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Using Chlorophyll a Fluorescence Imaging to Select Desiccation-Tolerant Native Moss Species for Water-Sustainable Green Roofs

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Abstract: Green roofs have been more thoroughly investigated in the last few years due to the potential benefits they offer to ecosystems in urban areas (e.g., carbon sequestration, particle retention, heat island effect attenuation). However, current climate change models predict an increase in desertification, with an increase in temperature and decrease in rainfall, which means there is an increasing demand for green roofs with lower water consumption. Vegetation with very little water requirements, such as desiccation-tolerant mosses, has shown a potential to complement or substitute for vascular species, increasing the sustainability of lower water use in green roofs. In this study, we use chlorophyll a fluorescence imaging to screen for bryophytes with adequate physiology to be used in green roofs placed in at-risk areas with prolonged drought episodes. Apart from Hypnum cupressiforme, all selected species presented a high potential for use in those conditions, particularly Didymodon fallax, Grimmia lisae, Pleurochaete squarrosa, and Targionia hypophylla. Chlorophyll a fluorescence imaging technology proved to be a simple and non-invasive tool for a fast screening of these poikilohydric organisms, to be used in future studies of bryophyte biology, but more importantly in the green roof industry.

Keywords: green roofs; mosses; chlorophyll a fluorescence; water use; sustainability; Mediterranean; biological soil crusts

1. Introduction

Green roofs consist of plant-based structures that are placed on a waterproof layer on top of residences, factories, offices, and other buildings increasing the services provided by green spaces in urban areas [1–6]. In addition to an improvement of urban aesthetics, green roofs attenuate flood effects, increasing the retention time of rainwater [5,7]. Furthermore, they contribute to the thermal regulation of buildings and reduce the urban heat island effect, as well as mitigating heat loss during the winter [8,9]. Other important contributions include biodiversity conservation, increased carbon sequestration, air quality improvement, soundproofing for building interiors, increased roof durability, and an ability to slow down the spread of potential fires [10–13].
The increased popularity of green roofs began in northern Europe, in areas with cold and humid climates (e.g., Germany, Netherlands). These environments enabled easier maintenance of these structures at a low cost without resorting to irrigation [9]. However, in the Mediterranean area or in regions with similar latitudes (e.g., California, south-western Australia, South Africa, Chile), characterized by hot and dry summers, the use of classical green roofs requires irrigation, to maintain both plant survival and aesthetic quality [14].

However, the need for watering can make green roofs economically non-viable, jeopardizing the objective of optimizing energy consumption for building acclimatization [15]. The possible economic advantages of green roofs depend on them being low cost, sustainable, and absent of high maintenance requirements. Moreover, converting older buildings to support green roofs would be extremely difficult, due to the overwhelming costs of structural adaptations to include irrigation systems.

In desert areas, it is very difficult for plants to survive due to low precipitation. However, these areas are covered with biological soil crusts (biocrusts), a complex mosaic of poikilohydric organisms, including cyanobacteria, green algae, lichens, mosses, microfungi, and bacteria. Cyanobacteria weave filaments to create a matrix that aggregates, protects, and stabilizes soil surfaces, creating conditions that allow the establishment of other higher organisms such as lichens and mosses [16,17]. The green covers arose as a potential solution, allowing green roofs to be compatible with the harsh conditions of urban centers. Although lichens are, at present, impossible to produce artificially and cyanobacteria are highly dynamic throughout time, mosses appear to be the most stable option for these covers. Mosses from these harsh environments can photosynthesize when water is available but, in drought conditions, they undergo extensive desiccation and their entire metabolism ceases. Desiccated mosses remain dormant for months and years, returning to normal function upon rehydration, presenting a spectrum of desiccation tolerance (DT) [18–21]. Moreover, mosses can retain water up to 8–10 times their dry weight, allowing for a self-sustained growth [7,22]. More importantly, this water retention makes them a viable candidate for attenuating the effects of flash floods, which are becoming more frequent due to climate change. Furthermore, mosses do not have roots but instead grow in a very thin substrate layer, decreasing the weight load on architectural structures, and allowing them to be tested on steeper surfaces, such as walls. They can also be used in traditional green roofs acting as a filler in between vascular plants, increasing the overall system performance of green roofs regarding water consumption [23,24].

