipoxygenase pathway volatiles (LOX volatiles) decreased during desiccation together with the rate of photosynthesis, albeit with a slower rate. However, LOX volatiles and methanol, were emitted with the greatest rate during rehydration, suggesting that ROS accumulated during desiccation. In rehydrated fern leaves, the burst of LOX emission preceded the burst of methanol emission, and thus, ROS likely accumulated first in the symplast leading a LOX burst there, followed by ROS diffusion into cell walls and generation of the cell wall methanol burst. The total amount of LOX volatiles and methanol emitted during rehydration were inversely associated with the fern photosynthetic capacity and with the sensitivity of photosynthetic decline, i.e. the upper canopy species that had a greater rate of photosynthesis and sustained photosynthesis for a longer time period were subject to a greater degree of oxidative stress. Thus, the capacity to tolerate higher levels of oxidative stress might be the key adaptation of upper-canopy epiphytic ferns species to more severe environmental stress. On the other hand, methanol emission increased with progression of desiccation stress in lower-canopy species, suggesting that cell-wall modifications are quantitatively more pronounced during desiccation in these species. It

currently remains unclear whether and how upper-canopy species cope with the greater level of oxidative stress and we suggest that further experimental work should focus on long-term physiological responses after rehydration, in particular on how different durations of desiccation stress alter photosynthesis, stress volatile kinetics and antioxidant levels at different times during recovery.

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Summary statement

Sequential desiccation and rewatering cycles in filmy ferns lead to major emission bursts of lipoxygenase pathway volatiles and methanol. Volatile emission kinetics and magnitude indicate that rewatering rather than desiccation leads to the most severe endogenous stress level in filmy ferns, and that filmy fern species strongly differ in the severity of stress generated by rapid increases of frond water status.

Fig. 1.

Representative images of the hydrated, desiccated and rehydrated fronds of the four studied filmy ferns species, *Hymenoglossum cruentum* (A), *Hymenophyllum dentatum* (B), *H. caudiculatum* (C) and *H. plicatum* (D). The experiment was conducted according to the protocol described in Fig. 2.

Fig. 2.

Illustration of estimation of the first-order rate constant (k) for the reduction of the emission rate of volatile products of lipooxygenase pathway (Φ_{LOX} ; A) and net assimilation rate (A; B) upon desiccation of the filmy fern *Hymenoglossum cruentum*. Time $t = 0$ corresponds to the start of the measurements in steady-state conditions (2–3 min since leaf enclosure). In the gas-exchange chamber, the fern was exposed to dry air (average $\pm$ SE vapor pressure deficit of 3.029 ± 0.002 kPa, relative humidity of $4.75 \pm 0.05\%$) at 25°C under incident quantum

flux density of 300–400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 4 h, after which the leaf was rapidly rehydrated again. Only the desiccation part of the whole response was fitted.

Fig. 3.

Sample relationships of net assimilation rate (A) and relative fern water content (RWC, Eq. 1) in four filmy fern species through a desiccation cycle (0-4 h since the start) and after rehydration (4-5 h). Two parallel experiments were conducted under similar conditions, one for estimation of changes in RWC and the other for measurements of continuous changes in net assimilation rate and volatile emission (experimental conditions as in Fig. 2). In the case of RWC measurements, the samples were taken out in every 30 min and their mass was estimated (see Material and methods). At time 4 h, the plant was rehydrated by spraying with distilled water and the experiment was continued after the surface water had evaporated (typically rehydration and surface evaporation took 10-15 min). In the case of net assimilation, the measurements were carried out continuously and averaged for 10 min., and the rehydration was done as in the case of RWC experiment. In both cases, there is a 10-15 min hiatus in data after rehydration at 4 h (not shown in the figure). Due to a certain variability between experimental runs, we estimate that RWC and A synchronization is accurate to 5-10 min.

Fig. 4.