Photosynthesis is the key metabolic process for photoautotrophic organisms. The in vivo measurement of the chlorophyll a fluorescence provides a long-established and non-invasive method for assessing photosynthetic activity [25]. The introduction of imaging technologies to the in vivo chlorophyll a fluorescence measurement allowed to obtain spatial (topographic) representations of photosynthetic activity over a biological surface. Furthermore, this combined approach is an effective method, fostering for screening organisms/samples with different photosynthetic performance, as well as providing a lower-cost and faster tool for establishing user-made systems [26,27].

Under the current climate change, water will be available based on location, seasonality, and/or intensity of rainfall, with the Mediterranean region being the most cause for concern [28]. In our previous approach [29], we used Ellenberg’s ecological preference values, which is a system based on the ordinal classification of plants, according to the position of their actual ecological niche along an environmental gradient (light, temperature, and humidity) [30]. These preference values were used to select moss species most adequate for Mediterranean green roofs. Selecting the Ellenberg’s values typical for the Mediterranean climate (light: 8–9 (light-loving and full-light plants); temperature: 8–9 (Mediterranean and sub-Mediterranean plants); and humidity: 1–3 (extreme dryness to moderately dry sites)) and analyzing their life form, growth form, and lifestyle [31,32], a table of moss species with potential use in Mediterranean green roofs was generated [29]. From that list of potential bryophytes that naturally occur in harsh environmental conditions, we selected some of the most cosmopolitan species in the Mediterranean. Those species were tested with the non-invasive chlorophyll a fluorescence imaging technique, allowing the analyses of the photosynthetic fitness over desiccation and rehydration cycles.
2. Materials and Methods

2.1. Biological Material

Bryophytes were selected and collected from harsh environmental conditions (roadsides, sun-exposed walls) in dry sub-humid (Alegrete, Parque Natural de São Mamede) to semi-arid (Zebreira, Beira Baixa; Estremoz, Alto Alentejo; Barreiro, Setúbal; and Ermidas-Sado, Baixo Alentejo) areas, in both natural and urban locations (Figure 1A). Bryophyte selection included isolating colony patches of four different mosses that were selected from our previous work [29] (Table 1): Didymodon fallax (Hedw.) R. H. Zander (high (Barreiro) and low (Ermidas-Sado) human population densities), Grimmia lisae De Not., Pleurochaete squarrosa (Brid.) Lindb., and Tortella nitida (Lindb.) Broth. from urban and natural environments (Figure 1B). Although not on the list the mosses [29], Hypnum cupressiforme Hedw. and the liverwort Targionia hypophylla L. were selected as a less DT and more DT species, respectively, to explore their potential use in green walls, since they can grow on any surface regardless of structural orientation.

![Figure 1.](image)

**Figure 1.** (A) Collection sites in southern Portugal (a. Zebreira; b. São Mamede; c. Estremoz; d. Barreiro; e. Ermidas-Sado) where (B) the bryophyte species were collected (Didymodon fallax (Hedw.) R. H. Zander, Grimmia lisae De Not., Hypnum cupressiforme Hedw., Pleurochaete squarrosa (Brid.) Lindb., Targionia hypophylla L., and Tortella nitida (Lindb.) Broth.). Map of Portugal represents the two climate types in Portugal (Csa: Mediterranean hot summer (yellow); Csb: Mediterranean cool summer (green)) [29].

**Table 1.** Bryophyte species collected in the present study (clade, growth form, location, aridity index (1980–2010) (A.I.), natural (N), or urban (U) site, coordinates).

| Species                  | Plant Clade | Growth Form | Location          | A.I.* | N/U | Coordinates         |
|--------------------------|-------------|-------------|-------------------|-------|-----|---------------------|
| Didymodon fallax (Hedw.) | Bryophyta (mosses) | Acrocarpous | Ermidas-Sado      | Semi-arid | U   | 38°00′24.6″ N 8°25′03.2″ W |
| R.H.Zander               |             |             | Barreiro          | Dry sub-humid | U   | 38°39′56.0″ N 9°04′05.9″ W |
| Grimmia lisae De Not.    | Bryophyta (mosses) | Acrocarpous | Zebreira          | Semi-arid | U   | 39°50′33.4″ N 7°04′07.2″ W |
| Hypnum cupressiforme Hedw. | Bryophyta (mosses) | Pleurocarpous | Alegrete (Parque Natural de São Mamede) | Dry sub-humid | N   | 39°15′14.6″ N 7°18′05.0″ W |
| Pleurochaete squarrosa (Brid.) Lindb. | Bryophyta (mosses) | Acrocarpous | Zebreira          | Semi-arid | U   | 39°51′06.9″ N 7°04′22.9″ W |
Table 1. Geographical location of samples of *Targionia hypophylla* L. (liverworts) and *Tortella nitida* (Lindb.) Broth. (mosses) used in the experiment.

| Species                  | Location                      | Sub-humid U      | N      | W      |
|--------------------------|-------------------------------|------------------|--------|--------|
| *Targionia hypophylla* L.| Barreiro                      | Dry              | Sub-humid | 38°39'56.0" N | 9°04'05.9" W |
| *Tortella nitida* (Lindb.) Broth. | Alegrete (Parque Natural de São Mamede) | Dry | Sub-humid | 39°15'14.6" N | 7°18'05.0" W |
|                          | Estremoz                      | Semi-arid        |        | 38°48'01.8" N | 7°39'41.9" W |

* Aridity Index (1980–2010) according to [33].

Samples were collected dry and stored in paper bags until analysis (3 months later) as per normal practice [34].

2.2. Experimental Design

Throughout the experiment, samples were kept in 8 cm Petri dishes in a growth chamber (ARALAB, Portugal) under controlled conditions of light (circa 100 µmol m$^{-2}$ s$^{-1}$), temperature (18 °C), relative humidity (50%), and photoperiod (16 h/8 h, day/night). After dry samples were weighed (T0), chlorophyll $a$ fluorescence was measured (for details see the section below) bryophytes were rehydrated afterward with 50 mL of distilled water. Measurements were also taken after 3 (T3), 6 (T6), and 10 (T10) days, keeping the samples hydrated in Petri dishes partly closed during this period, avoiding water evaporation and, thus, desiccation. Afterward, samples were dried for 3 days with the Petri dishes fully opened. When samples reached the same weight as T0, i.e., at the end of the drying process (T13), measurements were taken and then the mosses were rehydrated with 50 mL of distilled water. Measurements were taken after 1 hour (Rh1h) and 1 day (Rh1d), keeping the Petri dishes partly closed and samples hydrated (Figure 2).

![Figure 2](image_url)

**Figure 2.** Representation of the experimental design of this study, in this case, *Pleurochaete squarrosa*, showing representative chlorophyll $a$ fluorescence images ($F_o$ (minimum fluorescence); upper row) and correlated RGB images (low row), at selected times.

2.3. Imaging Pulse Amplitude Modulated Chlorophyll $a$ Fluorescence

Imaging pulse-amplitude-modulated chlorophyll $a$ fluorescence measurements were performed using the Mini Version of Imaging-PAM M-Series (Walz GmbH) which comprised an IMAGE-K5 1/2” CCD camera (640 × 480 pixel resolution) with a 16 mm objective (Allied Vision Technologies GmbH, Stadtroda, Germany). The stage works at a fixed working distance and comprises a 24 × 32 mm area, illuminated by a Luxeon Light-Emitting Diode (LED) array (460 nm) of 12 high-power LEDs divided into four groups and equipped with short-pass filters and providing the measuring beam, the actinic light, and the saturation light pulses. After 5 minutes of dark-adaptation, a saturation pulse with an intensity of 6000 µmol photons m$^{-2}$ s$^{-1}$ for 0.8 s and the measuring pulse frequency of 8 Hz was applied, allowing the measurement of the dark-adapted samples for their maximum ($F_m$) and minimum ($F_o$) fluorescence values, respectively. In turn, the maximum photochemical efficiency of photosystem II (PSII) ($F_v/F_m$), a ratio that can be used as an indicator of the photosynthetic fitness,
was calculated [35]. Furthermore, the electron transport rate (ETR), which reflects the energy used in photosynthesis, and the non-photochemical quenching (NPQ/4), which provides information on the dissipative mechanisms of photosynthesis (mostly through heat) [35–37], were determined by applying a blue actinic light (111 µmol photons m⁻² s⁻¹) for 5 minutes, followed by a new saturating light pulse. Rapid light curves (RLCs) were constructed by calculating the relative electron transport rate (rETR) for each level of actinic light (E) using the formula rETR = E × ΔF/Fm'. The RLC consisted of exposing the samples to ten incremental intensities of actinic light: 0, 32, 61, 111, 145, 223, 320, 402, 624, 1270, and 2000 µmol photons m⁻² s⁻¹, each with a 30 s irradiance step. Afterward, the model of Platt and colleagues [38] was applied to characterize the light response of the rETR vs. E curves, estimating the parameters of α (initial slope of the light curve indicative of photosynthetic efficiency), β (photoinhibition), rETRmax (maximum rETR), and E₅₀ (light saturation value). The model was fitted through the application of Microsoft Excel Solver (curve fits with r > 0.95). Numerical values and images of the chlorophyll a fluorescence parameters were obtained from the digital images using analytical software (Imaging Win v.2.41, Walz GmbH, Effeltrich, Germany). For each species, five shoots were selected with the software.