Representative time-dependent changes in the emission rates of volatile products of the lipoxygenase (LOX) pathway (A, Φ_{LOX} , mainly various C6 aldehydes and alcohols, also called green leaf volatiles) and methanol (B, Φ_{methanol}) overlaid on relative water content data in four filmy fern species during desiccation (0-4 h) and upon rewatering (4-5.5 h). Volatile emission measurements were conducted continuously, while RWC measurements were carried out every 30 min (Fig. 2 and Fig. 3 for experimental protocols). Data presentation and accuracy of data synchronization as in Fig. 3.

Fig. 5.

Comparison of representative changes in LOX volatile (A) and methanol (B) emissions with changes in net assimilation rate (A, B) and changes in LOX volatile and methanol emissions (C) through the desiccation (0-4) and rewatering (4-5.5 h) cycles in four filmy ferns. The same data as in Figs. 3 and 4. As the measurements of net assimilation and volatile emission rates were conducted simultaneously, there are no errors in data synchronization.

Fig. 6.

Time constants for the reduction in relative water content (k_{RWC}), net assimilation rate (k_A) and LOX emission rate (k_{LOX}) through the desiccation period (A), and the correlations of k_A with k_{LOX} (B) and k_{LOX} with LOX emission rate at 1 h since the start of desiccation (C) in four filmy ferns. Representative time-courses of net assimilation rate, LOX emission rate and relative water content through the desiccation cycle are demonstrated in Figs. 3 and 4. Estimation of the time constants is shown in Fig. 2.

Fig. 7.

Total amount of LOX volatiles (A) and methanol (B) released during the emission burst after rewatering in four filmy ferns. Representative time-courses of rewatering are shown in Figs. 4 and 5 and the data were integrated from the rewatering at $t = 4$ h until the emission rates reached a steady-state at ca. 4.5 h. Data among species were compared with ANOVA followed by Tukey's post-hoc test and means with different letters are significantly different at $P < 0.05$.

Table 1

Average ($\pm$ SE) net assimilation rate and total emission rate of lipoxygenase pathway volatiles at different times since the start of desiccation

Species	Time (h)		
	0	0.5	1
Net assimilation rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$)			
<i>Hymenoglossum cruentum</i>	1.91 $\pm$ 0.21 ^a	1.15 $\pm$ 0.13 ^a	0.54 $\pm$ 0.08 ^a
<i>Hymenophyllum caudiculatum</i>	2.68 $\pm$ 0.32 ^{ab}	1.85 $\pm$ 0.12 ^{ab}	0.99 $\pm$ 0.05 ^{bc}
<i>Hymenophyllum dentatum</i>	3.64 $\pm$ 0.63 ^b	1.74 $\pm$ 0.28 ^{ab}	0.83 $\pm$ 0.06 ^{ab}
<i>Hymenophyllum plicatum</i>	3.98 $\pm$ 0.58 ^b	2.66 $\pm$ 0.27 ^b	1.06 $\pm$ 0.07 ^c
LOX emission rate ($\text{nmol m}^{-2} \text{s}^{-1}$)			
<i>Hymenoglossum cruentum</i>	1.19 $\pm$ 0.05 ^a	1.08 $\pm$ 0.08 ^a	0.84 $\pm$ 0.06 ^a
<i>Hymenophyllum caudiculatum</i>	1.94 $\pm$ 0.08 ^b	0.69 $\pm$ 0.06 ^b	0.52 $\pm$ 0.06 ^b
<i>Hymenophyllum dentatum</i>	1.99 $\pm$ 0.14 ^b	1.35 $\pm$ 0.05 ^c	1.19 $\pm$ 0.07 ^c
<i>Hymenophyllum plicatum</i>	1.83 $\pm$ 0.08 ^b	1.37 $\pm$ 0.05 ^c	1.27 $\pm$ 0.08 ^c

Means with different lowercase letters are statistically different among different measurement times among species according to ANOVA followed by Tukey's post-hoc test ($P < 0.05$).