2.4. Statistical Analysis

For each variable considered in this study, differences among species within each assayed day were evaluated through one-way ANOVA with Tukey’s multiple comparisons test (GraphPad Prism 6.03 for Windows, GraphPad Software, San Diego, CA, USA) and are presented in Supplemental Data.

3. Results

Chlorophyll a fluorescence analysis shows that, in general, recovery was very similar for all bryophytes, except for H. cupressiforme, which had a slower recovery, reaching their optimal performance at T6. Although still in good condition, at T10 the differences among species increased in number and complexity (see statistical differences among species in Tables S1–S7 in Supplemental Data). When the samples were dry at T0 and T13 chlorophyll a fluorescence was absent in all samples.

Regarding the maximum quantum efficiency of PSII (Fv/Fm; Table 2A), a widely used parameter to evaluate photosynthetic fitness, at T3 there were four species (D. fallax (B), G. lisae, P. squarrosa, T. hypophylla) that presented values between 0.6–0.7 corresponding to 80–90% recovery of the reference unstressed value of 0.75 for mosses [18,39]. The exceptions were T. nitida and H. cupressiforme, which only recovered to those values at T6 and T10, respectively.

In the case of the electron transport rate (ETR; Table 2B), the trend was very similar, but as observed previously, H. cupressiforme showed the lowest performance at T3. Nevertheless, at T6, the ETR of all the analyzed species was fully recovered, with the liverwort, T. hypophylla, presenting the highest value (36 µmol m⁻² s⁻¹). However, after 10 days of constant rehydration, some of the most tolerant species (D. fallax (B), G. lisae, T. hypophylla, P. squarrosa) started to demonstrate a statistically significant decrease in ETR (circa 20 µmol m⁻² s⁻¹), compared with the values at T6 (circa 16 µmol m⁻² s⁻¹).

Non-photochemical quenching (NPQ/4; Table 2C) presented the highest values in T. hypophylla and P. squarrosa at T3, decreasing drastically at T6, but increasing to more typical values at T10. A similar pattern was also observed for D. fallax, whilst for the remaining species, NPQ/4 increased throughout the hydration period.

The parameters measured in the rapid light curves (RLC) allowed further analysis of the response of the selected bryophytes in the hydration–dehydration cycles. The photosynthetic efficiency (α; Table 3A) pattern was very similar to the one observed for Fv/Fm in all species. The lower photosynthetic performance of H. cupressiforme at T3 seems to be due to the higher photoinhibitory response (β; Table 3B), which decreased over time, in contrast with the other species, where it consistently increased over time.
Regarding the maximum relative electron transport rate (rETR\textsubscript{max}; Table 3C), an increase between T3 and T6 was observed, but, after 10 days of rehydration, the six species presented very similar values (circa 20 µmol m\textsuperscript{-2} s\textsuperscript{-1}).

This previously observed photoinhibitory effect (Table 3B) is further confirmed by the general decrease of saturating irradiance (E\textsubscript{k}; Table 3D) over the hydration period (again, except for \textit{H. cupressiforme}). At T10, most bryophytes reached their saturating light at values around 100–125 µmol m\textsuperscript{-2} s\textsuperscript{-1}, but \textit{D. fallax} (Barreiro) and \textit{G. lisae} (Zebreira), which grow in full light-exposed sites, showed values closer to 175 µmol m\textsuperscript{-2} s\textsuperscript{-1}.

Table 2. Chlorophyll \textit{a} fluorescence parameters (A, maximum photochemical efficiency of photosystem II (F\textsubscript{v}/F\textsubscript{m}); B, electron transport rate (ETR); and C, non-photochemical quenching (NPQ/4)) in different bryophyte species, during a cycle of 10 days hydration followed by a 3 days dry event and a recovery period of 24 hours (average ± standard deviation, n = 5, different letters (a,b,c) indicate significant differences at p < 0.05 between times within the same species). Sites: E-S—Ermidas-Sado, Baixo Alentejo; Br—Barreiro, Setúbal; Z—Zebreira, Beira Baixa; E—Estremoz, Alto Alentejo; SM—Parque Natural de São Mamede, Alto Alentejo.

|          | F\textsubscript{v}/F\textsubscript{m} | T0      | T3      | T6      | T10     | T13     | T13 + 1h | T14     |
|----------|----------------------------------|---------|---------|---------|---------|---------|----------|---------|
| Didymodon fallax (E-S) | 0.570 ± 0.026 | 0.605 ± 0.016 | 0.680 ± 0.017 | 0.589 ± 0.025 | 0.569 ± 0.026 |
|           | a                                | a       | b       | a       | a       |
| Didymodon fallax (Brr) | 0.626 ± 0.058 | 0.628 ± 0.016 | 0.545 ± 0.062 | 0.367 ± 0.101 | 0.565 ± 0.036 |
|           | a                                | a       | a       | b       | a       |
| Grimmia lisae (Z) | 0.583 ± 0.051 | 0.665 ± 0.013 | 0.625 ± 0.026 | 0.606 ± 0.018 | 0.623 ± 0.022 |
|           | ac                               | b       | abc     | c       | abc     |
| Hypnum cupressiforme (SM) | 0.281 ± 0.051 | 0.592 ± 0.048 | 0.655 ± 0.014 | 0.450 ± 0.048 | 0.620 ± 0.022 |
|           | a                                | b       | b       | c       | b       |
| Pleurochaete squarrosa (Z) | 0.618 ± 0.015 | 0.641 ± 0.019 | 0.690 ± 0.002 | 0.527 ± 0.004 | 0.633 ± 0.004 |
|           | a                                | b       | c       | d       | ab      |
| Targionia hypophylla (Brr) | 0.716 ± 0.008 | 0.744 ± 0.007 | 0.747 ± 0.002 | 0.666 ± 0.047 | 0.700 ± 0.015 |
|           | ac                               | a       | a       | bc      | c       |
| Tortella nitida (SM) | 0.520 ± 0.030 | 0.613 ± 0.014 | 0.615 ± 0.025 | 0.491 ± 0.040 | 0.584 ± 0.013 |
|           | ac                               | b       | b       | c       | b       |
| Tortella nitida (E) | 0.352 ± 0.145 | 0.645 ± 0.047 | 0.714 ± 0.017 | 0.000 ± 0.000 | 0.572 ± 0.016 |
|           | a                                | b       | b       | c       | d       |

|          | ETR (µmol m\textsuperscript{-2} s\textsuperscript{-1}) | T0      | T3      | T6      | T10     | T13     | T13 + 1h | T14     |
|----------|-------------------------------------------------------|---------|---------|---------|---------|---------|----------|---------|
| Didymodon fallax (E-S) | 18.5 ± 1.7 | 20.2 ± 1.4 | 17.3 ± 1.9 | 13.9 ± 2.5 | 14.0 ± 1.8 |
|           | a                                | a       | ab      | b       | b       |
| Didymodon fallax (Brr) | 20.3 ± 3.6 | 18.2 ± 2.7 | 13.2 ± 1.7 | 8.0 ± 5.3 | 18.7 ± 1.2 |
|           | a                                | a       | ab      | bc      | c       |
| Grimmia lisae (Z) | 21.1 ± 2.5 | 20.9 ± 1.4 | 14.7 ± 1.2 | 19.1 ± 3.5 | 19.7 ± 1.7 |
|           | ab                               | a       | b       | a       | a       |
| Hypnum cupressiforme (SM) | 3.0 ± 2.3 | 13.1 ± 2.8 | 16.1 ± 3.2 | 10.5 ± 1.7 | 16.6 ± 1.9 |
|           | a                                | bc      | b       | c       | b       |
| Pleurochaete squarrosa (Z) | 15.5 ± 1.2 | 19.2 ± 1.0 | 17.6 ± 0.8 | 13.7 ± 0.3 | 14.5 ± 0.7 |
|           | a                                | b       | c       | d       | ad      |
| Species                      | A                  | B                  | C                  | D                  |
|-----------------------------|--------------------|--------------------|--------------------|--------------------|
| Targionia hypophylla (Brr)  | 18.6 ± 2.3 a       | 36.0 ± 0.6 b       | 14.3 ± 1.9 c       | 17.8 ± 0.9 a       |
| Tortella nitida (SM)        | 16.1 ± 1.0 a       | 14.1 ± 0.6 a       | 12.2 ± 2.9 b       | 17.0 ± 1.1 a       |
| Tortella nitida (E)         | 17.2 ± 1.5 a       | 18.7 ± 2.2 a       | 0.0 ± 0.0 b        | 14.0 ± 0.7 a       |

Table 3. Rapid light curve parameters (A, photosynthetic efficiency (α); B, photoinhibition (β); C, maximum rETR (rETRmax); and D, light saturation value (Ek)) in different bryophyte species, during a cycle of 10 days hydration followed by a 3 days dry event and a recovery period of 24 hours (average ± standard deviation, n = 5, different letters (a,b,c,d) indicate significant differences at p < 0.05 between times within the same species). Sites: E-S—Ermidas-Sado, Baixo Alentejo; Brr—Barreiro, Setúbal; Z—Zebreira, Beira Baixa; E—Estremoz, Alto Alentejo; SM—Parque Natural de São Mamede, Alto Alentejo.
|                          | Targionia hypophylla (Brr) | Tortella nitida (SM) | Tortella nitida (E) |
|--------------------------|-----------------------------|----------------------|---------------------|
|                          | 0.284 ± 0.014               | 0.182 ± 0.014        | 0.178 ± 0.058       |
|                          | a                           | ab                   | a                   |
|                          | 0.285 ± 0.021               | 0.185 ± 0.006        | 0.240 ± 0.020       |
|                          | a                           | ab                   | b                   |
|                          | 0.257 ± 0.013               | 0.213 ± 0.027        | 0.251 ± 0.045       |
|                          | ac                          | a                    | b                   |
|                          | 0.150 ± 0.006               | 0.175 ± 0.015        | 0.000 ± 0.000       |
|                          | b                           | b                    | c                   |
|                          | 0.247 ± 0.016               | 0.254 ± 0.019        | 0.192 ± 0.013       |
|                          | c                           | ab                   | ab                  |

**B**

| β            | T0            | T3            | T6            | T10           | T13           | T13 + 1h       | T14            |
|--------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------|
| Didymodon fallax (E-S) | 57.9 ± 13.4  | 59.1 ± 9.5    | 102.1 ± 20.9  |               | 205.1 ± 82.6  | 106.7 ± 17.4  |                |
| Didymodon fallax (Brr)  | 60.0 ± 18.6  | 64.0 ± 16.2   | 86.1 ± 13.3   |               | 121.0 ± 42.2  | 65.9 ± 8.3    |                |
| Grimmia lisae (Z)       | 46.9 ± 4.3   | 71.0 ± 16.5   | 89.5 ± 3.4    |               | 89.9 ± 27.3   | 62.4 ± 1.8    |                |
| Hypnum cupressiforme (SM) | 448.6 ± 296.2 | 155.1 ± 69.0 | 123.6 ± 15.0  |               | 195.6 ± 33.4  | 158.4 ± 29.8  |                |
| Pleurochaete squarrosa (Z) | 86.4 ± 3.6  | 88.0 ± 10.1   | 120.5 ± 15.2  |               | 128.6 ± 1.0   | 113.3 ± 1.0   |                |
| Targionia hypophylla (Brr) | 98.8 ± 19.2  | 79.7 ± 11.9   | 123.2 ± 13.2  |               | 262.0 ± 100.9 | 116.1 ± 17.0  |                |
| Tortella nitida (SM)  | 79.6 ± 2.7   | 97.3 ± 0.7    | 98.3 ± 14.2   |               | 185.8 ± 120.3 | 112.4 ± 18.9  |                |
| Tortella nitida (E)    | 86.1 ± 28.6  | 143.6 ± 15.0  | 143.6 ± 15.0  |               | 0.0 ± 0.0     | 126.5 ± 4.5   |                |

**C**

| rETRmax (µmol m⁻² s⁻¹) | T0         | T3         | T6         | T10        | T13        | T13 + 1h      | T14         |
|-------------------------|------------|------------|------------|------------|------------|--------------|-------------|
| Didymodon fallax (E-S)  | 37.6 ± 9.0 | 43.1 ± 6.4 | 26.5 ± 6.7 | 14.0 ± 5.9 | 23.6 ± 4.7 | b            |            |
| Didymodon fallax (Brr)  | 44.9 ± 15.8| 37.5 ± 14.2| 20.7 ± 5.1 | 14.9 ± 6.7 | 40.5 ± 4.9 | b            |            |
| Grimmia lisae (Z)       | 53.0 ± 9.2 | 42.9 ± 9.4 | 27.8 ± 0.6 | 33.9 ± 13.4| 40.9 ± 2.4 | b            | ab          |
| Hypnum cupressiforme (SM) | 3.2 ± 1.8  | 18.2 ± 9.4 | 20.7 ± 6.5 | 10.3 ± 2.3 | 18.6 ± 3.1 | c            | bc          |
| Pleurochaete squarrosa (Z) | 24.5 ± 3.9 | 29.7 ± 3.0 | 22.2 ± 2.9 | 15.9 ± 0.7 | 16.4 ± 0.3 | c            | bc          |
| Targionia hypophylla (Brr) | 30.1 ± 5.4 | 37.0 ± 6.4 | 21.5 ± 2.0 | 6.5 ± 2.1  | 22.1 ± 4.1 | c            | bd          |
| Tortella nitida (SM)    | 23.3 ± 1.9 | 19.4 ± 0.7 | 22.6 ± 5.0 | 11.9 ± 4.6 | 23.6 ± 4.3 | b            | a           |
| Tortella nitida (E)     | 21.3 ± 5.0 | 17.3 ± 3.4 | 19.7 ± 8.6 | 0.0 ± 0.0  | 15.5 ± 1.4 | b            | a           |
Following a 3 days desiccation event, again no photosynthetic activity was detected in bryophytes, with no measurable chlorophyll a fluorescence (T13). After 1 hour of rehydration (T13 + 1 h), all parameters showed values lower than those measured at T10, except for T. hypophylla which presented a higher rETR$_{max}$ (Table 3C), and T. nitida (E) which presented no photosynthetic activity at this recovery point (Tables 2 and 3, T13 + 1 h). However, after 24 hours of recovery, most species have recovered up to 90% the values measured at T6, regarding F$_v$/F$_m$ (Table 2A, T14) and α (Table 3A, T14), but with decreases in ETR (Table 2B) and E$_a$ (Table 3C), and, thus, showing higher values of non-photochemical quenching (Table 2C) and photoinhibition (Table 3D). Overall, the species that showed greatest differences were H. cupressiforme and T. nitida (E) (see statistical differences among species in Tables S1–S7 in Supplemental Data) presenting the lowest performances when compared with the remaining species.

4. Discussion

In recent years, commercial green surfaces have included mosses such as Tortula muralis and Bryum argenteum, selected intuitively without scientific criteria [40]. Although these mosses have been described as desiccation tolerant, not all bryophytes perform equally in the face of desiccation. The varying degrees of tolerance exist along a large spectrum, where the velocity of desiccation plays a major role, and where highly desiccation-tolerant species could withstand higher rates of water loss [41]. This might be due to the presence of constitutive desiccation tolerance mechanisms in these species, in contrast with the inducible tolerance mechanisms in less tolerant species [20,42]. On the other hand, desiccation-tolerant species are dependent on the alternation of dehydration–rehydration cycles, in a manner still not fully understood [21,43]. Nevertheless, their presence in green roofs contributes to sustainable irrigation of green roofs, particularly in areas with Mediterranean-like climates [23,24].

The most desiccation-tolerant species in this study (T. hypophylla and P. squarrosa) can maintain high values of photosynthetic fitness after desiccation/rehydration events, to which they are daily exposed in natural environments. These conditions require them to survive the excess irradiance, which indicates that these are good candidates to survive the harsh conditions of a Mediterranean green roof with lower irrigation requirements. In the case of P. squarrosa, the data further confirms its high adaptability and tolerance to sparse water supply, which has been observed previously in green
roof-like conditions [23,24]. For the other species, photoinhibition increases with time, which indicates a high relative acclimation to the optimal growth chamber conditions and possibly a concomitant decrease in the effectiveness of their desiccation tolerance mechanisms. This information could also indicate that an increase in the growth rate may be achieved from within the growth chambers’ optimal conditions, as found in other bryophytes [44], which may allow for an increase in the production speed of green roof installations. However, the use of growth chambers may also require a later increase of the dehydration/rehydration acclimation cycles before the organisms can be applied to green roof settings.

The species *H. cupressiforme* and *T. nitida* were the ones that negatively stood out from the other species. The first is a species that thrives in moist environments, taking more time to recover after desiccation (at least 10 days). Although this moss has a higher demand for irrigation, it could still be used for other architectural purposes such as shaded vertical walls, since it presents a pleurocarpic life form (matt-like structure) that is ideal for higher surface coverage. On the other hand, *T. nitida*, although presenting lower levels of photosynthetic performance than the more DT species, had a higher performance than *H. cupressiforme*, so it may still be considered as a possible desiccation-tolerant option for green roofs.

Furthermore, after 10 days of constant rehydration, some of the most tolerant species (*D. fallax, G. lisae, T. hypophylla, P. squarrosa*) started to demonstrate a decrease in photosynthetic activity (as shown by the decrease in ETR) indicating that hydration for more than a few days is not suitable for these species, increasing photoinhibition and non-photochemical quenching processes. In fact, during the hot and dry Mediterranean summers, these mosses lose their bright green color and turn dark-green-brown [24]. The color change of these mosses is caused by the loss of all cellular water and the consequential shutdown of their metabolic processes [18,20,41–44] that allow them to tolerate the high temperature and solar radiation of this season. Their tolerance of these extreme conditions makes them an ideal candidate for green roof application [21]. However, during the cold and wet winters typical of the Mediterranean these species could be prevented from going through the hydration–desiccation cycles that seem to be important in maintaining their photochemical performance. Nevertheless, this does not seem to hinder the ability of these species to be used in green roofs, since these structures are built with a draining system that prevents the accumulation of water on the surface [1].

The introduction of imaging technologies, such as the long-established chlorophyll a fluorescence measurement [25], a non-invasive technique that allows obtaining topographic representations of photosynthesis over a biological surface, allows the screening for organisms/samples with varied photosynthetic performances [26]. The recent development of the lower-cost user-made systems [27] facilitates the use of this technique for screening bryophytes most suitable for green roof technologies.

Green roofs provide many benefits to the ecosystem and are fundamental requirements for the creation of eco-friendly cities [45]. However, irrigation sustainability will be a requirement for future green roof instalments in a sustainable world [14]. The application of bryophytes to standard green roofs, as a standalone organism or in combination with other plants, is still in its infancy [24]. Expectedly, chlorophyll a fluorescence imaging is an effective non-invasive optical technology with the potential to rapidly select adequate species, which will increase the development speed of more sustainable green roofs. The application of green roofs to urban buildings may help cities adapt to the effects of climate change.

5. Conclusions

The non-invasive technique of chlorophyll a fluorescence analysis is a very fast tool to screen for species to be used in green roofs, allowing researchers to focus resources on promising species, such as *Didymodon fallax, Grimmia lisae, Pleurochaete squarrosa*, and *Targionia hypophylla*, which can undergo multiple cycles of dehydration/rehydration without losing photosynthetic performance. Green roofs
may benefit from their natural-based mechanism of water sustainability, which is built into their natural cycles. Therefore, this study could permit the selection of the desiccation-tolerant bryophytes by performing quick surveys, fostering the use of local species to reduce the dependence on the irrigation systems of green roofs, not only in the Mediterranean but all around the world.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4441/12/6/1748/s1, Table S1: Results of the statistical analysis comparisons of F_r/\text{ET}_m between species within each time (one-way ANOVA with Tukey’s multiple comparisons test); Table S2: Results of the statistical analysis comparisons of ETR between species within each time (one-way ANOVA with Tukey’s multiple comparisons test); Table S3: Results of the statistical analysis comparisons of F_r/\text{ET}_m between species within each time (one-way ANOVA with Tukey’s multiple comparisons test); Table S4: Results of the statistical analysis comparisons of \alpha between species within each time (one-way ANOVA with Tukey’s multiple comparisons test); Table S5: Results of the statistical analysis comparisons of \beta between species within each time (one-way ANOVA with Tukey’s multiple comparisons test); Table S6: Results of the statistical analysis comparisons of r\text{ET}_{max} between species within each time (one-way ANOVA with Tukey’s multiple comparisons test); Table S7: Results of the statistical analysis comparisons of Ex between species within each time (one-way ANOVA with Tukey’s multiple comparisons test).

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